BARLEY

Physical Properties, Genetic Factors and Environmental Impacts on Growth

KOHJI HASUNUMA
EDITOR

Agricultural Research Updates

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This book is dedicated to David D Perkins, who lived to see basic genetics by use of mutants of *Neurospora crassa*, in which ROS sensitive *ndpk-1*^{P72H} was detected. The mutants provided basic analytical method to dissect the process of detoxification of singlet oxygen. And to numerous investigators who contributed to the nature of barley, from the points of physical properties, genetic factors and environmental impacts on growth.

CONTENTS

PREFACE

Barley (*Hordeum vulgare* L) is one of the major crop plants sharing 4th in the quantity of the products, and from the quantity the first is wheat, the second is corn, and the third is rice. In a 2007 ranking of cereal crops in the world, barley was 4th both in terms of quantity produced (136 million tons) and in area of cultivation (566,000 km^2)[1]

Barley including the seeds and the straw provide the sources of fodder, those of alcoholic beverages and those of foods, sharing 65%, 30% and 1.5% of the product, respectively. Barley is 2n having 14 chromosomes of 1 to 7, designated as 7H, 2H, 3H, 4H, 1H, 6H and 5H to compare the numbering of homoeologus wheat chromosomes from the published report from the North American Barley Genome Mapping Project (NABGMP), on the cross (HT), performed between the two-rowed barley cultivars, Harrington (H) and TR306(T)[2]. The HT map was compared to the map made between the six-rowed barley cultivars, Steptoe (S) and Morex (M)[2]. Barley is most fitting to provide the sources of biofuels such as biogases and bioethanol, to reduce the usage of fossil fuels. In order to reduce the abnormal increase in the atmospheric CO_2 on the earth the high yielding by the mutational tecnique will be necessary. In the present book 10 outstanding contributions were collected from the authors of Czech Republic, Republic of Belarus, Greece, Spain, United Kingdom, the three groups in Australia, far east Russia and Japan. In each area people have grown historically different species of barley cultivars adapting different circumstances in the continents of Eurasia and Australia. Their reports widely covered from growth of differnt cultivars, for the purpose to supply fodder, to supply the source of brewing seeds, and to supply the source of bred mixed with wheat floure. From the points to keep helth barley contribute to supply healthy food including β-glucan. Hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) is a form of domesticated barley. Naked barley is an ancient food crop, but a new industry has developed useful hulless barley to increase the digestibility of the grain, for swine and poultry [1]. Further from Australia drought resistgant phenotype was analyzed with special consideration to reactive oxygen species (ROS). From Japan with narrow flat land area the method to obtain ROS resistant high yielding mutants were reported.

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Kohji Hasusnuma
May 28, 2013

INTRODUCTION

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Barley (*Hordium vulgare* L.) is a major cereals scientifficallly clasified as follows.

Kingdom: Plantae
(unranked): Angiosperms
(unranked): Monocots
(unranked): Commelinids
Order: Poales
Familiy: Poaceae
Subfamily: Pooideae
Tribe: Triticeae
Species: *Hordeum vulgare*

The major uses of barley including seeds and straw are animal fodder, sources of fermentable materials for beer and certain distilled bevarages, and components of various health foods. Barley is used in soups and stews, and in barley bread. Barley grains are commonly made into malt in a traditional and ancient method of preparation [1].

BIOLOGY

Barley is self-pollinating, diploid species (2n) with 14 chromosomes [2, 3]. The wild ancestor of domesticated barley, *Hordeum vulgare* subsp. *spontaneum* is abundant in grasslands and woodlands throughout the Fertile Crescent area of Western Asia and northeast Africa and is abundant along with roadside and orchard [1]. Outside this region, the wild barley is less common. However, in a study of genome-wide diversity markers, Tibet was found to be as additional center of domestication of cultivated barley [1].

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DOMESTICATION

Wild barley has a brittle spike and upon maturity, the spikelets separate, facilitating seed dispersal. Domesticated barley has nonshattering spikes, resulting in easier to harvest the mature ears. The nonshattering phenotype is caused by a mutation in one of two tightly linked genes known as Bt_1 and Bt_2. Many cultivars are mutants of both genes. The nonshattering phenotype is recessive, and so varieties of barley that exhibit these characteristics are homozygous for the mutant allele [1].

TWO-ROW AND SIX-ROW BARLEY

The spikelets are arranged in triplets, which alternate along the rachis. In wild barley, and other Old World species of Hordeum, only the central spikelet is fertile, while the other two are reduced. This characteristic is retained in certain cultivars known as two-row barleys. A pair of mutations, one is dominant and the other is recessive, result in fertile lateral spikelets to produce six-row barley. A mutation in one gene, *vrs1*, is responsible for the transition from two-row to six-row barley. Two-row barley has a lower protein content than six-row barley, thus more fermentable sugar content. High protein barley is best suited for animal feed. Malting barley is usually lower in protein content. Seeds with low grain nitrogen, which is usually produced without a late fertilizer application, will results more uniform germination, and they need shorter steeping having less protein in the extracts that can make beer cloudy [1].

HULLESS BARLEY

Hulless of naked barley (*Hordeum vulgare* L. var, *nudum* Hook. f.) is a form of domesticated barley with an easier-to-remove hull. Naked barley is an ancient food crop, but a new industry has developed around uses of selected hulless barley to increase the digestible energy of the grain [1].

CLASSIFICATION

In traditional classifications of barley, these morphological differences have led to different forms of barley, which are classified as different species.

Two-rowed barley with shattering spikes (wild barley): *Hordeum spontaneum* K. Kock.
Two-rowed barley with nonshattering spikes: *Hordeum disticum* L.
Six-row barley with shattering spikes: *Hordeum agriocrithon* Aberg.
Six-row barley with nonshattering spikes: *Hordeum vulgare* L.

Because these differences come from single gene mutations, most recent classifications treat these forms as a single species, *Hordeum vulgare* L [1].

HISTORY

Barley is one of the first domesticated grains in the Fertile Crescent, an area of relatively abundant water in Western Asia and the Nile river of northeast Africa.

The grain appeared in the same time as einkorn and emmer wheat. Wild barley ranges from North Africa and Crete in the west, to Tibet in the east. The earliest evidence of wild barley was dated to about 8500 BC.

GENETICS

The genome of barley was sequenced in 2012 [2, 3].

PRODUCTION

Top barley producers were, Germany, France, Ukraine, Russia, Spain, Canada, Australia, Turkey, United Kingdom, and United States, and the total million metric tons of barley in 2009, 2010, and 2011 were 151.8, 123.7 and 134.3, respectively, reported by FAO.

CULTIVATION

Barley is a widely adaptable crop. It is currently popular in temperate areas where it is grown as a summer crop and tropical areas where it is sown as a winter crop. Its germination time is one to three days. Barley grows under cool conditions, but is not particularly winter hardy. Barley is more tolelant of soil salinity than wheat, which might explain the increase of barley cultivation in Methopotamia from the second millennium BC onwards. Barley is not as cold torelant as the winter wheats (*Triticum aestivum*), fall rye (*Secale cereale*) or winter triticale (*Triticosecale Wittm*. ex A. Camus.), but may be sown as a winter crop in warmer areas of Australia and Great Britain [1].

PLANT DISEASES

This plant is known or likely to be susceptible to barley mild mosaic bymovirus, as well as bacterial blight. It can be susceptible to many diseases. However, it is very difficult for plant breeders to incorporate resistance. The devastation caused by any one disease will depend upon the susceptibility to the variety being grown and the environmental conditions during disease development. Serious diseases of barley include powdery mildew caused by *Blumeria graminis* f. sp. *hordei*, leaf scald caused by *Rhynchosporium secalis*, barley rust caused by *Puccinia hordei*, and variuos diseases caused by *Cochliobolus sativus*. Barley is also susceptible to head blight [1].

USES

Algicide

Barley straw, in England, is placed in mesh bags and floated in fish ponds or water gardens to help reduce algal growth without harming pond plants and animals. Barley straw has not been approved by the EPA (Environmental Protection Agency) for use as a pesticide and its effectiveness as an aglicide in ponds has produced mixed results during university testing in the US and the UK [1].

Animal Feed

Half of the United States' barley production is used livestoch feed. Barley is an important feed grain in many areas of the world not typically suited for maize production, especially in northern climates, for example, northern and eastern Europe.

Barley is the principal feed grain in Canada, Europe and in the northern United States. A finishing diet of barley is one of the defining characteristics of western Canadian beef used in marketing campaigns [1].

BEVERAGES

Alcoholic Beverages

A large part (about 25%) of the remainder is used for malting, for which barley is the best-suited grain. It is a key ingredient in beer and whisky production. Two-row barley is traditionary used in German and English beers. Six-row barley was traditionally used in US beers, but both varieties are in common usage now. Distilled barley from green beer, whiskey has been made primarily from barley in Ireland and Scotland, while other countries have used more diverse sources of alcohol, such as the more common corn, rye and wheat in the USA. In the US, a grain type may be identified on a whisky label if that type of grain constitute 51% or more of the ingredients and certain other conditions are satisfied [1].

Nonalcoholic Beverages

Nonalcoholic drinks such as barley water and barley tea, called "mugicha" in Japan, have been made by extract of roasted barley grain by hot water. In Italy, barley is also used as coffee substitute, called caffe d'orzo, which is obtained from ground, roasted barley [1].

FOOD

Nutritional value per 100g of barley grain is as follows.
Energy: 1,474 kJ (352 kcal)
Carbohydrates: 77.7 g
Sugars: 0.8 g
Dietary fiber: 15.6 g
Fat: 1.2 g
Protein: 9.9 g

Thyamine (vit. B1): 0.2 mg
Riboflavin (vit. B2): 0.1 mg
Niacin (vit B3): 4.6 mg
Pantothenic acid (B5): 0.3 mg
Vitamin B6: 0.3 mg
Folate (vit. B9): 23 µg
Vitamine C: 0.0 mg
Ca: 29.0 mg
Fe: 2.5 mg
Mg: 79.0 mg
Phosphorus: 221 mg

Barley contains eight essential amino acids. According to a 2006 study, eating whole-grain barley can regulate blood sugar (i.e., reduce blood glucose response to a meal) for up to 10 h after consumption compared to white or even whole-grain wheat, which have similar glycemic indices. The effect was attributed to colonic fermentation of indigestible carbohydrates [1].

Hulled barley (or covered barley) is eaten after removing the ineligible, fibrous, outer hull. The hull removed barley is called dehulled barley, pot barley or scotch barley.

Considered a whole grain, dehulled barley has its bran and germ, making it a nutritious and popular health food. The pearl barley or pearled barley is dehulled barley, which has been steam processed further to remove the bran. It may be polished, a process known as pearling. Dehulled or pearle barley may be processed into a variety products, including flour, flakes similar to oatmeal and grits [1].

DETOXIFICATION PROCESS OF ROS INCLUDING SINGLET OXYGEN

Detoxyfication processes of singlet oxygen was uncovered by the group of Hasunuma, K. [4]. The strong sunlight will exite chlorophyll to the exited states, which will give the energy to ambient ground state oxygen evolved by photolysis of water in the process of photosynthesis.

The ground state oxygen, triplet oxygen, will be activated to singlet oxygen upon acception of the energy, which will make flight outside of chloroplast. The singlet oxygen will be captured by catalases in the cytosol at the heam prosthetic group, and the catalase with

singlet oxygen will make interaction with nucleoside diphosphate kinase2 (NDPK2) , which has capacity to bind NADH. From NADH electron will be transferred to bound singlet oxygen releasing superoxide, which will be detoxyfied to hydrogen peroxide by superoxide dismutase (SOD). The hydrogen peroxide is catalysed by catalses to water, oxygen and thermal emission. Thus ROS could function to detoxify strong sunlight energy to thermal emission via the function of singlet oxygen, superoxide and hydrogen peroxide [4, 5, 6, 7, 8, 9].

NDPKs having capacity to bind NADH have function to detoxyfy ROS by providing electron to ROS leading to function of catalases releasing water, triplet oxygen and thermal emission. Further singlet oxygen and superoxide could react with unsaturated fatty acids constituting membrane systems of plasma membrane, chloroplasts, mitochondria, and nuclei remaining malondialdehyde as oxydized forms, thus protecting not only proteins but also DNAs in chloroplast, mitochondria and nucleus [4,5,6,7,8,9].

ROS was found to be a major factor for the circadian rythm, and ROS closely relate to histidine kinases including NDPK and also high molecular weight histidine kinases. Histidine kinase activity is known to be functioning as a factor for circadian rythm, showing clear circadian rythm [9]. Photosynthesis is known to be controlled by circadian rythm [10] indicating mutual regulation of photosynthesis and the evolution of ROS constituting circadian rhythm.

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Chapter 1

ORGANIC BREEDING AND CULTIVATION OF BARLEY. EFFECTS ON PHYSICAL AND CHEMICAL PROPERTIES

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ABSTRACT

Barley is one of the earliest crops to be domesticated and its cultivation since the beginning of the 21st century, provides new utilities for the nutrition and sustainable development. It is persisted as a major cereal crop due to its broad ecological adaptation, its utility as a feed and food grain and due to the superiority of barley malt for use in brewing. The most important barley gene pool is consisted of varieties, breeding lines and landraces that are a valuable source of germplasm to meet the future needs of a sustainable and low-input agriculture. Modern varieties that were released under conventional breeding probably could not meet the needs for sustainable development. Therefore the release of new varieties developed under organic breeding and suitable for organic agriculture is of arising interest. These varieties could be appropriate for low input cultivation systems showing high degree of adaptation to different stressful environments and high quality characteristics. The significant genetic variability provides high adaptability to biotic and abiotic stresses. Additionally, the implementation of proper rotation systems with legumes as well other cultural practices are aiming to improve the sustainable or ecologically-based field management. Also, barley, due to its

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high adaptability, is integrated with other crops to inhibit weed growth, through allelopathic interactions. The allelopathic effectiveness varies among the barley varieties, hence, selection programs might improve the allelopathic potential of new varieties used for weed management. Barley genome has a broad genetic base with significant variability to morphological and agronomic characteristics and thus is cultivated in a wider range of environments (latitude and height) than every other cereal; therefore, timely heading and appropriate crop duration are essential attributes to adaptation. Important physical properties that are affected are plant height, yield, plumpness and grain weight. From the chemical point of view, barley is an excellent source of soluble and insoluble dietary fibers and other bioactive constituents, such as vitamin E (tocols), B-complex vitamins, minerals and phenolic compounds that in some cases are affected by cultivation practice.

Keywords: Barley, organic breeding, physical properties, tocols, β-glucans

ABBREVIATIONS

T3	tocotrienol
T	tocopherol
L.S.D	Least Significance Difference
C1, C2	Controls
Al, A2	Field after legumes
Bl, B2	Field after cereals
USDA	United States Department of Agriculture
S_d	standard deviation
G	Genotype
E	Environment
GxE	interaction Genotype x Environment

INTRODUCTION

Barley (*Hordeumvulgare* L.) is ranked 5thworldwide in dry matter production in the first decade of the 21st century [1]. It is an ancient cereal crop domesticated from about 10,000 years ago in the Fertile Crescent of the Middle East [2]. Barley is considered the most widely adapted cereal crop which is successfully cultivated in a wide-range of latitudes and altitudes, showing high adaptability to different stressful environment such as drought or salinity [3, 4, 5, 6, 7, 8]. Additionally, it has considerable allelopathic potential ensuring significant competitiveness against weeds [9, 10] and shows positive reaction to low-input environments [11]. Today, barley is used mainly for animal feeding or as a brewing grain, although in antiquity was used as a main food source for humans.

Over the last 60 years, the rapid development of conventional agriculture resulted in a massive increase in cereal grain production, which has been dependent upon a large and continuous investment in plant breeding [12]. This evolution increased crop production which also contributed to the successful animal production [13]. Barley breeders were interested in releasing high yielding varieties with an ideotype with short and strong stem and high harvest

index [14]. These varieties were developed under conditions where artificial fertilizers and agro-chemical are widely used and therefore the conventional cultivation system is characterized by high input demands and high environmental cost [12].

Therefore, nowadays, there is an arising concern regarding the sustainability of agricultural production. In this framework, a lot of research is established aiming to consider the environmental effects and the energy cost of an agricultural system [15]. Consequently, farmers are requested to achieve high crop yields using conservation practices to ensure minimum environmental impact and reduce the dependency of agriculture on external, non-renewable fossil energy that cause greenhouse gas production [16, 17, 15]. Practices such as: integrated arable farming systems, conservation tillage practices, and low-input or organic farming could give a solution to the above issue [18, 19, 20]. Organic farming is based on the banning of synthetic biocides and fertilizers [21], and consequently promotes the use of renewable resources in production, the improvement of soil fertility and processing systems to prevent pollution and avoid waste [22]. Accordingly, barley would be a suitable crop for organic cultivation due to its significant genetic variability and adaptability potential to biotic and abiotic stresses.

Additionally, barley is important because is presenting interesting characteristics related to human nutrition. Recently, it has been recognized and reappraised the importance of incorporating barley into food products and their positive effects in lowering blood cholesterol due to the effectiveness of barley β-glucans [23]. The presence of constituents in barley known to prevent and to alleviate certain diseases reinforce the effort of the released of new barley varieties, including hull-less waxy genotypes, suitable for food uses [24]. Also, Hildermann [25] has demonstrated the need of specific breeding programs for organic farming. This issue is discussed with specific focus on barley crop, together with the evaluation of barley varieties for human consumption.

ORGANIC AGRICULTURE

According to the Brundtl and Report, released from the United Nations in 1987 [26], sustainable development is: “development that meets the needs of the present without compromising the ability of future generations to meet their own needs.” Sustainable management of agricultural land aims at maintaining or enhancing food production, reducing the level of production risk, protecting the potential of natural resources and preventing degradation of soils and water quality, while being economically viable and socially acceptable [27, 28]. In an organic farming system the management of soil fertility, weeds, diseases and pests differs from conventional practices, targeting to the development and enforcement of an agro-ecosystem [22]. Organic Agriculture is increasingly gaining recognition, in political and scientific level, as a solution for sustainable agricultural production [22]. According to Verhoog et al. [29] this system of cultivation not only refers to the avoidance of chemical inputs and to the application of agro-ecological procedures in practice farming but also emphasizes to the integrity of the farming system.

Within the concept of organic cultivation, the cultivated area (land) consist an entity, where interactions between biotic and abiotic factors influence productivity and are available to the producers [30, 31]. The use of chemical inputs is avoided due to the negative

consequences of the balance of this ecosystem [29]. A crucial factor that influences the viability of this system is the maintenance and improvement of soil fertility. The productivity of this farming system is highly depended on soil nutrient transformation processes and soil conditions [32, 33]. Therefore, a main principal is the implementation of methods that facilitate the nutrient elements cycling through the maintenance and increase of soil fertility, the omission of synthetic fertilizers and pesticides and the reduction of the use of high inputs to agricultural system [34]. Methods using organic by-products have reported that improve soil chemical properties such as the increasing of soil pH [35], the availability of nutrients to plants such as potassium [36], calcium and magnesium [37]. Finally, as reported to barley crop, organic amendments can also increase the yield [11].

Apart the available resources for agricultural production; another important parameter for organic farming is the cultivated crop and the selected variety. The genus *Hordeum* is widely used for studies in organic farming and low inputs systems of cultivation and it shows a high degree of adaptation to different stressful environments [15, 38].

The effectiveness of agro-ecosystem productivity depends on its high productivity efficiency under low inputs, showing yield stability. In order to achieve stability several good agricultural practices should be adopted although it is generally admitted that, in organic cultivation, significant variability has recorded to yield stability comparing to conventional agriculture [33]. It is important to take into account that organic farmers are mainly interested in varieties with high adaptability in adverse environmental conditions and not in those with high yielding potential [39]. However, the pressure to move towards more sustainable agriculture, is clearly indicates a need to access and exploit a broader germplasm resource [7].

Murphy et al. [40] had recorded indications that the most effective way to increase yield in organic systems through breeding, is by applying direct selection within organic systems rather than indirect selection under conventional systems. Nowadays, organic cultivation depends strongly on conventionally bred and produced varieties with narrow genetic base. The conventional agriculture utilize elite cereal varieties originated from a relatively narrow germplasm pool, which are predominantly well adapted to high input agriculture [7]. These varieties are developed under conditions where artificial fertilizers and agro-chemical are widely used, and therefore there are not the optimal solution for the conditions of organic agriculture. Future progress in yield and quality of organic products requires the use of varieties suitable for organic systems of cultivation with broad genetic base.

In order to improve barley yields with high adaptability to adverse conditions and low-input environment, the evaluation and use of landrace's diversity would be a powerful means [41]. The careful study of genetic structure of landrace collections, the geographical and environmental data of origin, may reveal genomic signatures of selection which provide valuable information for breeding for specific environments, farming methods and users. The study of collection derived from Syria and Jordan, sampled from a gradient of agroecological conditions, showed a wide range of responses to drought stress as established by extensive field trials [42]. Additionally, preliminary morphological evaluation revealed significant variation between and within collection sites for many agronomically important characters [4] and disease reactions [43].

Finally, organic agriculture is contributing to the improvement of soil characteristics for these integrated production systems. Soils play a key role in the definition of sustainable land management since they represent the basis of food production. If soils are eroded or degraded to a larger extent, a society may lose its fundament of safety and self-sufficiency [44].

Fließbach. [34] have recorded data on the changes in soil organic carbon and pH over 21 years of organic, biodynamic and conventional farming at reduced and normal fertilization intensity. The study that focuses on yield and soil fertility on long-term trial treatments is crucial for a productive organic agricultural system [33]. In organic farming systems, the soil quality and microbial biomass and activities were enhanced as compared to conventional practices. This emphasizes the significant role of element cycling process that is promoted by and active soil biological community [34].

PHYSICOCHEMICAL PROPERTIES OF BARLEY

In general, physical properties that are important and affect several processes (malting, pearling, milling) are grain size and shape, colour, hull, crease, bran, flake formation and hardness [45]. The physical properties of barley grain, like those of other grains and seeds, are essential for the design of equipment and the analysis of the behavior of the product during agricultural process operations such as handling, planting, harvesting, threshing, cleaning, sorting and processing (pearling, malting, milling). Principal axial dimensions of barley grain are useful in selecting sieve separators and in calculating grinding power during size reduction. They can also be used to calculate surface area and volume of grains, which are important during modeling of grain drying, aeration, heating, and cooling [46].

Bulk density (or hectoliter weight), true density, and porosity (the ratio of inter granular space to the total space occupied by the grain) play an important role in many applications such as design of silos and storage bins, transportation separation from undesirable materials, separation and grading as well as maturity evaluation. Flow-ability of barley grain is usually measured using the angle of repose (a measure of the internal friction between grains) that will be useful in hopper design, since the hopper wall's inclination angle should be greater than the angle of repose to ensure the continuous flow of the materials by gravity. The need for knowledge of friction coefficient of agricultural materials on various surfaces has long been recognized by engineers who are concerned with rational design of grain bins, silos and other storage structures [46].

Also, barley grain is an excellent source of soluble and insoluble DF and other bioactive constituents, such as vitamin E (including tocotrienols), B-complex vitamins, minerals, and phenolic compounds [47].

More specifically, barley grain consists of about 65–68% starch, 10–17% protein, 4–9% β-glucan, 2–3% free lipids and 1.5–2.5% minerals. Total dietary fiber content is ranging from 11 to 34% and soluble dietary fiber is varying from 3 to 20%. Hulless or de-hulled barley grain contains 11–20% total dietary fiber, 11–14% insoluble dietary fiber and 3–10% soluble dietary fiber. The pearling procedure is reducing insoluble fiber content, protein, ash and free lipids, but in contrast an increase of starch and β-glucan by the removal of outer layers is observed, including the hull, bran (pericarp, testa) and germ (embryo). The latter are richer in insoluble fiber, protein, ash and lipids and poorer in starch and β-glucan than the endosperm. Protein content plays an important role in foam production (stability in beer) and a minimum of 10-13% is required for brewing Baik and Ullrich [45]. Amylose content is an important chemical characteristic as it is affecting the cooking and the quality of a final barley product [45].

Additionally, barley is a rich source of tocols, including tocopherols and tocotrienols [45] β-glucans and araboxylans (3.9-6.1 %) [47, 48].

Arabinoxylans and mixed linkage (1-3)(1-4)-b-D-glucans, commonly known as β-glucans, are the major non-starch polysaccharides present in various tissues of barley. The β-glucans [linear homopolymers of D-glucopyranosyl (Glcp) residues linked mostly via two or three consecutive β-(1-4) linkages that are separated by a single β-(1-3) linkage] are the major fibre constituents of barley and they have been reported that they are lowering plasma cholesterol, reducing glycaemic index, and reducing risk of colon cancer [45, 47]. Total β-glucans in barley are varying from 4.4 to 8.9 % and as for the water soluble β-glucans, those are ranging from 1.2 to 2.9 %. Arabinoxylans [linear chain backbone of ί-D-xylopyranosyl-(Xylp) residues linked through (1-4) glycosidic linkages] in barley also depends is affected by genetic and environmental factors, but appears to be less variable than that of β-glucans. Total arabinoxylans are varying from 3.98 to 6.05 % and as for the water soluble arabinoxylans, those are varying from 0.4 to 1.0 % [47]. The localization of β-glucans and arabinoxylans in the barley grain and their interaction with other constituents are important from technological perspectives. More specifically, arabinoxylans are affecting cereal based processes (milling, brewing, and breadmaking). Furthermore, arabinoxylans offer nutritional benefits of soluble and insoluble fibre, and, because of the presence of phenolic moieties in their molecular structures, they may also have some antioxidant properties [47].

Tocopherols (T) and tocotrienols (T3) are bioactive phytochemical compounds that have an important role in the food industry as lipid stabilisers and in medicine as suppressors of free radicals, which potentially lead to cancer and aging. More specifically, tocopherols inhibit lipid peroxidation, while tocotrienols exert a hypocholesterolaemic effect by lowering both cholesterol levels in serum through inhibition of cholesterol synthesis and atherogenic lipoprotein levels in plasma, and they also show cancer-suppressive activity by inhibiting tumor cell proliferation [48].

NEW CHALLENGES IN CEREALS BREEDING

Conventional Breeding

Genetic improvement has significant contribution to the increase of barley grain yield per hectare mainly at the second half of 20th century [49, 50] e.g., 33% in United Kingdom 1947-1983, [51], 40% in Norway (1960-1992) [52], 35% in Argentina 1944-1988 [53]. Genetic gain in grain yield was higher for 2-rows varieties compared to 6-rows varieties [54, 55] while other results support that was similar [56].

Breeding programs have followed three main strategies in barley [57]. The first strategy has as selection criterion the grain yield per se. The second focused on eliminating yield defects e.g., improve lodging resistance, tolerance to biotic stresses as a way to increase yield potential. The third strategy used the concept of "ideotype" e.g., in identifying and breeding the traits worthy of selection because it has great influence on grain production [58, 59].

The selection for grain yield or for factors that reduce yield losses (e.g., resistance to lodging) had more significant contribution to the increase of barley yield per hectare compared to the selection for morphological or physiological characteristics [60]. Selection

for spike number per m^2 in Mediterranean result in yield increases since specifies kernel production [61].

While plant breeding has led to the release of high yielding modern varieties in cereals, restricted results were recorded for yield stability [62, 63] and negative response was reffered under stressful environments [64, 65]. There are several differences in yield stability in barley varieties. The variety 'Atlas' was cultivated in whole California and had a good yield performance even in stress environments. Another variety called 'Vaghn' had higher yield compared to the variety 'Atlas' in favorable environments, but in stress environments had unpredictable yield [66]. So far, in Europe [67] and Canada [68, 69], there are few barley varieties with high and stable yield in a wide range of environments. As far as farmers are concerned, they would prefer to cultivate high-yielding varieties which characterized by stability, and probably would "sacrificed' a portion of yield and would choose a variety with high yield stability between years [70].

About the factors that affect the improvement of yield, the genotypes contribute 30-60% to yield increase in United Kingdom (1946-1977), the environment 10-30% and the interaction GxE (25-45%) [71]. Abeledo et al. [72] found significant Genotype x Fertilization (Nitrogen) interaction when he has compared newer and older varieties and also found that the newer varieties showed better response to increased nitrogen levels. The new varieties were developed and released for high inputs fields e.g., fertilizers, water and herbicide. According to Ceccarelli [41] since experiments were conducted in high inputs environments, breeding has systematically missed the chance to exploit genetic differences at low levels of inputs, particularly in the case of fertilizers. These differences do exist according to theory and experimental data, and can be identified if selection is conducted under the target level of input (low) or stress condition [41].

Nowadays, low input crop production systems are of gaining interest in countries with traditionally high input agriculture [73], and according to Ceccarelli [41], plant breeding should release varieties suitable for low input agriculture.

Organic Breeding-New Challenges for High-Yield Producing and Quality Products

Although organic agriculture is increasingly accepted from consumers and producers there is a lot of skepticism that as cultivation system, is less productive efficient compared to conventional agriculture [74]. One of the most important obstacles to the transition from conventional to organic farming in cereal production is the possible reduction in yield [75]. Therefore, an important challenge of organic agriculture is to achieve high yield with excellent quality, using cultivation systems with low inputs, applying sustainable practices and meet the need for sustainable development [76]. Another considerable challenge for breeders is the diversity of agro-ecological target environment and the different cultural practices [77]. Breeding for organic agriculture requires specific strategies that use genetic diversity to support the wide-ranging conditions and farmer practices [12].

Organic agriculture is enhancing specific plant breeding activities to meet its requirements for varieties better adapted to the specific organic environment and low input systems of cultivation [78]. Ceccarelli et al. [79] highlighted the fundamental problem in plant breeding related to the relationship between the selection environment and the target

environment. Generally, direct selection in target environment is always most efficient [80]. In order to identify the best genotypes for organic agriculture, direct selection was more efficient in organic systems than indirect selection in conventional systems [12]. The effect of cultivation system to varieties performance was recorded, as important interactions were found by Legzdina et al. [81] for yield in barley genotypes grown under organic and conventional conditions.

Traditional breeding mainly is based on a combination of a bulk-pedigree method of selection, in combination with applied selection in the presence of stress and use of adapted germplasm [65].The selection of germplasm resources is crucial for successful organic plant breeding. Barley has a broad genetic base and specific agronomic characters (low transpiration rate, allelopathic ability) that establishes it as an adequate crop for this purpose. Barley gene pool has mainly three components: 1. Varieties and breeding lines, 2. Landraces and 3. Barley wild progenitors (*Hordeumvulgare* ssp. *spontaneum*) which would be contributed to drought tolerance of cultivated barley [82]. The possible alternative strategies could consist of the following options:

I. Evaluation of used varieties in conventional agriculture with desirable characteristics. The evaluation of currently grown commercial varieties under organic cultivation will be the first germplasm that could be used by plant breeders. This resource represents the best available gene combination in conventional cultivation [83] and there is a possibility to adapt also in organic cultivation. Current varieties could be used direct in organic cultivation or as a starting material in organic plant breeding programs. Others resources that can be used as starting material are obsolete cultivars and elite breeding lines.
II. Breeding programs starting from landraces where selection under organic cultivation will be held. The replacement of landraces by modern varieties has as consequence a significant loss of genetic variation for resistance to biotic and abiotic stresses [7]. Barley landraces are a mixture of pure lines, selected for many years under low-input fields where nutrient availability is low. Therefore, this option consists a very good genetic resource that possess a combined resistance that modern varieties do not have and may therefore represent a source of variation for selection of varieties adapted to low input cropping systems [7].

The high adaptability of barley and its broad cultivation to different conditions contributed to the release of varieties resistant to abiotic and biotic stresses [84]. Drought is one of the most severe abiotic stresses limiting barley production. Barley is well adapted to arid environments, comparing to other cereals, and the immediate progenitor of cultivated barley *H. vulgare* ssp. *Spontaneum* could be used for the adaptation of cultivated under drought conditions [85]. The study on identified landraces yield under drought stress [86] may be very valuable for combating it. Additionally, it was identified significant variability in barley genetic resources for salinity tolerance [87, 88].

Also, plant breeders could identify tolerance to diseases on available barley genetic resources [89, 90]. In a comparison of wild and cultivated barley genotypes with powdery mildew-infected leaves, the wild barley leaves showed a slower rate of disease-associated senescence [91]. Several landrace accessions appear amongst the accessions screened for disease tolerance in barley [92].

Apart, abiotic and biotic stress tolerance, Bertholdsson [10] identified traits related to the adaptability of spring barley crop (*Hordeumvulgare*). The most important crop traits were early crop biomass, early shoot height, straw length, early heading and maturity. Plant heightis one significant characteristic for organic farming related to competitiveness against weeds. Barley varieties with relative stable plant height had relative high-stable grain yield in Mediterranean conditions [93]. However, grain yield was found to correlate differently to plant height under organic and conventional growing conditions [94].

Additionally, the allelopathic potential of barley increases crop competitiveness. Model predictions suggested that new varieties with increased early vigour and allelopathic activity offer a potential to further reduce weed interference [10]. Barley due to its allelopathic effectiveness has great adaptability and wide use for sustainable agriculture systems aiming to weed management. The variability of this characteristic among different varieties could be an important research area [95].

Criteria for organic plant breeding and propagation strategies include adaptation to soil fertility based on lower and organic inputs, better root system and ability to interact with beneficial soil micro-organisms, weed supressiveness, contributing to soil, crop and seed health, high product quality, high yield level and high yield stability. It differs from the conventional farmers ideotype in priorities set for the different characteristics [39]. According to Lammerts van Bueren [39] organic crop ideotype may benefit not only organic but also conventional farming systems, due to the possible reduce of dependence on external inputs.

APPLICATION OF BARLEY ORGANIC BREEDING IN GREECE

The presented breeding program lasted eighteen years, since 1994, and was held in the Cereal Institute of Thessaloniki, Greece under conditions of organic systems of cultivation and aimed to release new varieties adapted to low input conditions. This program takes place in a nursery field with the whole genetic material from the parents of the crosses, F_1, F_2, F_3, F_4, F_5 and $F_6$where rotation of cereal - legumes was applied. There were also used organic and biodynamic methods [96].

During the experimentation process the following assumptions were made:

I. Crop rotation with family *Fabaceae* species,
II. Avoidance of agrochemicals inputs, i.e., chemical fertilizers, herbicides and pesticides, replacing them by natural compounds,
III. Selection of genotypes in segregating generations on their interactions on different levels of biological pest control, nutrient cycling, water and soil conservation in order to increase output, stability and resource-use efficiency.

The major stages of a common breeding program, i.e., "generation of variability", "selection", and "testing of experimental varieties" differ from the specific organic breeding program in the following points:

- Crosses with adapted varieties or selected lines of adapted populations were took place in the research station.

- The second stage is longer and consists firstly of the evaluation of the breeding value of segregating generations F_2 till F_6. Also the second stage usually takes place on the research station on a secondary research field where organic farming is applied for more than five years. During the second stage the breeding material is exposed to biotic and abiotic stresses on different research stations.
- The end product of the second stage is usually tested in more than three locations under organic conditions. Also these products were evaluated on organic farms with farmers who know the crop, their environment and the interactions between the two well. At this stage participatory plant breeding is applied at a small scale [96].

Stages of organic breeding: 1. Generation of variability. Consists of crossing and producing a number of segregating populations, and selection within the superior populations based on the following selection criteria: Criteria for parents: grain yield, general adaptability, high quality, tolerance to diseases. Criteria F_1: vigor of crosses of variety. *2. Selection.* Evaluation of the breeding value of the different segregating populations. (Methods ear to row in F_3, F_4, and F_5. Selection intensity about 10%. Criteria: Tolerance to biotic and abiotic stresses in organic conditions, homozygosis of lines. *3. Testing of breeding lines.* The breeding material is exposed to relevant biotic and abiotic stresses, in different research station and in farmer's field [96].

The organic matter at the breeding nursery was increased up to 50-100% from the beginning of organic breeding till now, because of the straw and green manure from rotation (Table 1).

Soil samples were taken during years 2006, 2009 and 2012 from both experimental fields with cereal and legumes cultivation and compared with soil samples originated from field were conventional system of cultivation was applied. The parameters measured were: mechanical composition, pH (1:1), $CaCO_3$ (%), organic matter (%), electric conductivity and available phosphorus and potassium. All analyses were made at Soil Science Institute of Thessaloniki, Greece and all the above measurements were realized according to Standard Methods of Soil Science [97]. From this organic breeding program a number of barley varieties were released. The barley varieties released were: 'Thessaloniki' (1999), 'Dimitra' (2000), 'Persefoni' (2000), 'Palaiologos' (2003), 'Byzantio' (2003), 'Konstantinos' (2003), 'Andromeda' (2005), 'Ippolytos' (2005), 'Triptolemos' (2009), 'Makedonia' (2011) and 'Sirios' (2012). All of them are registered in the National and in European Common Catalog of cultivated varieties and are currently cultivated in Greece. The new varieties aim to substitute the commercial varieties in less favourable to marginal environments, in low input cultivation systems and in organic farming, but also in favourable environments due to their productivity that equals the one of commercial varieties.

The results show that the new varieties are productive and stable under the conditions of organic farming. This means that these varieties are very competitive to weeds, diseases, in fields with limited soil fertility, in low-input cultivation systems, as well as in conventional cultivation. An evaluation experiment with conventionally bred varieties and organically bred varieties (Table 2) [98] showed that the latter ones have equal productivity. Besides the organically bred varieties showed greater ability to exploit the input factors.

Table 1. Soil physical and chemical traits of the pedigree fields

Soil samples	Depth cm	Texture class USDA	pH 1:1	$CaC0_3$ %	EC Mmhos/cm	Organic matter %	P Olsen ppm	K exch/ble ppm
C1	0-30	SL	7.85	2.2	0.662	0.96	31.21	640
	30-60	SL	7.98	2.6	1.009			
C2	0-30	SL	8.16	3.1	1.042	1.02	32.25	710
	30-60	SL	7.92	6.6	1.141			
Al	0-30	SL	8.11	6.2	0.698	1.98	60.24	1200
	30-60	SL	8.26	8.4	0.515			
A2	0-30	SL	7.72	1.3	1.200	2.04	46.39	1150
	30-60	SL	7.82	1.3	0.652			
Bl	0-30	SL	8.01	2.6	1.856	1.82	64.32	1320
	30-60	SL	8.05	2.2	0.615			
B2	0-30	SL	8.13	2.2	0.812	1.90	58.19	1100
	30-60	SL	7.88	2.2	0.624			

C1, C2: Controls Al, A2: Field after legumes, Bl, B2: Field after cereals.

Table 2.Comparative trials between conventional and organic varieties in dense stand

	Cultivation system		Cultivation system[1]	
Applied procedures of breeding varieties	Organic	Conventional	Organic	Conventional
Conventional	4944[2]	4967	2301	2795
Organic	5187	5449	2413	2908
L.S.D. $_{.05}$	488		232	

[1]In this trial six conventional and six organic varieties were compared.

[2]Grain yield in kg/ha.

From the above organic breeding program resulted to the release of 11 barely varieties adapted to organic cultivation and low-input farming systems. The above varieties were included to the National and Common Catalogues and 9 of them are already cultivated in Greece and the EU. In case of tocols in barley, research studies revealed that genotype has a significant effect in tocols concentration [48, 99] as well as location [99] and cultivation method [48]. For the genotype and cultivation method effect, a study for tocols concentration in barley revealed an apparent increase in all tocotrienols combined with a decrease in tocopherols under organic cultivation (Table 3). The aforementioned was accompanied with an overall increase in total tocols and a clear increment in the tocotrienol-to-tocopherol ratio (T3/T ratio) (Table 3). The T3/T ratio has been reported as an index of the different distribution of tocols within the kernel [100] as well as an important criterion of nutritional quality and as a quality factor when new varieties/or cultivation techniques are developed and tested [101]. The results showed a higher increase of T3/T ratio in all the organically cultivated varieties when compared to the conventionally cultivated ones [48]. Those results could indicate that the tocols content is a beneficial nutritional factor and the organic cultivation could positively affect their content in barley. The increment in organic cultivation could be explained by two possible hypotheses.

Table 3. Mean values of tocols content in 12 barley genotypes grown under conventional and organic cultivation

Cultivation	Variety	Tocols(mg/kg)									
		α-T3*	(β+γ-)-T3*	δ-T3*	α-T*	(β+γ-)-T*	δ-T*	Total Tocopherol (mg/kg)	Total Tocotrienol (mg/kg)	Total Tocols (mg/kg)	T3/T ratio
Conventional	Persefoni	11.64±0.32	1.66±0.01	0.85±0.03	7.78±0.27	1.05±0.82	0.30±0.01	9.06	14.14	23.20	1.56
	Thermi	13.98±1.05	2.48±0.06	2.23±0.16	8.34±0.23	0.82±0.01	0.30±0.01	9.32	18.68	28.00	2.01
	Thessaloniki	13.26±2.52	2.52±0.06	1.80±0.18	8.52±0.59	1.50±0.08	0.26±0.01	10.27	17.57	27.84	1.71
	Dimitra	15.93±0.13	1.88±0.07	0.91±0.28	8.49±0.12	0.88±0.01	0.25±0.01	9.52	18.71	28.23	1.97
	Athinaida	9.81±0.17	1.96±0.06	0.59±0.04	5.39±0.13	0.59±0.02	0.18±0.00	7.11	12.36	19.47	1.74
	Ippolytos	12.41±0.08	1.80±0.06	0.72±0.02	7.20±0.08	1.06±0.04	0.26±0.01	8.07	14.92	22.99	1.85
	Byzantio	11.46±0.04	2.05±0.01	1.16±0.01	7.07±0.02	1.04±0.01	0.32±0.01	8.42	14.67	23.09	1.74
	Cyprus	16.37±0.02	2.48±0.01	1.36±0.01	9.40±0.01	1.65±0.01	0.35±0.01	11.40	20.20	31.60	1.77
	Kos	13.68±0.04	2.33±0.02	1.18±0.01	7.81±0.01	1.44±0.02	0.29±0.01	9.53	17.18	26.71	1.80
	Palaiologos	14.76±0.18	2.16±0.05	2.42±0.11	6.87±0.12	1.23±0.05	0.39±0.02	8.49	19.34	27.83	2.28
	Konstantinos	13.00±0.23	1.74±0.06	0.91±0.03	7.33±0.23	1.27±0.01	0.22±0.01	8.79	15.65	24.44	1.78
	Niki	13.04±0.49	2.15±0.04	1.72±0.06	6.60±0.27	1.11±0.04	0.25±0.03	7.96	16.91	24.87	2.12
Organic	Persefoni	13.55±2.37	2.34±0.15	1.91±0.11	5.47±0.88	1.01±0.21	0.23±0.08	6.78	17.79	24.57	2.62
	Thermi	15.29±0.61	3.01±0.08	4.69±0.35	7.22±0.51	0.64±0.04	0.16±0.10	8.16	22.99	31.15	2.82
	Thessaloniki	14.61±1.45	2.91±0.08	3.65±0.18	6.02±0.59	0.80±0.04	0.21±0.02	7.02	21.16	28.18	3.01
	Dimitra	16.41±0.52	2.27±0.04	1.38±0.06	5.72±0.60	0.86±0.22	0.16±0.04	7.02	20.05	27.07	2.86
	Athinaida	12.05±0.86	2.55±0.10	1.75±0.16	6.45±0.32	0.80±0.06	0.21±0.02	6.44	16.35	22.79	2.54
	Ippolytos	15.77±0.06	2.46±0.04	1.58±0.02	6.89±0.04	0.78±0.01	0.22±0.01	8.24	19.81	28.05	2.40
	Byzantio	15.68±0.77	2.59±0.07	2.00±0.13	6.42±0.08	0.78±0.06	0.18±0.04	7.38	20.27	27.65	2.75
	Cyprus	18.00±0.03	2.97±0.02	2.90±0.02	5.98±0.02	1.28±0.01	0.26±0.01	7.51	23.86	31.37	3.18
	Kos	14.20±0.01	2.69±0.02	1.86±0.01	5.84±0.02	0.91±0.02	0.21±0.01	6.95	18.75	25.70	2.70
	Palaiologos	17.52±0.51	2.91±0.05	3.16±0.19	5.72±0.29	1.13±0.09	0.30±0.04	7.15	23.60	30.75	3.30
	Konstantinos	14.79±0.35	2.37±0.05	2.14±0.11	6.77±0.41	1.00±0.03	0.22±0.03	7.99	19.29	27.28	2.41
	Niki	13.89±0.39	2.72±0.08	2.79±0.21	6.09±0.25	0.70±0.03	0.15±0.01	6.94	19.40	26.34	2.80

* Average concentration ± S_d(standard deviation).

Table 4. Kernel quality characteristics of the twelve barley varieties grown under conventional and organic cultivation

Variety		Ash (% Dry matter)	1000 Kernel weight	Hectolitre weight	Plumpness >2.5mm	Protein content (%)
Persefoni	Conventional	2.74	43.38	66.36	86.38	12.52
Thermi		2.79	42.5	63.68	76.70	14.28
Thessaloniki		2.59	43.38	62.44	79.91	13.91
Dimitra		2.98	42.75	62.64	72.36	13.32
Athinaida		2.83	44.88	50.56	74.79	14.68
Ippolytos		2.86	33.25	57.82	45.54	14.01
Byzantio		2.66	40.38	59.42	62.34	13.47
Cyprus		2.98	36.13	57.5	41.69	13.83
Kos		2.83	36.13	59.79	57.98	14.05
Palaiologos		3.17	33.5	55.77	40.93	14.2
Konstantinos		2.61	35.25	59.37	51.56	13.91
Niki		2.57	41.63	63.38	80.9	13.67
Persefoni	Organic	1.94	42.88	64.88	86.73	10.87
Thermi		1.83	43.25	68.6	90.38	11.63
Thessaloniki		1.78	43.5	64.51	87.85	12.16
Dimitra		1.77	42.75	64.36	75.51	10.88
Athinaida		1.92	41.88	54.24	66.63	12.43
Ippolytos		1.8	35.13	65.78	58.76	11.53
Byzantio		1.71	40	65.18	70.26	11.1
Cyprus		1.54	37.5	61.74	45.54	11.14
Kos		1.77	34.38	65.38	61.36	11.04
Palaiologos		1.98	38.25	60.44	47.09	11.6
Konstantinos		1.79	34.63	65.78	61.89	11.49
Niki		1.7	44.38	67.96	92.5	11.21

The first hypothesis is attributed to fertilization of the conventional systems, where growth acceleration is succeeded contrary to a decrease in secondary plants metabolites. The second hypothesis refers to the stressful environments in organic cultivations, where the plant devotes greater resources to synthesize their own chemical defence mechanisms [102]. Absence of nitrogen fertilization in organic cultivation clearly decreased protein content in all barley varieties. Additionally the variation of the bran to endosperm ratio, (as expressed by the ash content), between the two cultivation methods was studied. In case of conventional growing conditions, higher ash content was reported [48]. The concluding remarks from the above study are the followings:

1. The implementation of the organic breeding program released 11 barley varieties, during the period 1994-2012, suitable for organic cultivation.
2. Soil fertility was improved after 18 years of the experimentation where the organic cultivation system was applied. The organic matter increased by 80-100% (Table 1) and phosphorus and potassium availability improved.
3. The selected genetic material under organic cultivation showed excellent nutrition value for feed. Additionally, many released barley varieties showed exceptional characteristics for human consumption [48]. Recent studies revealed a positive effect of organic cultivation and breeding in tocols concentration.

CONCLUSION

Until recently, a minority of plant breeder was interested in breeding for organic cultivation. However, the increase of agricultural input prices, the impact of climate change and the need for sustainability are creating a framework where the need for the specific breeding objectives is obvious.

Many of the required characteristics in new varieties are similar to conventional and organic systems of cultivation; however there are several those have a higher priority in organic agriculture. These include traits relevant to the farming system and crop rotation. Also, the early plant vigour shows positive interactions with desirable characters such as weed competitiveness, nutrient uptake and disease and pest resistance.

Barley organic varieties seem to be suitable for cultivation towards a sustainable development based on the aforementioned advantages. Selected barley varieties under organic cultivation showed a positive effect in tocols concentration, something that upgrade its nutritional value for food and feed.

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Chapter 2

BIOLOGICAL CHARACTERISTICS OF SPRING BARLEY VARIETIES IN THE FAR EAST OF RUSSIA

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ABSTRACT

In the first section the history of breeding varieties of spring barley in Primorsky Krai (the Pacific Coast of Russia) is traced. Promising varieties of feed and malting lines are presented. Data on development of the cultivation technology of spring barley in the local environment are demonstrated. The second section describes the main harmful fungal and viral diseases of spring barley on the Pacific Coast of Russia.

Keywords: Spring barley, variety, selection, virus disease, fungal diseases

ABBREVIATIONS

FEB RAS	Far Eastern branch of Russian Academy of Sciences
RAAS	Russian Academy of Agricultural Sciences
ARRIP	All-Russian Research Institute of Plant named after NI Vavilov
BAS	biologically active substances
RPC	Russian Pacific Coast
BSMV	barley stripe mosaic virus
BYDV	barley yellow dwarf
WWMV	winter wheat mosaic virus
CMV	cereal mosaic virus

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OMtV	oat mosaic tenuivirus
NCMV	northern cereal mosaic virus

AGROBIOLOGICAL FEATURES AND TECHNOLOGIES OF CULTIVATION OF SPRING BARLEY IN PRIMORSKY KRAI

Breeding of barley in Primorsky Krai began in 1937. Originally cultivated varieties from other areas (eg, Wiener), and local, obtained by mass selection (Primorsky 20). The vast majority of local barley did not meet the requirements of the production because, being brought by immigrants from different parts of Russia, they had brittle ears and were not resistant to lodging and diseases. After 1940, barley breeding was carried on by V.G. Reifman and A.V. Zaytseva. They selected a sample of barley (k-14163) from the collection of the All-Russian Research Institute of Plant named after NI Vavilov (ARRIP) that was characterized by high productivity and resistance to lodging. Individual selection of the best plants of this sample resulted in a barley variety Ussuriysky 8 regionalized in 1956 (Moiseenko, L.M., Klykov, A.G. and Timoshinov, R.V., 2008; Klykov, A.G., et al., 2010).

In the following years selection of this culture was minimized, most attention being paid to seed farming of the regionalized variety Ussuriyski 8. Full-scale spring barley breeding was resumed in 1967 by Smirnova Z.G. While creating new hybrid material she used hybridization with broad involvement of the world collection of samples of different ecological and geographical origin from the collection of ARRIP. By crossing the Dutch variety (k 19660) with Ussuriysky 8, a cultivar Primorsky 89 (brewing line) was got and then regionalized in 1983. Later the method of individual selection of the hybrid combination Birgitta (Sweden) x Krasnodarsky 35 was used to obtain the cultivar Primorsky 44 (feed line).

Starting from 1996 the work was continued by Semernina V.J., who studied samples of winter and spring barley to determine the feasibility of using them for hybridization in Primorsky Krai. She estimated the studied varieties based on their major economically valuable features and sorted out valuable sources. The results of the study demonstrated that varieties of winter barley in Primorsky Krai have a very low yield, its main reason being their strongly developed laxial ear (Moiseenko, L.M., Klykov, A.G. and Timoshinov, R.V. 2008; Klykov, A.G., et al., 2010).

The study of spring barley varieties from the collection nursery identified the most valuable cultivars with various selection and economic features. These features and cultivars are as follows: productive tillering – in Priazovsky 9, Nutans 642, Musson, Aga, Sobolek (Russia), Berezinsky (Belarus), Dera, Salome (Germany) and MV-50 (Hungary); the number of grains per ear - in Narymansky 6, Musson, Volgar (Russia), Zazersky 85, Visit (Belarus), St-126 (Czechoslovakia), Uta Brobsdorfer (Austria) and Ellice (Canada); ear productivity - in Murash (Russia), Sobolek, Rosava (Ukraine), Glenn (the USA) and Dorset (Germany); productivity and yield in Priazovskiy 9, Sobolek (Russia) and Berezinsky (Belarus). A group of cultivars was shown to have a complex of economically valuable traits (Klykov, A.G., et al., 2010). They are: Aga, Sobolek, Priazovsky 9, Berezinsky, MV-50 and Dera. The cultivars Odessky 100 (Ukraine), Orest (the USA), Dorina (Germany) and Samson (Canada) were characterized by high productivity (Figure 1).

The highest productivity of the plants was observed in hybrids involving the following varieties: Primorsky 44 x Orest (11.0 g) and Primorskiy 44 x Dorina (11,8 g). In the Russian Far East common are the following cultivars of spring barley: Pervenets, Aga and Andrew in Amur Oblast, Yerofei and Musson in Khabarovsk Krai, and Primorsky 89, Primorsky 44 and Primorsky 98 (below) in Primorsky Krai.

Figure 1. The barley cultivars competitive test nursery.

Characteristics of Spring Barley Varieties Selected in the Primorskii Agricultural Research Institute (Catalogue of cultivars, 2005)

Primorsky 89. (Figure 2). The cultivar was bred in the Primorskii Agricultural Research Institute by crossing varieties k-19660 (the Netherlands) x Ussuriysky 8 followed by individual selection.

Variety nutans. The ear is yellow, fusiform, slightly tapering towards the top, of medium density (for 4 cm of spike rod there are 10-11 spikes). The ear length is 7-12 cm; the ear is slightly brittle, medium drooping. Aristae are straw-yellow, equal to the length of the ear, slightly divergent, moderately rough in dry years and gentle and slightly jagged in humid years. The grain is yellow, elliptical, of high brewing qualities. 1000 grain weight is 38-44 g. The cultivar with slight seed shedding, slightly scarious (8-9%). Glumes narrow, smooth; palea thin and finely wrinkled. Transition of palea into spine is gradual. The main grain stubble is long-hairy. The stalk of medium height (80-90 cm), medium decumbent in humid years and highly stable in ordinary ones.

The barley variety Primorsky 89 is mid-season, with the growing season of 75-80 days. Resistant to root rot, moderately susceptible to the mesh and striped Blight, *Fusarium* head blight, susceptible to loose smut. The average yield is 3.5 MT / ha, the potential up to 6.0 MT / ha. Since 1983 is included in the State Register for the Far East region.

Primorsky 44. (Figure 3). The cultivar was bred in the Primorskii Agricultural Research Institute by intervarietal hybridization (Birgitta (Sweden) x Krasnodar 35) followed by individual selection.

Variety *nutans*. The double row spike is of medium density and length (7-10 cm), yellow, erect or slightly drooping, with soft spine, slightly serrated, spikes are weakly or moderately divergent, in the phase of full earing are gray-purple. Floral scales enters spine gradually. The grain is yellow with a faint greenish tint, large and lined; its shape is closer to rhombic, slightly rugose, fineness above average and high. 1000 grain weight is 42-50 g, slightly scarious (8-10%). The main grain stubble is long-hairy.

Figure 2. The cultivar Primorskii-44.

Figure 3. The cultivar Primorskii-89.

The plant height is of 75-85 cm. In contrast to Primorsky 89, this cultivar tillering is less (average tillering is from 2 to 4 productive stems), the ear hardly wilts, is upright; starting from the phase of tillering the plants are bright green, while those of Primorsky 89 are dark green. The cultivar is middle-grade, with 73-80 days of the growing season, of feed line; the content of protein in grain is 11.5-13.5% and that of starch is 60%. Resistant to lodging. Against the natural background the cultivar is resistant to root rot, moderately susceptible to stripe blight and *Fusarium* head blight, susceptible to loose smut. The average yield is 35-40 MT / ha, the potential up to 6.0 MT / ha. Since 1990 this cultivar is regionalized for the Russian Far East.

Primorsky 98. (Figure 4). Cultivar was bred in the Primorskii Agricultural Research Institute by intercultivar hybridization (k-19362 Sumerimoti, Japan x Primorsky 3474) x (K-2938 Shikokunadaka № 1, the Japan x Primorsky 3541), followed by individual selection.

Figure 4. The cultivar Primorskii-98

Variety *submedicum*. The ear is of medium length (8-10 cm), cylindrical, soft, non-friable, with a strong waxy coating. Awns are of ear length, located along the ear, persistent. The grain is elliptical, large, light yellow. 1 000 grain weight is 40.6-53.1 g. The aleurone layer is white. Palea slightly rugose. There are no teeth on nerves palea. Palea transition into is awn gradual. The bristle at the base of the grain is long-hairy. The ventral furrow is light yellow, glabrous.

The plant height is 59-93 cm. The culm is quite thick, the lower internodes are shortened. The bush is of semi-erect type. The cultivar is characterized by good tillering (up to 4 productive stems per plant), is highly resistant to drought, especially in the first half of the growing season, and resistant to lodging.

The cultivar is mid-season, with the growing period of 72-78 days. In the field is moderately resistant to the mesh and striped blights, moderately susceptible to loose smut. As compared with Primorsky 89, this cultivar is more resistant to germination of seeds in the ear in humid years.

Crude protein content in the grain, on average, is 9.9 % that of starch is 60%, the extract content is 81.1%, volume weight is 678 g, slightly scarious – 11%. The average yield is 4.0 MT / ha, potentially up to 6.5 MT / ha. Since 2004 the cultivar is included in the State Register of the Far East Region. (Klykov, A.G. et al., 2004).

Tikhookeansky. (Figure 5). The cultivar was created by hybridization followed by individual selection from a complex hybrid combination: Chernigovsky 90 x (Ussuriysky 8 x Union) x Tredi. This is a cultivar of spring barley of brewing line.

Figure 5. The perspective cultivar Tikhookeansky (brewery direction).

Variety *nutans*. The spike is dense, yellow, bearded, serrated spine, equal to the length of the ear, pressed to the ear. The grain is light yellow, elliptical. The plant height is 50-62 cm; productive tillering is 3.4 stems. Palea transition into awn is gradual; there is no nervation of lemmas, no teeth on palea nerves.

Mid-seasonal, with the vegetation period of 73-77 days. The average productivity in the competitive variety trials in the years of studies was 4.6 MT / ha, the increase was 0.8 MT / ha. On average, the content of protein in grain is 9.8% that of starch is 61.3%. 1000 grain

weight is 37.9 g, the extract content is 81%, volume weight is 655 g, slightly scarious – 7.5%. Resistant to lodging. On the natural background this cultivar slightly affected by mesh and striped blight, moderately susceptible to loose smut. Yields in the farming environment averaged to 3.1 MT / ha. (Moiseenko, L.M. et al., 2011).

Vostochny. (Figure 6). The cultivar was created by hybridization (Primorsky 6216 x Erofei) followed by individual selection. The spring barley variety of feed line.

Figure 6. Vostochny - a new feed barley cultivar.

Variety *nutans*. The ear is loose, yellow, bearded, rough awns strongly serrated, equal to the length of the ear, slightly divergent. The grain pale yellow, large, elliptical. Productive tillering averages to 2-3 stems. Palea transition into awn is wide there is no nervation of lemmas, no teeth on palea nerves.

The plant height is 76-96 cm. The cultivar is mid-seasonal, its vegetation period is 73-79 days. The average productivity in the competitive variety trials in 2006-2008 was 4.7 MT / ha, the increase in comparison to the standard was 0.4 MT / ha. The average content of protein in the grain is 10.9%, of starch is 60%. 1000 grain weight is 46.5 g, 645 g of grain nature, slightly scarious – 8.8%. Resistant to lodging and shattering. On the natural background slightly sensitive to the mesh and striped blight, moderately susceptible to loose smut. Yields in the farming environment on average was 3.0 MT / ha (Moiseenko, L.M. et al., 2011).

For the cultivar to realize its potential it is necessary to sow seeds of high quality reproductions and apply cultivation technologies appropriate to the area.

The Primorskii Agricultural Research Institute has developed a technology of cultivation of spring barley for the conditions of Primorsky Krai. According to Moiseenko, A.A. (1999) the effect of crop rotation on the yield of barley is 12.2-15.0%, which is similar to that of organic fertilizers together with mineral ones. In Primorsky Krai the best precursors for spring barley are perennial herbs, green manure seeded fallow, row crops, soybeans and buckwheat. Of technological processes the most essential is tillage. In most areas the best results are provide by early autumn plowing.

It has been found that replacement of moldboard plowing (in autumn and spring) for the plane carved processing of different depths in the fields after soybeans does not affect the yield of barley. Moreover, the plane carving processing in favorable years will provide higher yield than deep tillage but promotes the growth of weed infestation by 15-28% (Moiseenko, A.A., 1999).

The traditional system of soil cultivation based on deep moldboard plowing increases spraying of soil aggregates, reduces the content of organic matter in the soil by increasing its salinity, excessive compaction of the underlying soil and loosening of its cultivated layer, increases the evaporation of accumulated moisture and erosion. These reasons make it necessary to differentiate soil cultivation taking into account plant characteristics, cost-effective reduction of energy consumption, and reduction of its erosion.

Barley demonstrates good reaction to complete mineral fertilizer; the best soils for it are fertile and non-acidic (pH 6.8-7.5). Acidic soils should be limed before sowing. According to the agrochemical station of the Far East Plant Protection Research Institute, long application of mineral fertilizers without liming leads to acidification of the soil: pHsol was 4.6, and with liming it was 5.5. Liming increases the amount of phosphorus digestible for plants 1.5-2.0 times, as well as the mobility of molybdenum, but reduces the amount of boron, potassium, copper and zinc. Due to this fact, the need for boron and potassium fertilizers increased. The need for liming is determined by pH of saline suspension and the dose of $CaCO_3$ lime by the largest acidity. First, it is necessary to calcify highly acidic soils (pHsol less than 4.5) and then soils with medium acidic level (pHsol 4.6-5.0). The optimal dose is calculated as half of the acidity value. Liming is repeated in 5-6 years.

In Primorsky Krai, the recommended doses for barley re as follows: ammonium nitrate – 45, superphosphate – 60 and potassium chloride – 45 kg of active ingredient per 1 hectare. Phosphate and potash fertilizers are used during basic soil cultivation or during pre-cultivation. The root system of barley uses relatively little phosphorus and potassium from the soil. At the same time, phosphorus and potassium are extremely important, especially in the cultivation of malting barley. Phosphate and potash fertilizers increase the yield, and they can be applied during autumn plowing or pre-sowing cultivation, nitrogen fertilizers can be applied during pre-sowing cultivation.

For sod-podzolic soils micronutrients are applied when the boron content is less than 0.3 mg that of copper is 1.5, of manganese 30 and of zinc 0.7 mg per 1 kg of soil. The need of plants for boron increases on limed soils, for molybdenum on acidic soils (pH below 5.2), for copper on peat, and for zinc on soils with a high content of phosphorus. For non-root fertilizing and seed treatment boric acid, copper sulfate, zinc and manganese are used. For seed treatment of 100 kg of grain it is necessary to use 10 g of boron, 30 g of copper, 18 g of manganese and 12 g of zinc. When barley is cultivated for brewing the nitrogen dose is reduced. It should not be more than 60 kg / ha in the case of predecessors of the continuous planting, and than 30 kg / ha after row crops where organic fertilizers were applied. Excessive

use of nitrogen fertilizer for barley, especially on soils poor in phosphorus causes delay in ripening and lodging of plants, which reduces yields.

Due to the high injuriousness of diseases of seeds sown in cold soil and propagation of leaf and seed infections during the growing season, barley cultivation technology stipulates seed treatment using system chemical and biological agents in combination with plant growth regulators. Before sowing it is necessary to make air-thermal and solar heating of seeds. An important addition to the chemical protection is increasing self-defense in plants by the use of small and miniscule doses of biologically active substances (BAS). Their use in crops helps reduce disease development or enhancement of fungicidal activity of chemical pesticides, which can reduce the dose in chemical treatments. To date, there appears a range of BAS authorized for use in the domestic crop sector as inducers of stability (trade names "Narcissus" “Fitohit” “Immunotsitofit”, etc.). New substances are being developed at the Pacific Institute of Bioorganic Chemistry, FEB RAS (Vladivostok). Their scientific and industrial tests are conducted at research institutions of the Far Eastern Scientific Center of Russian Academy of Agricultural Sciences. Using a new generation of biologically active substances that affect plants in super small doses (several milligrams per 1 ha of crops), is of great ecological importance. Researchers of the Far East Plant Protection Research Institute have found that the use of “Immunotsitofit” and "Narcissus" for pre-sowing seed treatment and for barley plants spraying can increase the yield by 15-30%.

The reaction of barley to changes in planting dates is not always the same and depends on the availability of soil moisture and temperature conditions during seed germination. It is necessary to sown barley as early as possible, which results in better developed root system, better tillering, plants are less susceptible to spring drought and less affected by diseases. Besides, when sown early, barley ripens earlier, and it can be harvested prior to monsoon rains. Lack of moisture and high temperatures during sowing – sprouting period accelerate phases of development and lower productivity. Moderate temperatures and normal soil moisture during this period, on the contrary, contribute to the formation of a sufficiently high yield even at later time of sowing. It was noted that in some years when sowing was made in mid-May the harvest was similar to that of April planting dates. Creating optimal conditions for the assimilation apparatus of plants is one of the most important conditions for the formation of the crop. It is believed that the realization of the potential of plants is feasible when the leaf surface is more than 40-50 thousand square meters.

The recommended seeding rate for recognized varieties of barley is 5.0-5.5 million germinating seeds per 1 hectare. Seeding depth primarily affects the field germination and productive tillering of barley. The reason for sparse crops is deep embedding of seeds during sowing. Seeds sown at a depth of more than 7 cm hardly give any crops. Adjustment of the depth of sowing in seeding machines should be based on soil texture and moisture, uniformity of field microrelief, and topsoil looseness. Optimal depths for germination are 4-5 cm in heavy and 5-7 cm in light soils and ridges. The recommended method is drill sowing with row-spacing of 15 cm. Effective in Primorsky Krai is presowing packing and harrowing. In the phase of full shoots of barley, when annual weeds are in the stage of "white thread", light harrows are applied. Barley crops are harrowed crosswise or diagonally.

In the Russian Far East, abundant heat and moisture, especially in the second half of the summer, promote intensive growth of weeds. Control of them is extremely difficult because of a great variety of biological weed groups that fundamentally differ in their life spans, reproduction ability, and in time of germination. Weed spreading in the fields is caused by

huge amount of seeds and organs of vegetative propagation of weeds, along with neglect of crop rotation and alternation of herbicides. Monitoring of crops revealed that about 50% of surveyed fields in the Russian Far East have a strong and a very strong degree of infestation.

In Primorsky Krai the following weeds are common in grain crops: ragweed, yellow and pink sow thistle, mugwort, barnyard grass, and couch grass. The Far East Plant Protection Research Institute has developed a technology of chemical protection of crops against one of quarantine weeds, ragweed. In the Russian Far East, harvesting often takes place under unfavorable weather conditions, so it is important to gather barley before monsoon rains. Among the early grain barley is the first to ripen.

Especially large grain losses can arise from incorrect choice of technology and machinery for the conditions of harvesting. There are two main methods of harvesting: a single-phase method (direct combine harvesting), when thrashing immediately follows mowing, and a two-phase method (split combine harvesting). Both methods should be used to complement each other. The second method allows harvesting to begin 5-7 days earlier and the main part of the grain to be harvested up to August, 20, the beginning of the period of unfavorable weather conditions.

FUNGAL DISEASES OF BARLEY ON THE RUSSIAN PACIFIC COAST (RPC)

The main work on identification of fungal diseases of barley and other cereals in the Far East of Russia was done in the 1960s-1980s. The review of the results of these studies is given by a team of authors in the book "Pathogens of crops in the Far East" (1980). The most dangerous diseases of cereal crops on the RPC are caused by rust fungi *Puccinia*, but they cause less damage in barley. Most harmful for this crop are dwarf rust of barley, linear or stem rust of cereals and brown leaf rust of cereals.

Dwarf rust of barley caused by *P.hordei* Otth is most harmful in the northern areas of the RPC (Amur Oblast and Khabarovsk Krai) because it is sensitive to the spring drought characteristic of the south of the region. The pathogen is a full-cycle one and has different hosts, but in the Far East of Russia its intermediate hosts *Ornithogalum* spp.and *Dipcadi* spp. do not occur, so the fungus develops only part of the cycle (Azbukina, Z.M. et al., 1980). Linear or stem rust of cereals caused by *P. graminis* Pers. affects leaves, leaf sheaths, glumes and floral scales, and spine. The fungus is a full-cycle different-host one (its intermediate host being *Berberis amurensis* Rupr.). In the areas where barberry does not grow of great importance are wild cereals: *Hordeum jubatum* L., *Eletrigia repens* (L.) Nevski, *Elymus dahurius*Turcz., *E.sibiricus* L. (Azbukina, Z.M. et al., 1980).

Brown leaf rust of cereals caused by *P.persistens* Plowr. also affects the leaves reducing the assimilative surface and decreasing the efficiency of physiological processes. This is a full-cycle different-hosts fungus, but the intermediate host is not found in the area under study, so in this case wild grasses are also important in the spread of the pathogen (Azbukina, et al., 1980).

Among smut fungi there are three specific pathogens affecting barley on the RPC (Govorova, O.K., 1990).

First, this is barley loose smut *Ustilago nuda* (Jens.) Rostr. If it affects the plant, all the parts of the ear, except for the main stem, turn into a mass of spores. Loose smut of barley is spread throughout the RPC and is very harmful. In addition to obvious losses in the form of infected seeds it causes significant damage to crops. In some years, the harvest was reduced by 8%. Infection occurs during flowering. Spores fall to the pestle and germinate there (Govorova, O.K., 1990).

Another disease is a solid stone or smut of barley caused by the fungus *Ustilago hordei* (Pers.) Lag. It is also widespread in the region, though not as much as loose smut (1-2%). The fungus destroys the ovary, turning it into a tight dark ball covered with thin dilapidated glumes. Infection occurs at the stage of seedlings. The fungus develops on many kinds of wild grasses, thus persisting in nature (Govorova, O.K., 1990).

Black loose smut caused by the fungus *U.nigra* Tapke is rarer, but according to some authors the damage may reach 6-7%.

Most harmful, especially under the conditions of low and unstable moisture that are characteristic of the RPC, is root rot of cereals (it is often a complex of fungi *Bipolaris sorokiniana* (Sacc.) Schoem., *Fusarium* spp). In some years in the spring drought the level of death of barley seedlings may be as high as 15-20% and accompanied by a significant decrease in overall productivity through the root system damage. In addition to the underground organs *B.sorokiniana* lesions can develop on all above-ground organs, first causing small, 1-2 mm in diameter, slightly elongated brown spots that merge later in ocher spots up to 1 cm or more in length. Most dangerous is the spikelet form when the affected ears are covered with black velvet coating of spores ("black germ") (Azbukina, Z.M. et al.).

The most common is striped leaf blight of barley caused by the fungus *Drechslera graminea* (Rab.) Ito (*Helminthosporium gramineum* Rab.). The disease appears on shoots in early spring. First, light green spots gradually lengthening appear. Then, the bands darken, the leaf tissue dies, and ruptures develop. Primary infection is caused by either overwintered conidia or mycelium infected kernels. Secondary infection occurs during the propagation of conidia from infected barley plants. Thus, the main sources of the fungus are infected seeds with the inner infection or those with the outside infection by spores, and affected plant residues (Azbukina, Z.M. et al., 1980).

Another disease is net blotch of barley caused by the fungus *Drechslera teres* Ito (*Helminthosporium teres* Sacc.). The primary symptoms of the disease develop at the first embryonic leaf in the form of oval brown spots with a yellow ring. Spots gradually expand and form characteristic mesh pattern on the leaf surface. In humid conditions a plaque of spores develops on the stain surface. The fungus survives in crop residues as mycelium and can overwinter in the seed and on their surface. The optimum temperature is 22-24°C. It spreads in the same way as D. graminea (Rab.) Ito. , but unlike it, this pathogen is more sensitive to heat and humidity (Malakhova, N.M. and Mylnikov, N.M., 2003). In humid years barley is significantly infected by bordered spot caused by *Rhynchosporium graminicola* Heins. The fungus is highly specialized and almost exclusively affects barley and, occasionally, rye. The symptoms are typical: elongated, oval or irregular diamond-shaped blue-green spots on the leaves and sheaths. In the case of strong development of the disease the spots cover most parts of the leaves that dry up prematurely, thus reducing the assimilative surface.

A very common disease on barley is Fusarium caused by the fungus *Fusarium graminearum* Schw. *Fusarium* on the barley is highly severe, not only because it leads to a

significant reduction in yield (especially in the case of early infection) but also because of the appearance of the phenomenon called "drunk bread" in food and fodder grain. In some years the prevalence of Fusarium, especially in the southern regions, increases dramatically. This is due to climatic conditions (Azbukina, Z.M, et al., 1980). Symptoms of the disease appear during the ear ripening. They are early yellowing of the whole ear or of some individual spikelets. The mycelium affects straw immediately near the ear causing browning and, apparently, blockage of the vascular system, which prevents grains from full forming and ripening.

VIRAL DISEASES AFFECTING THE BARLEY ON RPC

Active study of viruses cereals was conducted in 70-80 years.

In general, on the Pacific coast of Russia (Kamchatka Krai, Khabarovsk Krai, Primorsky Krai, Amur Oblast, Sakhalin Oblast and Magadan Oblast) several viruses have been revealed that affect cereals, including barley (Gnutova, R.V. et al., 1995).

Then the interest in viral diseases declined. The reason is that the winter cereal crops in these regions is almost grown, spring cereals grown only feed direction, and increased focus on soybean production. Currently, began to restore the rice cultivation, cultivation of barley with good brewing qualities. There was a need to study viral diseases. Diagnostic antisera were obtained in a number of viruses and with their help we tested the degree of infection of the cereal crops (Sibiryakova, I.I. et al., 1994). To obtain the most effective diagnostic tools were used antigen- active strains and well-proven methods of injection of the antigens to immunized animals (Kakareka, et al., 2013).

The thirteen-year monitoring of viral diseases in cereal collections and nurseries of the Primorskii Agricultural Research Institute and the first survey of cereal crops in Primorsky Krai conducted in 2000 gave reasons to believe that phytovirology situation in this region is relatively favorable. Overall prevalence of cereal viral diseases in different geographical areas of the Russian Far East may differ significantly. Thus, in the steppe zone of Primorsky Krai the rate of diseases is slightly higher (0.37 - 0.42%) than in the forest-steppe zone or in the southern taiga (0.15 - 0.25%) (Dyakonov, K.P. and Sapotsky M.V., 2004). The most widespread disease in this crop is stripe mosaic. The first description of the disease in the Russian Far East dates back to 1966. The disease is most common in Primorsky Krai. The symptoms that are similar to those caused by blight are as follows: alternating light green stripes and strokes from the base and the top sheet. The pathogen of the disease is a virus of the genus Hordeivirus, with rod-shaped virions of 130x30 nm. The properties of the virus, which is a typical representative of the genus, are well documented (Atabekov J.G. and Novikov V.K., 1971). Barley stripe mosaic virus (BSMV) is quite contagious and easily transmitted mechanically. BSMV is characterized by a very high level of vertical transmission, by seeds (up to 90-100% for some barley cultivars). This peculiar biology of the virus along with the presence of the host plants from the wild species, especially weeds such as *Setaria viridis* and *Avena fatua*, that long remain viable, allows BSMV to survive in agricultural and biological communities for a long time.

The affection degree is 0.36-0.54% for barley field crops and up to 8.3% for plot crops. The share of stripe mosaic among other viruses is up to 45%. The disease level goes up and

down through years and depends on barley cultivars and growing conditions. Drought, lingering cloudy weather and other similar factors increase the level of the disease. If barley stripe mosaic virus is transmitted artificially, the amount transmission varies from 8 to 86% depending on cultivars.

The infection is caused by affected seeds. Barley shoots show 0.08 - 0.1% of infected plants. The disease can be transmitted from the initial nidus of infection to healthy plants mechanically. It has been proved experimentally that heavy rain together with strong wind could result in infecting up to 16% of healthy neighbors (Dyakonov, K.P. and Sapotsky, M.V., 2004).

Since in the seeds the virus remains active for several years usually, in the collection nurseries there usually is a mixture of many strains introduced from different regions. The collections of varieties of the RPC are no exception. At present active work to systematize the BSMV strains is in progress.

Another important virus detected on barley and other cereals on the RPC described by Mamaev and others is the cereal mosaic virus (CMV) (Mamaev, P. Y., 1998; Sapotsky, M.V. and Kakareka, N.N., 2004; Kostin, V.D., Mamaev, P.Y. and Volkov, Y.G., 1989). External symptoms on barley are dwarf, increased tillering, and mosaic on leaves and sheaths. Most widespread the virus is on oats in Amur Oblast. The pathogen is transmitted by dark leafhopper *Laodelfax striatellus* Fall. Transovarial transmission has not been revealed (Mamaev, P. Y., 1998). The level of infection of barley is usually negligible. This is due to the fact that barley is less populated by leafhoppers. Previously it was thought that the disease is caused by oat pseudorozette virus, of the genus Phytorhabdovirus (Razvyazkina, G.M 1975). Further investigations showed that this is a different virus from the same genus (Minskaya, L.A. et al., 1987; Mamaev, P. Y., 1998). Bacilliform virus particles measure 420x67 nm (in oat pseudorozette virus they are 160x60 nm). Besides, in leafhoppers cereal mosaic virus is not transmitted transovarially, but pseudorozette oat virus is transmitted in this way. More detailed studies have identified a strain of the northern cereal mosaic (NCMV) (Sapotsky, M.V. and Artyukova, E.V., 2002). Mamaev has shown that the boundary of the virus spread is the basin of the Ingoda River (Transbaikalia). On the other side of the mountains another phytorhabdovirus circulats, namely, oat pseudorozette virus (Mamaev, P. Y., 1998; Razvyazkina G.M., 1975). Northern mosaic virus is more common in Amur Oblast; it is transmitted by dark leafhoppers *Laodelphax striatellus* Fall. Its population in Primorsky Krai is negligible because lack of winter crops in the territory breaks down year-round feeding of its individuals.

In the barley plants with symptoms similar to stripe mosaic but without necrotization Bromus mosaic virus of the genus Bromovirus has been identified (Volkov, Y.G., Sybiryakova, I.I. and Gnutova R.V., 1998.). This is isometric virus with a diameter of 28-30 nm, easily transmitted mechanically as well as by adults and larvae of *Oulema melanopus* L., large grain flea *Chaetocema aridula*, and the striped bread flea *Phylotreta vittula*. The level of infection of barley is usually small (less than 0.1%), but according to Dyakonov, K.P. and Sapotsky, M.V. (2004) in some years infestation may be as high as 14%.

Recently, barley yellow dwarf (BYDV) has been identified on the RPC. Earlier to Dyakonov, K.P. and Sapotsky, M.V. (2004) suggested that there is a possibility of the spread of the pathogen as carriers of the virus, the aphid of genus *Macrosiphum*, colonize barley and other cereals. Using the ELISA the virus has been detected in plants with yellow spot (Volkov Y.G. et al., 2011). This dangerous pathogen is transmitted by aphids, namely, by *Sitobion*

avenae F., *Schizaphis gramina* Rond., and *Rhopalosiphum padi* L. All the three species are common in the south of the region, which creates more opportunities for re-infection of cultivated cereals (Dyakonov, K.P. et al., 2005; Volkov, Y.G., Kakareka N.N. and Dyakonov, K.P., 2010).

It has been found that in Primorsky Krai and in the region of the middle- and downstream of the Amur River (Priamurye) the optimal conditions for the existence of the above-mentioned insects are: air temperature between 20-25ºC, its relative humidity of 55-75%, wind speed up to 2 m/s, and increased cloudiness (Dyakonov, K.P. and Sapotsky M.V., 2004).

The development of symptoms is as follows. At the early stage of infection the edges of the barley leaves turn yellow. Plants are stunted. The affected leaves are yellow-golden in color, more rigid and erect. The infection rate is low, which suggests that the virus has been introduced only recently. However, in 2004 oat crops in the central districts of Primorsky Krai were affected by BYDV by 12% (Dyakonov, K.P. et al., 2005). Introduction of winter crop forms is likely to greatly enhance the degree of damage of cereal crops by this pathogen. The pathogen is an isometric virus with a diameter of 30 nm and it is a member of the genus Luteovirus. Its vectors are aphids of several species; the virus is not transmitted mechanically (Brunt, A.A. et al., 1996).

One more virus detected in small amounts on barley is wheat streak mosaic virus, a member of the genus Tritimovirus. The pathogen is spread by the mite *Aceria tulipae* Schev. Several plants infected by the virus were found during the immunochemical examination of a collection nursery near wheat plots where the pathogen was detected in larger amounts (1-1.5%). The virus is well-known and well-documented (French, R. and Stenger, D.C., 2002).

In Primorsky Krai Sapotsky, M.V. identified a pathogen of the genus Tenuivirus, first in oat and then in barley plants with symptoms of bushy dwarf. The author called it oat (Russian) mosaic tenuivirus (Sapotsky, M.V. and Kakareka, N.N., 2004). Apparently, it is a mixed infection with CMV (see above). At the same time, plants with these symptoms from Amur Oblast did not have this pathogen.

Although direct comparison of oat mosaic and winter wheat mosaic tenuiviruses has not been made, we have no reasons to suppose that these viruses are identical, because WWMV is transmitted exclusively by vector *Psammotettix striatus* L. (family *Cicadellidae*) (Razviazkina, G.M., 1975), but oat mosaic tenuivirus is transmitted by vector *Laodelphax striatellus* Fallen (family *Delphacidae*). The circulation of the WWMV infection in agrobiocenoses is closely connected with winter cereal crops, whereas in the south districts of Prymorsky Krai, where OMtV was detected, only spring crops are planted. It is reasonable to suppose that OMtV is close to rice stripe tenuivirus (RSV), because they have similar structure and the same vector, and because RSV was detected on rice crops in this region earlier.

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Chapter 3

CONTRIBUTION OF BARLEY IN THE BOVINE METHANE PRODUCTION IN FEEDLOTS. WHAT IS CONSIDERED TO BE TRUE AND WHAT IS A MYTH?

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ABSTRACT

The world population of ruminants contributes approximately 12-15% of the total atmospheric methane emission (Moss et al., 1995, 2000). Methane is one of the most important greenhouse gases, second position going to carbon dioxide in its contribution to global warming, and is considered to be one of the eminent greenhouse gases needing to be controlled.

However, barley grain is one of the energetically more efficient materials in producing feedlots. It is well-known that barley-based diets have resulted in the carcasses with firmer, and with whiter fat than the carcasses of animal fed with corn-based diets, although theses diets give a higher risk of ruminal disturbances compared to those with other cereals (Castillo et al., 2012).

It is clear that we are facing two opposite aspects: the economic benefits required in intensive farms and its balance to the environmental effect. Studies on barley based diets about the production of methane came from several decades ago. One of the first reports (Moe and Tyrrell, 1979) concluded that the methane production by adult cattle could be predicted adequately from dry matter or totally digestible carbohydrate, and other recent studies (Murray et al., 2007) focus on grazing ruminants, considering interactions between soil, plants and animals.

The main purpose of our study is to focus on barley and its connection with methane emission in feedlots; for the purpose of reviewing our experience in relation to the effect of barley on animal health and the results of animal performance, which will influence the feedlots on the emission of green-house gases that were mentioned previously, and finally we will present alternative methods to control or minimize emission of methane.

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Thus, our contribution will be divided into three different parts:

1. Why has barley been the favorite cereal in bovine feedlots? Review was made on the effects on bovine health, and the production and quality of the meat.
2. We review why barley contributes to the largest emission of methane compared to other cereals. Description of how feedlots contribute to the emission of greenhouse gases is included.
3. We review controlling strategies for the emission of methane from barley given as diets. The results achieved to date were discussed.

Keywords: Barley; feedlot; methanogenesis; greenhouse effect

ABBREVIATIONS

ADF	Acid Detergent Fiber
ADG	Average Daily Gain
BW	Body Weight
CCC	Committee of Climate Change
CH4	Methane
CO2	Carbon dioxide
CP	Crude Protein
DE	Digestible Energy
DM	Dry Matter
DMI	Dry Matter Intake
EU	European Union
F/G ratio	Feed DMI:gain ratio
FA	Fatty Acid
FAO	Food and Agricultural Organisation
GE	Gross Energy
IPCC	Intergovernmental Panel of Climate Change
IR	Infrared Radiation
MUFA	Monounsatured Fatty Acids
NDF	Neutral Detergent Fiber
NEFA	Non Esterified Fatty Acids
NRC	National Research Council
NS	No Significant
OECD	Organisation for Economic Co-operation and Development
OGTR	Office of the Gene Technology Regulation
OM	Organic Matter; PDL: Plant Derived Liquid
PUFA	Polyunsatured Fatty Acids
RDR	Rapidly Degraded Protein
SDR	Slowly Degraded Protein
SFA	Saturated Fatty Acids
VFA	Volatile Fatty Acids
YDS	Yeast Derived Surfactant

INTRODUCTION

Animal production in general, and one of ruminants in particular, is suffering a series of pressures from diverse nature that generate technical and economic tensions, for which the technicians and professionals of the sector are working to find solutions. But they are also generating other types of tensions that originated from the social demands concerned with the animal health and welfare or the respect to the environment.

Society in general has a negative perception of the animal production. Somehow, the popular culture wishes a respectful production with the environment and with the animals, as well as obtaining a quality product from a low cost and associated to the traditional image of the cows grazing in green pastures. In fact, on a European survey, (Eurobarometer, 2008) "global warming/climate change" rank second after "poverty, the lack of food and drinking water" on what the respondents consider to be the most serious problems, and maintained this position on a more recent questionnaire (Eurobarometer, 2011).

Global warming and the accumulation of greenhouse gases have created a social alarm and have mobilized politicians and scientists to study and establish measures of control. However, nowadays it is not easy to have a clear picture of what the situation of the agriculture in this context is: its global contribution, the efficient strategies for mitigation and the impact of their implementation in resolving the problem (Calsamiglia, 2012).

CARBON FOOTPRINT AND CLIMATE CHANGE

The carbon footprint is the amount of gases with greenhouse effect that are produced in a system. In this calculation, not all the gases have the same effect, e.g., CO_2 has an equivalent value of 1 (standard), methane (CH_4) has an equivalent value of 25 units of CO_2, and N_2O of 298. The environmental impact of greenhouse effect gases is determined following international recognized normative and is expressed in equivalents of CO_2 (CO_2eq). The calculation of the carbon footprint is essential to identify weak points and implement strategies for reduction or compensation of emissions.

The greenhouse effect is thought to be due to the absorption of solar infrared (IR) radiation by gases (mainly CO_2, water vapor, CH_4 and N_2O) and the earth's surface, which, as a result, are heated and then remit IR radiation at low frequency with a high absorptive power (Moss et al., 2000). Indeed, greenhouse gases are essential for maintaining life on earth, since otherwise the planet would be permanently frozen because the incoming heat from the sun would be radiated back into space by the earth's surface (Moss, 1993). However, the cut-off point at which the concentration of greenhouse gases should be minimized is not known, but it is accepted worldwide that their concentrations in the atmosphere should not be allowed to continue rising; and the Intergovernmental Panel on Climate Change (IPCC) have asked developed nations to evaluate the amount of gases they are producing and to develop research to limit emissions further.

CONTRIBUTION OF ANIMAL PRODUCTION TO THE GREENHOUSE EFFECT

In 2006 the Food and Agriculture Organization of the United Nations (FAO) published a report entitled "Livestock's long shadow" (FAO, 2006), in which animal production was considered one of the main causes of global warming through the emissions of greenhouse gases (responsible for 18 percent of greenhouse gas emissions measured in CO2 equivalent) from enteric fermentation in ruminants, the great majority from manure; ahead of the emissions associated with transport. However, other studies (CCC, 2008; Gill et al., 2010) have indicated that the contribution of agriculture/stockbreeding is about the 8-12% of the total production of greenhouse gases. From which, approximately a 5.1% is attributable to livestock and their manure. In fact, the FAO published later a corrective report (FAO, 2010), including this data; although this report was not as widespread as the first one.

It is well-known that the problem exists, and, as suggested by Calsamiglia (2012) efforts must be made to identify and implement strategies to reduce the production of greenhouse gases rather than discussing the total contribution of each sector; as it is much more effective and responsible. Therefore the animal production sector must identify the key points within the production systems that are susceptible to improvement in terms of greenhouse gas emissions, in order to propose and implement strategies that could help to achieve the goal of reducing the production of gases with greenhouse effect.

1. WHY HAS BARLEY BECOME SO IMPORTANT IN THE DIET OF FEEDLOT?

Barley is one of the oldest cultures of mankind (Castro, 2001); in fact, it is believed to be one of the first domesticated plants at the beginning of Agriculture.

> This cereal was first domesticated in Southwest Asia, in the area known as Mesopotamia, where it is still possible to find a wild progenitor *Hordeum vulgare* subsp. *spontaneum* in natural habitats and around the edges of fields.

Originally, barley was mainly cultivated and used for human food, but it is now used primarily for animal feed and to produce malt, with smaller amounts used for seeds and direct human consumption. Barley is also used in the production of starch, either for food or for the chemical industry (OECD, 2004). In addition, barley has some useful by-products, the most valuable being the straw which is used mainly for bedding in developed countries, but also for animal feed in developing (Castillo et al., 2009) and under-developed countries (Akar et al., 2004).

Currently, it is a crop adapted to various conditions, from the Mediterranean-type climate to cool areas. Barley varieties, like other cereal grains, have been developed by plant breeders for their climatic conditions, thus yielding barley of high economic and feeding or malting value (Bull and Bradshaw, 1995).

Approximately three-quarters of global production is used for animal feed, 20% is malted for use in alcoholic and non-alcoholic beverages, and 5% as an ingredient in a range of food products (Blake et al., 2011).

The importance of barley and their products in the world today can be understood because there are 94 countries that produce it, 87 that export it and 162 that import it worldwide (Mashayekhi et al., 2005). The European Union (EU) ranks first as the greatest producer of barley with 46.1%, followed far behind by Russia, Canada, Australia and Ukraine; together representing 73% of world barley production. Thus, barley is the world's fourth most important cereal crop both in terms of area of cultivation and in quantity of grain produced (http://faostat.fao.org/). In the EU, predictions for barley production are 147 million tons in 2014/15, more than 14 million tons more compared to 2007/08 (Maluenda, 2010).

High grain diets are common in beef cattle feedlots because of the large supply of feed quality grain. Barley has traditionally been the primary grain used in feedlot rations in the northern hemisphere as an energy and protein source. The ease of transport, storage, and mixing of grains makes them more attractive than forages.

The nutrient content compares favorably with that of corn, oats, wheat, and milo. The crude protein (CP) content of barley is higher than corn and the energy content of barley is slightly lower than corn due to a higher acid detergent fiber (ADF) and neutral detergent fiber (NDF) content. Barley grain is characterized by 19-21% NDF and 52-73% starch. However, nutrient composition is highly variable and may be affected by geographical location, growing conditions, year grown, variety (two-row or six-row), feed or malting type, and season planted (see review of Hussey, 2012).

In the last few years, barley trade has been linked to the spectacular rise in prices; the main causes are due to 1) strong demand for cereals, mainly in emerging countries like China and India; 2) rise in transport costs; 3) new commodity future markets; 4) devaluation of the dollar against the euro; 5) adverse meteorology in some producing countries; 6) introduction of restrictive measures by major countries in the cereals sector and 7) the increased production of biofuels, especially in the United States (Maluenda, 2012).

Despite all these factors, barley remains a great interest from the point of view of animal nutrition, having a significant effect on the profitability of feedlot. Then we describe the most important characteristics of this grain, which led it to have a key role in the animal nutrition industry worldwide.

TAXONOMY

Barley belongs to the genus *Hordeum* in the tribe Triticeae of the grass family, Poaceae (also known as Gramineae). The Triticeae tribe is a temperate plant group containing several economically important cereals and forages as well as about 350 wild species. The genus *Hordeum* is unusual among the Triticeae as it contains both annual species, such as *H. vulgare* and *H. marinum*, and perennial species, such as *H. bulbosum* (OGTR, 2008).

There are 32 species within the *Hordeum* genus, all with a basic chromosome number of x=7. Cultivated barley *Hordeum vulgare* L. ssp. *vulgare*, and its wild progenitor *H. vulgare* L. ssp. *spontaneum* are diploid species with 2n=2x=14 chromosomes. Other *Hordeum* species are diploid, tetraploid or hexaploid.

The two species *H. vulgare* and *H. bulbosum* are considered to share a common basic genome I which is not related to any other genome in the genus. On the other hand, the genomes of the two annual Mediterranean species *H. marinum* and *H. murinum* seem not to be closely related to the other genomes in *Hordeum* and have been designated as X and Y, respectively. The remaining diploid *Hordeum* species are all closely related and share the H genome (von Bothmer, 1992; OGTR, 2008).

This extraordinary genetic variety is what has made the barley one of the most used species in agricultural genomics.

CHARACTERISTICS OF BARLEY KERNEL

The structure of barley kernel (seed), like other seeds, reflects their biological function: protection and transport of the embryo, complimented with an energy source to aid ingermination, and early growth. Barley kernel can be subdivided into three basic parts: *hull*, *endosperm*, and *germ* (embryo, see Figure 1):

The *hull* portion, composed of the lemma and palea, contains most of the fiber.

> The hull-less cultivars, where the hull is easily removed during threshing, have relatively low fiber content as compared with hulled cultivars.

Hulls accounts for 7 to 17% of the kernel weight. Therefore, a reduction in the percent of composition of the hull results in a higher percentage of endosperm and germ. These components contain more available energy because they are rich in starch and protein.

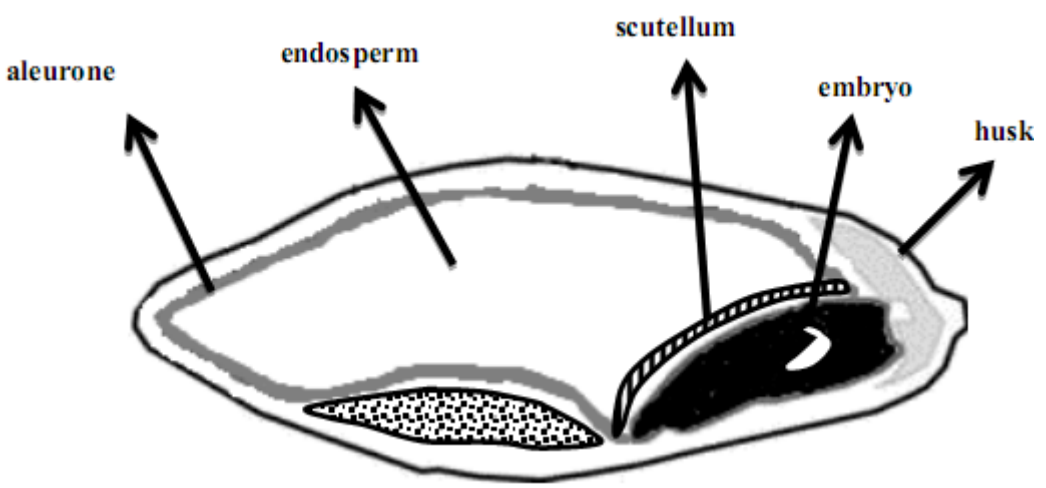


Figure 1. General structure of barley kernel. The *embryo* and the *endosperm* provides a store of carbohydrates (mainly starch) and protein to support the initial growth of the germinating embryo. The grain is protected by an outer husk.

Barley *endosperm*, which contains most of the starch (Huntington, 1997), consists of several layers of tissue: aleurone, starchy endosperm, and depleted cell layer. Seven to 13% of the kernel weight is in the aleurone layer which contains essential enzymes and enzyme

inhibitors. It contains 60 to 85% arabinoxylan, up to 20% ß-glucans, 8% cellulose, and 6 to 16% proteins.

Starchy endosperm consists of starch granules of varying sizes and shapes. Approximately 63 to 65% of kernel starch is contained in the starchy endosperm layer (Hunt, 1995).

The *germ* content only makes up to 3% of the kernel weight, but it contributes to the total nitrogen (N) content of barley.

However, barley protein content is highly variable, ranging from 9.1 to 24.1% of the barley kernel weight (Hunt, 1995).

NUTRITIONAL CHARACTERISTICS OF BARLEY. DIFFERENCES WITH OTHER GRAIN SOURCES

Some of the most economically important cereal grains grown around the world and used in feedlots include not only barley (*Hordeum vulgare*), but also corn (*Zea mays*), wheat (*Triticum aestivum*), rye (*Secale cereale*), sorghum (*Sorghum bicolor*), oats (*Avena sativa*) and triticale *(×Triticosecale*). (Huntington, 1997; Gibb, 2000; Lawrence, 2010).

Nutritional value of grains is determined by several factors: 1) Grain structure and size; 2) Starch content; 3) Protein characteristics; 4) Protein/Starch interaction and 5) Grain water absorption.

Compositional differences between the most common cereal grains can be found in Table 1.

Table 1. Gross compositional fractions of different cereal grains (values based on % dry weight, adapted from Pascoe, 2009)

Cereal grain	Protein	Lipid	Starch	Fibre	Ash
Barley	10.9	2.3	73.5	4.3	2.4
Corn	10.2	4.6	79.5	2.3	1.3
Oats	11.3	5.8	55.5	10.9	3.2
Rice	8.1	1.2	75.8	0.5	1.4
Rye	11.6	1.7	71.9	1.9	2.0
Shorgum	11.0	3.5	65.0	4.9	2.6
Wheat	12.2	1.9	71.9	1.9	1.7

Not only is barley higher in protein than corn, their higher level of ruminal starch digestion results in more microbial protein production (Surber and Bowman, 1998). In recent years genetic manipulations have provided increased non-starchy polymers to barley varieties, including elevated β-glucans and pentosans (Fulcher and Rooney, 2002), a very large and softer, kernel exhibiting hulless and waxy starch characteristics, with exceptionally high antioxidant levels (Andreasen et al., 2000a,b; 2001).

The cell walls in barley bran contain many small phenolic and polyphenolic compounds that provide antimicrobial and antifungal activity, as well as insect anti-feedant activity (OGTR, 2008; Pascoe, 2009).

Thus, phytic acid may reduce the bioavailability of trace elements in animal diets through chelation of minerals such as Fe, Zn, P, Ca, K and Mg. This anti-nutrient is of particular interest in monogastric animals, whereas ruminants possess digestive enzymes which degrade phytate and release the chelated minerals (OGTR, 2008).

The phenolic compounds proanthocyanidins and catechins are found in barley seed coats and these can form insoluble complexes with proteins inhibiting nutrient utilization (Newman and Newman, 1992). Currently proanthocyanidins-free barley has been produced and released commercially (von Wettstein, 2007)

Ruminal starch digestion appears to be the key to efficient utilization of grain by ruminants. In these animals, starch in cereals also serves as an important source of energy for microbial growth and has therefore great impact on the feed protein value. Microbial digestion of starch granules from barley radiates from a central point of microbial attachment on the surface of the granule, in contrast with microbial corn starch digestion (McAllister et al., 2006).

The apparent inefficiency of ruminal digestion of starch in animal feed barley-based diets seems to be compensated through greater microbial protein synthesis (Surber and Bowman, 1998).

Nonetheless, there are studies that point out that the degree of digestibility of barley would be determined not so much for its variety, as by its way of administration: when steers do not consume diets *ad libitum*, a relatively low feed intake resulted in longer retention time in the digestive tract; longer retention time, in turn, may afford to lower quality barley more opportunity to be digested. *Ad libitum* system (with potentially greater intake), improves nutrient availability; thus a higher quality barley source may be of greater benefit for cattle performance (Fife et al., 2008).

Finally, barley contains 2-3% minerals, although the mineral content can vary markedly with variety, growing conditions and fertilizer application. The major minerals are mg, P, K, Ca and Na (OGTR, 2008).

It is clear that based on current knowledge, future barley breeding programmes should consider nutritional quality to a greater extent than in the past.

BARLEY VARIETIES

Until the late nineteenth century, all barleys existed as highly heterogeneous landraces adapted to different environments. Over the past 100 years, the landraces have mostly been displaced in agriculture by pureline varities with reduced genetic diversity. Extensive cultivation, intensive breeding and selection have resulted in thousands of commercial varieties of barley. For commercial purposes, barley varieties are classified into broad classes that are used as a basis for world trade.

Thus, there are many criteria to classify barley, so we can find multiple classifications depending on; 1) hulled or hulless (naked); 2) feed or malt type; 3) height (dwarf); 4) seed color (colorless, white, yellow, blue) and so on.

The major factors to distinguish barley varieties are feed or malting barley; winter or spring growth habit; starch amylose/ amylopectin ratio; hull or hulless barley, and six-, four-, two row varieties (OECD, 2004).

Six row barley can produce 25-60 grains, while two-row barley produces 25-30 grains (4). Four-row barley is actually a loose six-row barley (Gomez-Macpherson, 2001), so many sources only differentiate between two- and six-row varieties. Wild barley is two-row, and most cultivated barley is of the six-row type (Figure 2).

According to the Alberta Agriculture and Rural Development (2011) barley varieties use to vary with respect to: 1) bushel weight; 2) crude protein (CP) content; 3) acid detergent fiber (ADF); 4) kernel hardness; 5) dry matter (DM) degradability after 0 - 24 hours in the rumen; 6) and total tract digestibility of: DM, organic matter (OM) and nitrogen.

Figure 2. Two-row and six-row barley varieties.

As a diploid, inbreeding, temperate crop, barley has traditionally been considered a model for plant genetic research. The basic objectives of this breeding have been oriented towards the creation of resistant lines to local pathogen population.

Therefore, the search for new resistance sources and introduction of new germoplasm sources into breeding programs as well as pathogen virulence and variability studies remains an important research field. A successful breeding strategy highly depends on correct choice of parent genotypes, therefore it is important to identify the most resistant varieties and exclude the susceptible ones (Statkevičiūtė and Leistrumaitė, 2010).

The barley genome is almost twice the size of that of humans and determining the sequence of its DNA has presented a major challenge (The International Barley Genome Sequencing Consortium, 2012). Traditionally, morphological traits, cytological characters, biochemical tests, and pedigree information are used to assess genetic diversity and classify barley germplasm. However, these methods are always associated with various limitations

and are insufficient to reveal the whole information within barley resources (Matus and Hayes, 2002). Many types of molecular markers have been used to characterize crop resources (Manninen, 2000; Wang et al., 2010). Breeding new barley varieties is based on creating new allele combinations and subsequent testing and selection of the desirable phenotypes during the selfing generations. Heritable variation is created mainly by controlled crosses between adapted high yielding cultivars and breeding lines. Spontaneous mutations, as well as mutations induced by radiation or chemical treatments, have also been used. Transgenosis has been added to the tools for creating new variation in barley (Manninen, 2000). Enriching its broad natural diversity, extensive characterized mutant collections containing all of the morphological and developmental variation observed in the species have been generated, characterized and meticulously maintained. The major impediment to the exploitation of these resources in fundamental and breeding science, has been the absence of a reference genome sequence, or an appropriate enabling alternative. Providing either of these, has been the primary research challenge to the global barley community.In ruminant nutrition, three varieties are the most used for feeding an also reported in literature:1) Gunhilde barley; 2) Harrington barley and 3) Medallion barley (Boss and Bowman, 1996a).

> Gunhilde was developed as a two-row feed variety in Europe; Harrington is a two-row malting variety; and Medallion is a high-yielding six-row feed variety, genetically related to the variety Steptoe.

Studies about the influence on these differences on animal performance are controversial, whereas Boss and Bowman (1996b) concluded that differences in digestive characteristics found between barley varieties may contribute to differences in animal performance, other studies (Alberta Agriculture and Rural Development, 2011) did not find differences in the feeding value of different samples of barley grain (Table 2)

> Nevertheless, it has been suggested that head morphology (two-row vs. six-row) had the most influence on nutrient composition and in most cases the composition of two-row barley is superior to six-row barley.

Recently, the genome of this cereal has been completely sequenced, providing a new molecular and cellular insight into the biology of the species; and a platform to advance genome-assisted crop improvement (The International Barley Genome Sequencing Consortium, 2012).

BARLEY PROCESSING FOR INCLUSION IN FEEDLOT DIETS

Grain processing (grinding, steaming, pelleting, flaking, etc.) is a key component of modern cattle-feeding operations. Feed can be the single most costly item in cattle production, representing 70–80% of the total cost of weight gain (Boyles et al., 2001). Several studies have compared feeding whole barley versus processed barley. Whole barley kernels with an intact pericarp are very resistant to bacterial attachment and digestion in the rumen (Beauchemin et al., 1994; McAllister et al., 1994) then, some form of processing methods should be appropriate. Techniques such as *grinding* or *dry-rolling* increase digestibility of

barley grains for cattle (Owens et al., 1997; Hussey, 2012) Nevertheless, many of the larger feedlots using barley prefer *temper-rolling* over *dry-rolling* because it is easier to control the resulting kernel thickness and minimize the amount of fines (Beauchemin et al., 2001).

Currently, the industry has developed numerous methods of grain processing including physical cold, hot physical processing (wet or dry), chemical and enzymatic processing, or the addition of surfactants (see review of Castillo et al., 2012).

Extensive processing maximizes digestibility but also increases the incidence of bloat, acidosis, laminitis, and liver abscesses and causes variation in feed intake related to digestive disturbances. Beauchemin et al. (2001) studied the effects of barley grain processing on the site and the extent of digestion of beef feedlot finishing diets.

> Effects of extent of barley *rolling* on chewing activities, ruminal fermentation, and site and extent of digestion were evaluated for feedlot cattle finishing diets. Barley grain was temper-rolled to four extents: coarse, medium, medium-flat, and flat.
>
> Authors found that digestibility of organic matter (OM) in the rumen and in the total tract were numerically lower for steers fed coarsely rolled barley than for steers fed more extensively processed barley. Digestibility of starch in the total tract has linearly increased with grain processing. In addition, flow of microbial nitrogen to the duodenum was one-third lower for steers fed coarsely rolled barley than for steers fed further rolled barley; and increased grain processing tended to decrease rumination time without affecting eating time. The main conclusion from this study was that optimal degree of rolling for barley fed to feedlot cattle corresponded to a *Processing Index* of 75% or lower, and that coarsely rolled barley is not recommended because it resulted in the lowest digestibility and lowest microbial protein synthesis.

Therefore, ideal grain processing depends on striking a balance between optimizing grain utilization and minimizing expenditures associated with processing. Many factors, such as moisture, kernel uniformity, kernel hardness, and roller setting, can affect barley processing, and these factors often vary substantially in barley purchased from commercial sources. Consequently, achieving optimal processing of this highly variable grain represents a significant challenge to producers. Wang et al. (2003) evaluated the effects of *tempering* and of surfactant applied during tempering on the ruminal degradation in feedlot cattle. Authors concluded that tempering did not affect the proportion of the soluble or degradable fractions of barley but reduced ruminal effective degradability. Tempering with surfactant had no effects on degradation parameters.

BARLEY ON ANIMAL HEALTH

Designing high-grain diets for feedlot cattle involves balancing the opportunity for enhanced growth with maintenance of good health. However, much research into the effects of dietary starch source (e.g., barley versus other grains) has hitherto taken into account only production parameters, such as weight gain, and has not considered indicators of metabolic status and health, such as blood parameters (possibly due in part to these variables being more expensive to monitor than production data). Although it is widely recognized that the starch contained in small cereal grains (such as barley) is degraded more rapidly in the rumen than

the starch in maize, little is known about whether different grains have different effects on metabolism.

> Berry et al. (2004) found that feeding higher-energy diets decreases the percentage of calves with *Pasteurella multocida* and *Haemophilus somnus* pathogens in calves that receive one or more antimicrobial treatments.

Most studies have been focused on the negative effects of feeding high grain diets on different metabolic parameters, but not on the specific effects of each one. The main nutritional characteristic of cereals, such as barley, is that they provide starch, glycogen precursor. Therefore, barley consumption provides theoretically higher levels of blood glucose. However, this effect has not always been reflected in beef cattle fed with different types or mixed of cereals (Castillo et al., 2011) and is related to rumen VFA profile as well as the average intake animal associated with each grain through changes in rumen pH.

> Tiffany and Spears (2005) reported plasma glucose to be lower in Angus steers fed barley-based diets than in those fed maize-based diets, which they attributed to the lower ruminal propionate levels of barley-fed steers limiting post-absorptive metabolism and gluconeogenesis.

Another aspect to be considered is the decrease in ruminal pH associated to barley consumption, causing ruminal disturbances and metabolic acidosis (Owens et al., 1998). It is well known that lactic acid accumulates when the abrupt introduction of easily fermentable carbohydrates stimulates proliferation of the fast-growing lactic acid producer *Streptococcus bovis*, so that its growth rate exceeds that of bacteria that consume lactic acid.

> In fact, blood L-lactate will be higher in animals fed a barley-based diet than in those fed mainly corn, in accordance with the ruminal fermentation pattern of barley (Huntington, 1997); this is reflected in the lower blood pH values found in beef cattle fed barley compared with those fed corn (Castillo et al., 2009).

Nevertheless, the acidogenic digestion of barley seems to be conditioned by grain variety or genotype, which can alter the site and extent of starch digestion (Boss and Bowman, 1996 a,b) or the nutritional management of the ration (in terms of CP and quality of straw in the ration, Castillo et al., 2011).

> Boss and Bowman (1996b) observed that steers fed Gunhilde and Harrington barley varieties showed greater microbial N flow than those fed the Medallion variety. On the other hand, ruminal acetate: propionate ratio was lower in steers fed Harrington variety than in those fed Gunhilde and Medallion barley varieties.

Regarding other metabolic parameters, Castillo et al. (2011) notes the lack of differences with respect to the values of non-esterified fatty acids (NEFA), urea or serum total proteins between steers consuming a barley-rich diet and others consuming a corn-rich diet.

BARLEY ON ANIMAL PERFORMANCE AND MEAT QUALITY

Currently most feedlots procure barley based on physical attributes such as plumpness, weight, and moisture. Based on these physical characteristics, some feedlots have pricing mechanisms set in place for barley that does not meet site specifications. These pricing mechanisms may or may not correlate with the feeding value and animal performance in the feedlot (Hussey, 2012).

Table 2. Effect of barley variety and type on steer performance and carcass characteristics (adapted from Ramsey, 1994)

Barley Variety	Type	ADG	DMI	F/G ratio	Dressing%	Cutability Estimate
Duke	6 Row Feed	3.52	23.9	6.63	57.3	62.3
Condor	Hulless Feed	3.3	23.4	6.84	58.2	61.6
Ellice	2 Row Malt	3.54	21.7	6.38	57.9	61.7
Harrington	2 Row Malt	3.45	23.6	6.85	58.5	61.8
Virden	6 Row Feed	3.21	22.1	6.78	57.6	62.2
Bonanza	6 Row Feed	3.39	21.4	6.71	57.2	62.3
Abee	2 Row Feed	3.39	22.5	6.75	57.6	60.8
Leduc	6 Row Feed	3.43	22.8	6.69	57.8	61.7
Differences		N.S.	N.S	N.S	N.S	N.S
Effect of 2 *vs.* 6 row barley type						
	2 Row	3.41	22.4	6.64	58	61.6
	6 Row	3.39	22.7	6.67	57.6	62.2
	Hulless	3.3	23.4	6.84	58.2	61.6
Differences		N.S.	N.S	N.S	N.S	N.S
Effect of Malt*vs.* Feed barley type						
	Malting	3.41	22.1	6.66	57.9	61.9
	Feed	3.39	23	7.25	57.7	61.8
Differences		N.S.	N.S	N.S	N.S	N.S

ADG = average daily gain; DMI = dry matter intake; F/G = feed DMI:gain ratio; N.S. no significant.

As already mentioned in a previous section, contradictory data is available evaluating the effect of barley variety on performance by beef cattle. Some researchers have reported that barley variety have influenced gain and digestion, whereas others have reported no effect.

> The study performed by Boss and Bowman (1996a) observed that diets based on Harrington barley resulted in greater dry matter intake (DMI), average daily gain (ADG), and carcass quality grade compared with diets based on Gunhilde or Medallion barleys.

A trial performed by Ramsey (1994) measured the effects of several factors which might affect feedlot performance of barley such as barley variety and type (two-row *vs.* six-row; hulless *vs.* hulled) and malting *vs.* feed type. The author used eight varieties of barley (see Table 2), and concluded that no differences in dry matter intake (DMI), average daily gain (ADG), DMI: gain ratios or carcass characteristics were detected between steers fed the eight varieties of barley.

> Although the differences in feeding value among barley varieties for ruminants are small they are still economically important, especially in a period of high grain prices.

Some reviews (see Castillo et al., 2012) indicate that barley cereal is not exactly favoring higher rates of production, especially in the growing period, being more advisable to use corn, due to starch digestion in the small intestine is more energetically efficient than ruminal fermentation.

> It has been described that corn-fed steers were 23% more efficient than barley-fed steers. Corn-fed steers gained faster, consumed less DM, and had heavier final BW than barley-fed steers (Loe et al., 2006)

But barley is highly fermentable in the rumen and allows less starch to escape and be digested in the small intestine compared with corn (Owens et al., 1986; Nocek and Tamminga, 1991), favouring greater microbial N synthesis. This increase in microbial protein could potentially increase the amino acids available for production (Surber and Bowman, 1998).

> Some barley diets have resulted in animal performance similar to those of corn-fed animals (Nichols and Weber, 1988). In addition there are reports suggesting that barley variety can affect animal performance (Ovenell and Nelson, 1992), due to differences in site and extent of nutrient digestion among barley varieties. A study performed by Boss and Bowmann (1996 a,b) with three varieties of barley (Gunhilde, Harrington and Medallion) demonstrated no differences in the amount or site of starch digestion, but in N digestion, with a greater efficiency of microbial growth for steers fed Harrington than for those fed Gunhilde or Medallion barley.

Similar differences were observed in carcass quality grade: thus, steers fed Harrington variety has a better marbling score or quality grade than those fed Gunhilde or Medallion varieties (Boss and Bowman, 1996a).These results conflict with those of Bradshaw et al. (1992) and Ovenell et al. (1993), who reported barley variety had no effect on marbling score or yield or quality grade.

Considering the digestible energy (DE) of barley as a parameter which distinguishes the quality of cereal, Hussey (2012) studied their effects on feedlot performance and carcass quality. The results showed on a live weight basis, that final body weight, weight gain or ADG were no different. On a carcass-adjusted basis, similar lack of differences were found. Nevertheless, DMI tended to decrease as the DE of the diet increased. These performance results were consistent with those of Berry et al. (2004), who also observed no difference in ADG and animal performance when different energy and starch concentrations in barley were fed, and were in contrast to expectations based on the Nutrient Requirements of Beef Cattle (NRC, 1996) that suggest that as the energy density of the diet increases, feedlot performance would be expected to improve.

> Previously, Stock et al. (1990) postulated that the greater starch content of high energy barley limits improvements in cattle performance, because cattle consume more

starch or less fiber and likely experience more subacute acidosis challenges. Then, cattle offset these challenges by consuming smaller and more frequent meals, explaining the lower intakes associated to high DE of grains.

2. MECHANISMS OF METHANE PRODUCTION

As mentioned before, ruminants are a significant source of greenhouse gases emissions. Most of the CH_4 and CO_2 is released from animals; however, a smaller level of both gases together with N_2O and NO results from microbial processes in the excreta or after landspreading. Additionally, the indirect greenhouse gas NH_3 is emitted from animal waste after microbial activity and serves as precursor for N_2O and NO (Table 3).

Livestock are the most important source of anthropogenic methane emissions (FAO, 2006). Among domesticated livestock, ruminants (cattle, sheep, goats, buffaloes, and camels) produce significant amounts of methane as part of their normal digestive processes. In the rumen, the first fore-stomach of these animals, fibrous feed is converted into products that can be digested and utilized by the animal through microbial fermentations. This microbial fermentation process, referred to as enteric fermentation, produces methane as a by-product, which is exhaled by the animal. The anaerobic decomposition of organic material in livestock manure also releases methane. This occurs mostly when manure is managed in liquid form, such as in lagoons or holding tanks. Manure deposited on fields and pastures, or otherwise handled in a dry form, does not produce significant amounts of methane. Methane emissions from livestock manure are influenced by a number of factors that affect the growth of the bacteria responsible for methane formation, including ambient temperature, moisture and storage time. In this chapter we will focus solely on the fermentation processes that take place within the animals: how greenhouse gases are produced, how barley contributes to this emission and the mitigation options available.

Table 3. Different sources of greenhouse gases from animal husbandry (from Clemens and Ahlgrimm, 2001)

	Animals	Manure, excreta
CO_2	Metabolism	Microbial formation during storage and field application of manure
CH_4	Enteric from ruminants (major part of the emissions) + digestive system of monogastric animals (global: 14 Mt a^{-1}, IPCC, 1995)	Microbial formation during storage and field application of manure (global: 14Mt a^{-1}, IPCC, 1995) Food production: reduced microbial CH_4 oxidation in fertilized fields
NO and N_2O		Microbial formation during storage and field application of manure Food production: microbial formation in fertilised fields
(NH_3)	Very small amount from digestive systems of animals	Rapid microbial hydrolysis of urea; then the emission itself is a process dominated by physical factors (global: 20-35 Mt a^{-1}, Ahlgrimm, 1998)

Enteric Fermentation: How Are Greenhouse Gases Produced by Ruminants?

In the anaerobic environment of the rumen, organic material is decomposed by bacteria through the process of fermentation, where this material is broken down into, among others, Volatile Fatty Acids (VFA) and carbon dioxide. During this process, hydrogen is released and accumulated in the fermentation system. In aerobic environments, oxygen is the terminal electron acceptor and it would be reduced to H_2O, using the excess of hydrogen in the process. The lack of oxygen in anaerobic systems necesitates the use of other terminal electron acceptors to remove hydrogen from the fermentation system. There are several compounds that can be used for this purpose, although apart from CO_2, they are usually at a really low concentration in the rumen and therefore carbon dioxide functions as the main terminal electronacceptor for the remaining excess hydrogen. Carbon dioxide is reduced to methane in the fermentation system (equation 1), and the methane in gaseous form subsequently dissipates from the system (usually exhaled by burping).

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \text{Equation 1}$$

Methane production occurs during the fermentation of organic matter also present in other natural environments (Deppenmeier, 2002) and it is aimed to remove hydrogen from the site of fermentation by reducing carbon dioxide and therefore maintaining a low redox potential at the site of fermentation. Without the removal of hydrogen, re-oxidation of reduced cofactors (FADH, NADH and NADPH) would be inhibited by the accumulated hydrogen, and the production of VFA would be inhibited (Wolin, 1975). Methane is produced by a specific group of microorganisms, namely asmethanogenic Archaea (Chaban et al., 2006).

Fermentation and VFA production by the microorganisms of the intestinal tract of animals is accompanied by the production of methane. However, the extent of dependency on microbial fermentation for the digestion of feed varies among species and, therefore so does the amount of methane produced per animal. Compared to other species, ruminants rely on bacterial fermentation to a larger extent than other animals and that is the reason why methane emissions from ruminants (expressed in relationship to their body weight) are higher than non-ruminants species (Table 4).

Table 4. Estimated annual enteric methane emissions from the main domesticated livestock species (Sauvant, 1993)

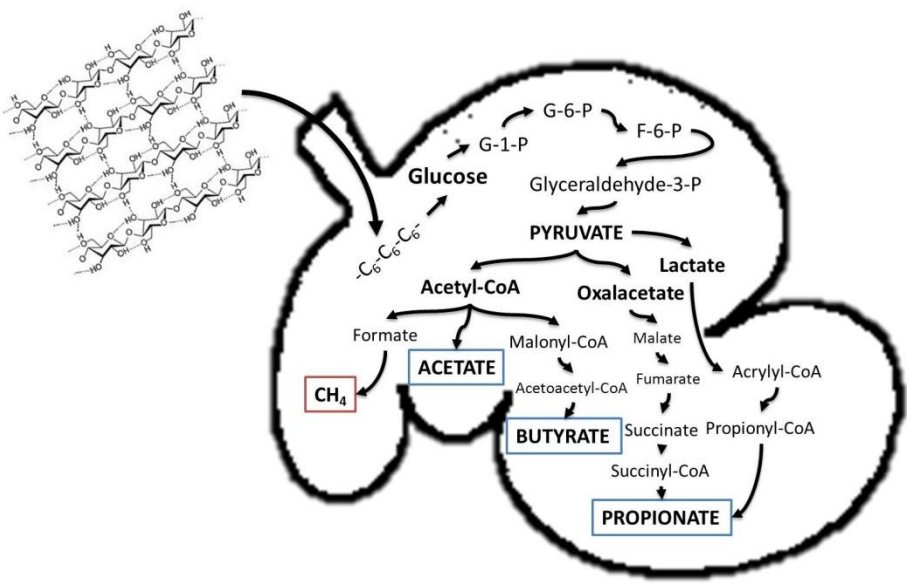
	Methane emission ($kg\ CH_4 \times animal^{-1} \times year^{-1}$)	Assumed average bodyweight (kg)	Methane emission ($g \times kg\ BW^{-1} \times year^{-1}$)
Non-ruminants			
Swine	1	80	13
Poultry	<0.1	2	-
Horses	18	600	30
Ruminants			
Dairy cows	90	600	150
Beef cows	65	400	163
Sheep	8	50	160
Goats	8	50	160

Microbial fermentation can occur in the ruminant animal both in the rumen and in the hindgut:

Fermentation in the Rumen

Ingested feed macro-molecules are degraded into small molecules in the digestive tract, so they can be transferred into the bloodstream through the digestive mucosa. This hydrolysis is mediated by enzymes of both endogenous and microbial origin. Depending on the animal species, their anatomy and physiology, the enzymatic digestion can take place at the beginning of the digestive tract, while the microbial digestion takes place at the end (carnivores, horses, rabbits…) or just the opposite as ruminants and some other animals considered as pseudo-ruminants (e.g., *camelidae*), which have large anaerobic fermentative chambers at the beginning of the tract.

Fermentation of glucose, released from plant polymers or starch, is an oxidative process under anaerobic conditions occurring in the Embden-Meyerhof-Parnas pathway, in which the three main VFA are produced: acetate, propionate and butyrate (Figure 3).



-C_6-C_6-C_6-: Carbohydrates. G-1-P: Glucose-1-phosphate. G-6-P: Glucose-6-phosphate. F-6-P: Fructose-6-phosphate. Glyceraldehyde-3-P: Glyceraldehyde-3-phosphate. CoA: Coenzyme A.

Figure 3. Diagram of the digestion of Carbohydrates in the rumen (Abuelo, 2011).

It is documented that the quantity of methane produced per unit of fermented feed is proportional to the pattern of the VFA produced, indeed Moss et al. (2000) proposed an equation (Equation 2) to estimate the amount of methane production, in base to the molar percentage of VFAs produced, since they are easier to quantify.

$$CH_4 = \frac{1.8 \times Acetate - 1.1 \times Propionate + 1.6 \times Butyrate}{4} \quad \text{Equation 2}$$

Source: Moss et al. 2000.

As it can be noticed from the equation, the levels of propionate have a negative effect on the production of methane, because during the production of acetate (equation 3) and butyrate (equation 4), hydrogen is produced, whilst the production of propionate (equation 5) results in the net uptake of hydrogen. And therefore a higher proportion of propionate in the VFA-profile results in reduced methane production (Ellis et al., 2008) and this property can be utilized in the manipulation of methane production. However, the production of acetate and butyrate always exceeds propionogenesis, resulting in a net surplus of hydrogen in the rumen.

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2CO_2 + 4H_2 + 2H^+$$ Equation 3

Production of acetate

$$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + 2CO_2 + 2H_2 + H^+$$ Equation 4

Production of butyrate

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2^- + 2H_2O + 2H^+$$ Equation 5

Production of propionate

According to the IPCC guidelines (IPCC, 2006), a lactating dairy cow loses typically 6.5% of the gross energy she ingests through methane emissions, but considerable variation around this number exists, partly depending on diet composition (Johnson and Johnson, 1995).

Fermentation in the Hindgut

Large amounts of organic matter can by-pass the rumen and be digested in the hindgut when there is no digestion in the small intestine or when the digestion is incomplete. So ground roughage diets and maize starch enriched diets can supply large quantities of digestible organic matter to the hindgut (Moss et al. 2000). It has been estimated that 10-30% of digestible organic matter can be digested there (Moss et al. 2000).

The anaerobic microbes in the hindgut are not quite different from those found in the rumen (Julliand, 1992). As in the rumen, methanogens of colonic fermentation use hydrogen to reduce carbon dioxide to methane (Miller and Wolin, 1986). Hydrogen can be used also to reduce CO_2 into acetate (Equation 6) when non-methanogenic fermentation occurs (Drake, 1994).

$$CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$$ Equation 6

This use of H_2 is of interest for animal nutrition, since acetate is absorber into the bloodstream and used as a major source of carbon and energy by ruminants, while methane is lost from the animal. Several attempts have been made by researchers to reduce CH_4 production and to increase acetogenesis by the ruminal microflora. However, unlike methanogens, acetogenic bacteria are able to use sources other than hydrogen for energy supply, explaining why the concentration of these bacteria can be high while acetogenesis is negible (Moss et al. 2000).

Contribution of Barley to Greenhouse Gases Emissions

Energy lost as enteric CH_4 from mature cattle ranges from 2 to nearly 12% of gross energy intake (Johnson et al., 2000). The range of emissions is due mainly to the level of feed intake and the composition of the diet (Johnson and Johnson, 1995; Moss et al., 2000; Benchaar et al., 2001). For beef cattle, intensive feedlot systems result in a lesser CH_4 production per unit of meat produced than extensive grazing systems (Clemens and Ahlgrimm, 2001). The estimation of energy lost as CH_4 from feedlot cattle from the IPCC is calculated from cattle consuming high-grain diets containing mainly corn grain, as methane emissions for barley grain were not extensively evaluated. However, it was speculated by Johnson et al. (2000) that high-grain barley diets result in greater CH_4 emissions than would be expected from corn enriched diets, based on previous reports of losses of 6.5 to 12% of gross energy for barley diets (Whitelaw et al., 1984).

Beauchemin and McGinn (2005) compared the CO_2 and CH_4 emissions of feedlot cattle fed either a high-corn or barley diet, at two different phases of the production cycle: backgrounding and finishing. As expected, they found a greater daily CH_4 emission when cattle were fed a backgrounding diet than when fed a finishing diet, due to the greater proportion of concentrate in the latter production phase. Comparing both grain diets, daily methane emissions per animal were 32% greater when a corn backgrounding diet was fed compared to feeding a barley backgrounding diet, but they didn't find an effect of grain source on CH_4 emissions from finishing diets. However, once they corrected the methane emission on the basis of DMI or gross energy intake, CH_4 emissions during backgrounding were not affected by grain source, whereas emissions were less for the corn finishing diet than for the barley finishing diet. However, as showed by Johnson and Johnson (1995) collecting data from 118 experiments, digestibility of dietary energy explains only 5% of the variation in proportion of gross energy lost as methane. Therefore, taking into account this small difference, barley should not be considered as a greater source of greenhouse emissions than other grains used for ruminant nutrition, especially when feeding roughage produces more methane than a high-grain diet.

3. Controlling Strategies for Methane Emissions in Barley-Based Diets

When expressed per kg of ruminant product (milk and meat), methane emissions vary widely between different parts of the world. This is largely a consequence of the level of productivity in different geographical regions in the world (FAO, 2010). In the developed world, genetic progress and improved feeding management have considerably improved animal productivity over the past decades. Although methane emissions per animal have generally increased, per unit of consumable product (milk and meat), methane emission has decreased significantly (see review of van Zijderveld, 2011a).

Research to specifically reduce methane emission from ruminants was first initiated in the early 60's to reduce the dietary energy losses that are associated with methane production. Much research has subsequently been published in the 60's and 70's to investigate the potential to reduce methane emissions and reduce the energetic losses from methane

production. After a period of less research activity on this topic in the 80's, methane investigation resurged in the 90's and it has been an active area of research ever since. The main focus however, has shifted in recent years from the reduction of methane emissions to benefit the animals´ energy utilization to the reduction of methane as a greenhouse gas.

Table 5. Specific measures to reduce methane emissions from ruminants, significant for Europe and research methods (Flachowsky and Lebzien, 2012)

Measures	Significance for Central Europe	Assessment of research need
Feeding high concentrate (high starch, low fibre) diets	Potential nearly exhausted	No research need
Feeding of fats, fatty acids, oilseeds	Potential nearly exhausted	Research need
Feed additives		
Halogen compounds (Cl, Br)	Not permitted in the EU	No research need
Ionophores	Not permitted in the EU	Research need
H_2 binding substances (e.g., fumaric acid)	Presently not significance	High research need
Phytogenic substances or plants containing such substances	Presently not significance	High research need
Further feed additives such as yeast, enzymes…	Presently not significance	Research need

Animal scientists have prioritized the need for further studies to reduce methane emissions considering different nutritional measures (e.g., Flachowsky and Lebzien, 2012; Table 5). In many cases, CH_4 reduction potentials by animal feeding and management, as well as feed additives permitted in the European Union are nearly exhausted. Therefore the reduction potential for CH4 under European conditions is estimated to be only 10–20% (Kreuzer, 2009). Larger reduction potentials exist globally (FAO, 2010) through use of metabolic modifiers such as trenbolone acetate, estradiol benzoate, monensin, tylosine and ractopamine, as permitted by country.

Currently, dietary strategies to reduce enteric methane emissions mainly revolve around one of the following principles (Martin et al., 2010):

- Direct inhibition of methanogenesis
- Lowering of the production of hydrogen during fermentation
- Providing alternative pathways for use of hydrogen in the rumen

The potential of dietary strategies to reduce methane emission by ruminants has been extensively reviewed (Tamminga et al., 2007, Beauchemin et al., 2008, Martin et al., 2010, Beechem, 2011; Eckard et al., 2011). In any case, reduction of methane production from ruminants is a complex challenge, because implementation of any strategy must take into account the possible consequences on the efficiency of the entire rumen system (Benchaar et al., 2001).

With respect to the composition of livestock diets, Kessel and Russell (1996) found that corn and soybean concentrate diets severely acidify cow rumen, with the effect of inhibiting methanogen activity. Their studies demonstrated the relationship between feed composition, rumen acidity, and methanogen activity. Indeed, forage-fed cattle–like

concentrate-fed cows–ingest a high amount of fiber. This leads to the copious secretion of saliva, which buffers the rumen (Parish, 2007). Concentrate-fed cows that do not secrete as much saliva experience dramatic reductions in ruminal pH with apparent toxicity to methanogens.

A diet high in corn and soybean concentrate appears to be useful for reducing enteric methane production. Sure enough, the very high grain diets (greater than 90% concentrate) often fed to feedlots result in strikingly reduced methane emissions: 2-3% of gross energy intake, relative to the approximately 6% of forage-fed cattle. Nevertheless, researchers have noticed that low pH levels for extended periods of time can shift the rumen microbial population in favor of bacteria that produce high levels of lactic acid, leading to acute acidosis (Parish, 2007).

In this scenario, Ceechem (2011) propose a heterogeneous feeding strategy that favors concentrate diets with occasional infusions of forage feed, that could provide the greatest reduction in methane production with minimal negative effects on cow health.

Prior suggestions on this matter (Moss et al., 2000) summarized different mitigation scenarios for methane emissions from ruminants (Table 6), and whose applicability depends on the laws of each country, as discussed later.

Basically, the review of Moss et al. concluded that methane generation would be decreased by promoting a shift in fermentation toward propionate production, but cannot be eliminated completely without adverse effects on ruminant production.

For the authors, the increase in animal productivity seems to be the most effective way of reducing methane release in the short term, although this method is only successful if overall production remains constant. There exists several ways to achieve this objective, but nearly all involve the increased use of feed containing higher quality/ lower fibre sources of carbohydrate.

Since then, authors indicated that the most promising areas for future research for reducing methanogenesis would be the development of new products/ delivery systems for antimethanogenic compounds or alternative electron acceptors in the rumen and reduction in protozoal numbers in the rumen.

Table 6. Mitigation scenarios for methane emissions from ruminants (Moss et al., 2000; Hino and Asanuma, 2003)

Strategy	Objective	Examples
CH_4 inhibition		
1. Direct inhibition	Direct inhibition of methanogenesis by halogenated methane analogues and related compounds	Chloroform, chloral hydrate, amichloral, trichloroacetamide, trichloroethyladipate, bromochloromethane, combination of bromochloromethane and α-cyclodextrin, 2-bromoethanesulfonic acid and 9,10-anthraquinone.

Table 6. (Continued)

Strategy	Objective	Examples
2. Ionophores	Based on negative relationship between methanogenesis and propionate production, the aim is to produce a shift in bacterial population with a concurrent shift in the fermentation from acetate to propionate	Monensin, salinomycin.
3. Propionate enhancers	Alternatives to antibiotics, due to consumer concerns. They may alter rumen fermentation in a manner similar to ionophores. Stimulate propionate production and inhibit methanogenesis	Malate, fumarate, acrylate.
4. Stimulation of acetogens	To re-channel substrates for methane production into alternative products	Ruminal acetogenic bacteria (e.g., *Acetitomaculum ruminis* as a daily fed feed additives
5. Methane oxidisers	Promote oxidation of methane to CO2 in the rumen	Methane oxidizing bacterium
6. Defaunation	Removal of protozoa from the rumen (defaunation), as responsible for between 9 and 25% of the methanogenesis in rumen fluid	Plant secondary metabolites (saponnins) or pure saponnin, fat inclusion in diet.
7. Probiotics	Is believed to be related to a reduction in the protozoal population	*Saccharomyces cerevisiae*, *Aspergillusoryzae.*
8. Inmunisation	It has been proposed to reduce the numbers of *Streptococci* and *Lactobacilli* in the rumen and thus, immunise ruminants against their own methanogens	---
Increase in animal productivity		
1. Diet type: *a) Changes in forage to concentrate ratio* *b) High concentrate diets*	a) Modifies the acetate:propionate ratio in rumen b) Produces a shift in fermentation pattern in the rumen which give rise to a more hostile environment for the methanogenic bacteria	
2. Forage type and supplementation	Increase in the microbial growth efficiency and digestibility, looking for optimizing rumen and animal efficiency.	

Later studies on this matter (Benchaar et al., 2001), and in line with the observations made by Moss et al. (2000) considered different simulated strategies such as 1) increase in the DMI; 2) increasing the proportion of concentrates in the diet; 3) replacing fibrous concentrate with starchy concentrate in the diet; 4) replacing rapidly degraded starch with slowly degraded starch in the diet; 5) considering the stage of maturity of the forage; 6) considering the forage preservation method (hay versus silage); 7) considering the forage species (legume versus grass); 8) taking into account processing of forage (long versus pelleted) or, 9) upgrading and supplementing with poor quality forages. The main effects of these strategies on CH_4 production (Mcal/day) are reflected in Table 7

That work demonstrated the usefulness of a mechanistic model of rumen digestion to understand and to assess the effectiveness of different existing dietary strategies, aiming at reducing methane emissions from ruminants in the future. Authors pointed out that CH_4 emissions from ruminants could be reduced by 9 to 40%, depending on the nature of

the intervention. The main conclusion obtained in this study was that ruminal methane production can be decreased by shifting fermentation towards propionate production and (or) by reducing the extent of ruminal fermentation. However, in some circumstances, CH_4 production cannot be reduced without negative effects on efficiency of digestion. The utilization of high starch diets drains away, reducing equivalents from methane production to propionate formation, but alters fiber digestion and microbial protein synthesis in the rumen. The best approach to reduce methane emissions from ruminants is one that could result in improving the productivity of the animal. In this way, methane production per kilogram of meat will be reduced. Increasing animal productivity should result, on a long-term basis, in a reduction of ruminant livestock size and, therefore, in a reduction of ruminants' contribution to the global methane budget.

Finally, authors aim to future strategies such as feed additives and lipids to mitigate methane production from ruminants.

Table 7. Summary of the effectiveness of the different strategies to mitigate methane production (Benchaar et al., 2001)

	CH_4production		
Nutritional-Dietary Strategy	Mcal/day	%GE[1]intake	%DE[2]
Increasing DMI[3] (Kg/day)			
a) With 100% alfalfa hay: from 9.0 to 17.0	1.64→2.81	5.33→4.85	8.43→7.82
b) With 30% alfalfa hay + 70% concentrate (45% corn meal + 55% soybean meal) from 9.0 to 17.0	1.54→2.25	4.21→3.25	5.6→4.65
Change in Forage/Concentrate ratio			
a) 100/0	2.55	4.98	7.99
b) 80/20	2.70	4.83	7.28
c) 50/50	2.61	4.43	6.3
d) 30/70	2.12	3.46	4.87
Replacement of fibrous concentrate with starchy concentrate			
a) Fibrous: beet pulp	2.72	5.07	7.05
b) Starchy: barley	2.34	3.86	5.47
Replacement of barley (RDR)[4] with corn (SDR)[5]			
a) barley	2.94	4.63	6.21
b) corn	2.53	3.91	5.16
Stage of maturity of forage			
a) Midbloom	1.73	3.17	6.71
b) Vegetative	1.80	3.64	5.71
Forage preservation method			
a) Hay	2.93	5.41	8.48
b) Silage	1.97	3.67	6.09
Forage species: legume vs. grass			
a) Grass: timothy hay	1.68	2.85	7.25
b) Legume: alfalfa hay	1.80	3.64	5.71
Forage processing			
a) Grinding	1.98	3.69	6.51
b) Pelleting	1.58	2.91	5.65
Chemical treatment or supplementation of cereal straw			
a) Untreated straw	1.07	1.66	6.10
b) Ammoniated straw	5.35	8.27	12.63
c) Supplemented straw	3.21	5.09	9.11

[1]GE: Gross Energy; [2]DE: Digestible Energy; [3]DMI: Dry Matter Intake; [4]RDR: rapidly degraded in rumen (barley); [5]SDR: Slowly degraded in rumen (corn).

It is clear that methane is an unavoidable by-product of anaerobic microbial fermentation of carbohydrates in rumen. However the importance of ruminants to mankind, in terms of their capacity to transform much of the world's biomass into high quality protein sources (i.e., meat and milk) for human consumption, necessitates the need to continue researching new alternatives, leaving others that are dead or who even oppose the legislation, particularly in the EU, which is very restrictive when it comes to animal nutrition and supplements.

Then we will address the current nutritional alternatives searching to reduce methane production in feedlot.

A. Ionophores and Methanogenesis

It has been believed that antimicrobial reagents such as antibiotics and bacteriocins are able to modify rumen fermentation. Antibiotics including ionophores and non-ionophores have been intensively studied for improvement of feed efficiency and animal health (Nagaraja et al., 1997; Mitsumori and Sun, 2008).

Inhibition of methane production is normally accompanied by an increase in propionate production, and a negative relationship between methanogenesis and propionate production has been clearly established in work on interspecies hydrogen transfer (see review of Moss et al., 2000).

Ionophoric antibiotics such as monensin have been shown to depress methane production by mixed rumen microbes *in vitro*. This decrease in methanogenesis is not due to a direct effect of the ionophores on methanogenic bacteria, but rather results from a shift in bacterial population from gram positive to gram negative organisms with a concurrent shift in the fermentation from acetate to propionate.

Van Nevel and Demeyer (1995) found that *in vivo* monensin depressed methane production by 25% when averaged over 6 studies, however unfortunately some long-term *in vivo* trials have shown that the inhibition of methanogenesis by monensin did not persist, due to monensin also depressing fibre digestion and protozoal growth (Hino and Asanuma, 2003). On the other hand, it has been known that prolonged application of monensin to steers lost its methane-suppressing activity (McCaughey et al., 1997) and rumenbacteria developed readily resistance to ionophores(Newbold et al., 1993).

A recent report indicated that long term administration of monens into dairy cattle stably reduced methane by 7% and that this reduction persisted for 6 months with no adverse effect on milk yield (Odongo et al., 2007). However, beef steers that had been given monensin only showed methane reduction during the first 4-6 weeks of administration. In this sense, the effect of salinomycin on methane production seemed to be more persistent.

The number of rumen protozoa is decreased byionophores and this decrease causes a reduction in methane, because rumen protozoa accommodate methanogens on their cell surface and within the cell (Tokura et al., 1999).

Ionophores are highly lipophilic ion carriers. They pass through the porous peptidoglycan layer of gram-positive bacteria and lodge in the lipid membrane. Therein,

> they destroy ion gradients at the expense of ATP, ultimately resulting in the depletion of energy reserves, impaired cell division, and the likely death of the microorganism (Tedeschi et al., 2003). Many formate and hydrogen-producing bacteria are gram-negative and sensitive to ionophores, effectively preventing the formation of the necessary substrates for methanogens. Bacteria responsible for cross-feeding hydrogen to methanogens are likewise inhibited, resulting in a dramatic reduction in methane production.

Many propionate-producing ruminal bacteria, on the other hand, are not inhibited by ionophores, resulting in an increased proportion of this volatile fatty acid (Callaway et al., 2003). Propionate is very efficiently utilized by ruminants, and thus may enable increased derivation of energy from feed (see review of Beechem, 2011).

> These data suggests that reduction of methane by monensin feeding is not due to a reduction in the population size of methanogens but is more likely due to the development of an alternative hydrogen-consuming pathway such as propionate enhancement by stimulation of the proliferation of propionate- and succinate-producing bacteria such as *Selenomonas* and *Megasphaera* (see review of Kobayashi, 2010).

The efficacy of ionophores in ruminant diets was examined by Guan et al. (2006).

> This study evaluated short- and long-term effects of feeding a single ionophore (monensin) or rotation of 2 ionophores (monensin and lasalocid) on entericCH_4 emissions in 36 Angus yearling steers over a 16-wk period. Their data suggested that the effects of ionophores on enteric CH_4 production are related to ciliate protozoal populations and that ciliate protozoal populations can adapt to the ionophores present in either low- or high concentrate diets. In addition, Rotation of monensin and lasalocid did not prevent ciliate protozoal adaptation to ionophores.

Studies such as those by Guan et al. (2006) proposed the use of ionophores in the list of mitigation strategies considered by the cattle industry because of the benefits provided through improved feed use.

> One peptide ionophore, aibellin, was able to increase propionate production in the rumen without significantly affecting production of total VFA, protozoal survival, or cellulose digestion (Hino et al., 1993; Hino et al., 1994). Apart from antibiotics, bovicin HC5, a bacteriocin from Streptococcus bovis HC5, has shown a capacity to reduce ruminal methane production in vitro (Lee et al., 2002).

But awareness over antibiotic residues in animal products and the threat of bacterial antibiotic resistance in the wider environment has led to an increasing interest in alternatives to antibiotics as growth promoters (Mitsumori and Sun, 2008; Beechem, 2011)

B. Malate and Methanogenesis

The potential of malic acid as inhibitor of methanogenesis is well documented *in vitro* (Carro and Ranilla, 2003; Newbold et al., 2005) and *in vivo* (Lila et al., 2004; Wallace et al., 2006).

> Malate and fumarate are propionate precursors in the succinate-propionate pathway, and by acting as alternative H_2 sinks in the rumen they have the potential to decrease ruminal methanogenesis (Newbold et al., 2005, Figure 4).

Nevertheless, responses to dietary supplementation *in vivo* are highly variable: studies have shown reductions in enteric CH_4 emission after malate supplementation with concurrent increases in molar proportions of propionate (Lila et al., 2004; Mohammed et al., 2004; Newbold et al., 2005). However, reductions in CH_4 emissions through malate supplementation are apparently more likely in diets with a greater concentrate than forage content (Foley et al., 2009).

This effect appears to be more questionable when it comes to fumaric acid. Similar to malic acid, *in vitro* trials have shown the merit of fumaric acid in reducing CH_4 production (Lopez et al., 1999; Newbold et al., 2005; Wallace et al., 2006). Other studies consider that in situations of an increased concentration of H in the rumen, fumarate may be an effective hydrogen sink (Ungerfeld et al., 2007).However, *in vivo* studies no effects of fumaric acid on CH_4 emissions was evident en feedlot (Beauchemin and McGinn, 2006; Kolver and Aspin, 2006; McCourt et al., 2008). Prior studies stated that this effect seems to depend largely on the nature of the fermented substrate, being more effective in forage-based diets than in high-concentrate diets (Carro and Ranilla, 2003). These results may explain why most literature about ruminal methanogenesis in barley-based diets is conducted with malic acid and not with fumarate.

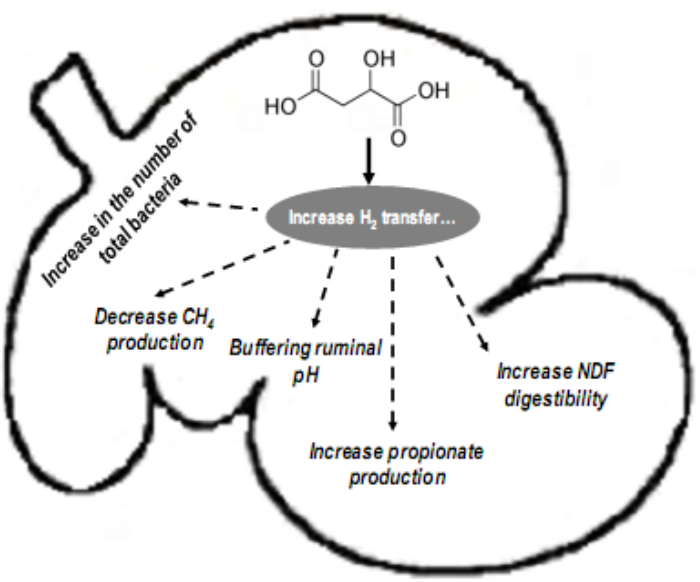


Figure 4. Main effects of malate on ruminal dynamics (from Castillo et al., 2012).

Recent studies (van Zijdervelt et al., 2011a) using Ca-fumarate in dairy cows concluded that effects of Ca-fumarate addition were negligible in a high-grain diet. The authors even concluded that taking into account the large quantity of fumarate that would be required to achieve substantial reductions in methane production, together with its costs and poor palatability precludes the use of this substance as a methane inhibitor in bovine.

C. Dietary Fats and Methanogenesis

Dietary fat is not fermented in the rumen and has been proposed as a promising strategy to reduce CH_4 emissions from ruminants (Eugene et al., 2008, Martin et al., 2010).

This decrease was hypothesized on basis to decrease total tract NDF digestibility (Martin et al., 2008); specifically NDF digestibility was considerably depressed in the rumen and this was partly compensated by the fermentation in the hindgut.

Other studies (Martinet al., 2010) consider that dietary unsaturated fat may affect methane production in several ways: indirectly, through decreased DMI or dilution of fermentable OM; through direct toxic effects on the rumen microflora; or by consumption of hydrogen during biohydrogenation

The net result is that less H_2 per unit of feed is produced when higher fat levels are included in the diets for ruminants.

In ruminants, the fat in the diet can affect rumen microorganisms. The rumen performs functions such as 1) directly absorb fatty acids of 14 carbon atoms (C) or shorter, 2) hydrolyze triglycerides and 3) hydrogenating unsaturated fatty acids and released., long chain PUFA (> 20 C) are more toxic to ruminal microorganisms than intermediate-chain fatty acids. Furthermore, short-chain fatty acids (<14 C) and long-chain (> 20 C) have a reduced digestibility.

It is clear that the anti-methanogenic properties of dietary fats will depend on the type of fatty acid employed (Czerkawski et al., 1966, Ajisaka et al., 2002, see Figure 5).

For the C18:0 fatty acids, inhibition of methane production appears to increase with the degree of unsaturation (Czerkawski et al., 1966).

Specific medium-chain fatty acids have been found to lower methanogenesis *in vitro* (Dohme et al., 2001). Ajisaka et al. (2002) observed significant methane reductions when cyclodextrin complexes of caprylic or capric acids were incubated with rumen fluid *in vitro*.

Recent studies conducted by van Zijdervelt et al. (2011b) where SFA (containing mainly palmitic acid, C16:0) was exchanged for a fat source containing C8:0 and C10:0 or a source containing extruded linseed (rich in C18:2 and C18:3), no significant effects were observed on methane production between treatments, although the results were significantly lower in comparison in those animals without fat intake in the diet.

Classification of fatty acids

One system of fatty acid classification is based on the number of double bonds:

0 double bonds: ***saturated fatty acids (SFA).***

Usually have between 12 and 24 carbon atoms. Examples of SFA are butyric acid (C4:0); caproic acid (C6:0); caprylic acid (C8:0); capric acid (C10:0); lauric acid (C12:0); myristic acid (C14:0); palmitic acid (C16:0);stearic acid (C18:0);arachidic acid (C20:0); behenic acid (C22:0); lignoceric acid (C24:0) or cerotic acid (C26:0)

These saturated fats are found mostly in some vegetable oils, such as coconut and palm oils (tropical oils).

With 1 (***monounsaturated, MUFA)*** or more (***polyunsaturated, PUFA)*** double bond: ***Unsaturated fatty acids.***

The two carbon atoms in the chain that are bound next to either side of the double bond can occur in a *cis* or *trans* configuration. A *cis* configuration means that adjacent hydrogen atoms are on the same side of the double bond; a *trans* configuration, by contrast, means that the next two hydrogen atoms are bound to opposite sides of the double bond.

Examples of MUFA are caproleic acid (C10:1 n-1); lauroleic acid (C12:1 n-3); palmitoleic acid (C16:1 n-7); oleic acid (C18:1 n-9); vaccenic acid (C18:1 n-7); gadoleic acid (C20:1 n-11); cetoleic acid (C22:1 n-11) or erucic acid (C22:1 n-9).

MUFAs fat is found in plant and products, such as olive oil, canola oil, peanut oil, nuts and seeds, and in some plant foods such as avocado. Also MUFAs can be found in fish oil.

Examples of PUFA are linoleic acid (LA, C18:2 n-6); α-linolenic acid (ALA, C18:3 n-3); γ-linolenic acid (GLA, C18:3 n-6); estearidonic acid (SDA, C18:4 n-3); arachidonic acid (AA, C20:4 n-6);eicosapentaenoic acid (EPA, C20:5 n-3); docosahexaenoic acid (DHA, C22:6 n-3); docosapentanoic acid (DPA, C22:5 n-3). Generally, PUFA fats are divided in two main types: *omega-3*(ALA, EPA and DHA) and *omega-6* fatty acids (LA, GLA and AA)

PUFA´s fat is found mostly in plant sources. (safflower, sunflower, soybean, corn, cottonseed).Omega-3 fatty acids are found in foods from plants like soybean oil, canola oil, walnuts, and flaxseed. Omega-6 fatty acids are most commonly found in liquid vegetable oils like soybean oil, corn oil, and safflower oil.

Figure 5. Main classification of fatty acids, and examples.

Other reports (Chung et al., 2011) concluded that anti-methanogenic role of linseed depends on the substrate employed; thus depressive effects on CH_4 emissions of linseed were less pronounced for the hay based diet. On the other hand, including ground linseed in a barley silage-based diet can mitigate enteric CH_4 emissions, but not in a grass hay based diet. Authors attribute these differences to the varied effects of linseed on ruminal fermentation, protozoal populations, ruminal digestion of forages and, possibly, total tract digestibility when silage versus hay is the diet forage.

Effectiveness of long-chain FA in suppressing methane is thought to be proportional to degree of unsaturation of the FA (Giger-Reverdin et al. 2003), although there is some uncertainty about this relationship (Johnson and Johnson 1995).

Medium chain (i.e., C12:0 and C14:0) FA are also effective at reducing methane emissions (Machmüller 2006), but these lipid sources (e.g., coconut oil, genetically modified canola oil) are often cost prohibitive for livestock producers.

Beauchemin et al. (2007) concluded that the addition about 3% lipid to high-forage diets in the form of saturated or unsaturated long-chain FA decreases methane emissions.

In barley-based diets Beauchemin et al. (2007) observed that the effects on methane emissions from growing cattle varied depending on the lipid source, although all were rich in long-chain fatty acids. Thus about 14% less methane was emitted per animal when diets contained tallow or sunflower oil and 33% less methane was emitted when diets contained sunflower seeds, compared with the control diet.

Digestibility of NDF in the total tract decreased by 15% with tallow and by 20% with sunflower seeds compared with the control, with only a numerical reduction from control for sunflower oil. Authors point out that, digestible energy intake was about 4% higher for sunflower oil, but 3% lower with tallow and 12% lower with sunflower seeds, compared with the control.

Therefore, the study shows that feeding saturated FA, such as tallow has no negative effects on fiber digestion. Furthermore, authors suggest that the negative effects of unsaturated long-chains FA on fiber digestion would be attenuated by feeding whole oilseeds that release lipids into the rumen more slowly than oil (Dhiman et al. 2000).

Sunflower seeds contain over 40% fat and, even when fed in the unprocessed form, much of this fat is bio-hydrogenated in the rumen (Gibb et al. 2004).

Another oil, coconut oil, is particularly effective (Dohme et al., 2000). The major component of coconut oil is lauric acid (C14:0), which is more potent in the reduction of methane in a semi-continuous fermenter that simulates the rumen (RUSITEC), more than other fatty acids including palmitic (C16:0), stearic (C18:0) and linoleic (C18:2) acids (Dohme et al., 2001; Kobayashi, 2010).

Lauric acid is inhibitory for Gram-positive rumen bacteria including cellulolytic ruminococci. Therefore, the addition of lauric acid to feed might decrease feed digestibility of a high roughage diet. However, a decrease in feed digestibility would be negligible with the high concentrate diet that is fed to beef cattle. Lauric acid was also shown to depress the metabolic activity of the saccharolytic bacterium *Streptococcus bovis* without affecting its maximal growth. The decreased lactate production by *S. bovis* in the presence of lauric acid may explain the preventive and curing effects of lauric acid on rumen lactic acidosis. These data suggest that lauric acid may not alter the size of a specific bacterial population but may modulate metabolic activity when it is fed over a long period of time (Kobayashi, 2010)

In conclusion, most of the oils and fatty acids that reduce methanogenesis also reduce the ruminal level of protozoa that are known to be *cosymbionts* of methanogens. Therefore, a reduction in protozoan numbers is partly responsible for the decreased methane production induced by oils and fatty acids (Kobayashi, 2010).

D. Yeast and Methanogenesis

Live yeast has not been extensively tested for their effect on CH_4 production (Martin et al., 2010), and the few reports available show contradictory results with increases, decreases or no effects reported (see Newbold and Rode, 2006).

> Oxygen consumption by respiring yeast in the rumen appears to be at least partly responsible for the probiotic activity of yeast cultures (Newbold et al., 1996; see review of Mitsumori and Sun, 2008)

In general terms, a meta-analysis performed by Sauvant et al. (2004) showed no effect of yeasts on CH_4 production.

> The mechanisms by which yeasts would decrease methanogenesis have been suggested by increasing microbial synthesis and by stimulating reductive acetogenesis.

The most widely used microbial feed additives (live cells and growth medium) are based on *Saccharomyces cerevisiae* (SC) and *Aspergillusoryzae* (AO).

> *A.oryzae* has been seen to reduce methane by 50% which was directly related to a reduction in the protozoal population (45%). On the other hand, addition of *S. cerevisiae* toan *in vitro* system reduced the methane production by 10% initially (Moss et al., 2000)

While current evidence for a significant decrease in methane emissions from productive livestock is limited, it is accepted that differences exist between products and *S. cerevisiae* strains and as yet, little or no strain selection has been based on an anti-methanogenic effect. However, yeasts are capable of showing great functional and metabolic diversity and some strains have been reported to decrease CH_4 production *in vitro* (Newbold and Rode, 2006).

Recently (Kobayashi, 2010) a yeast derived surfactant (YDS) has been proposed as a new tool for reducing rumen methanogenesis.

> The surfactant YDS disrupts bacterial cell walls in a selective manner depending on the structure of the bacterial surface. Gram-negative bacteria possess an outer membrane that minimizes bacterial cell damage from such a surfactant. Thus, YDS might also selectively inhibit Gram positive rumen bacteria. Indeed, YDS showed a similar antibacterial spectrum when tested against 13 representative rumen bacterial species. Propionate and succinate producers such as *Selenomonas ruminantium*, *Megasphaera elsedenii*, and *Succinivibrio dextrinosolvens* were tolerant to this material, while hydrogen and formate producers such as *Ruminococcus flavefaciens*, *Ruminococcus albus*, *Butyrivibriofibrisolvens*, and *Eubacterium ruminantium* were sensitive.

Therefore, YDS is believed to shift rumen fermentation toward more propionate and less methane production through selective anti-bacterial activities.

E. Plant Products and Methanogenesis

There is growing interest in the use of plant secondary compounds as a methane mitigation strategy. Very often, the effects on methanogenesis of phytochemicals are inconsistent between *in vitro* and *in vivo* data. The general decrease in methane production associated to plant products is not necessarily connected with the decrease in ruminal protozoa (Broudiscou et al., 2000; Patra et al., 2006). Most positive reports concern the use of chemical families of tannins and saponins, and the heterogeneous group of essential oils (Martin et al., 2010).

The wide variety of active ingredients characterizing these compounds (see Castillo et al., 2012) and their differing mechanisms of action on rumen dynamics, underlines the need for further research (Flachowsky and Lebzien, 2012)

> Windisch et al. (2009) reviewed effects of herbs and spices in animal nutrition and concluded that most studies were conducted with commercial products consisting of several phytogenic substances which do not allow definitive conclusions concerning the mode of action of single substances. Further weaknesses in the studies include very high variability of results because of variations in plant species, vegetation stage, harvest conditions and processing, as well as volatility of some ingredients (Kreuzer et al., 2009).

The anti-methanogenic activity for tannin-containing plants has been attributed mainly to the group of condensed tannins. Hydrolysable tannins, although they also affect methanogens, are usually considered more toxic to the animal and have not been extensively tested (Martin et al., 2010).

> Two modes of action of tannins on methanogenesis have been proposed *in vitro*: a direct effect on ruminal methanogens and an indirect effect on hydrogen production due to lower feed degradation (Tavendale et al., 2005).

In addition, tannins in many plants (e.g., legumes) may reduce ruminal protein breakdown and increase duodenal protein flow when provided at moderate doses (Carulla et al., 2005), increasing the interesting possibility as a mechanism to provide high value feed, whilst lowering methane production.

> However, when given to animals at higher doses they may also adversely affect animal performance, probably due to reduced organic matter and fibre digestibility (Tiemann et al. (2008)

Nonetheless, the effect of condensed tannins cannot be generalised and testing is necessary as in the case of high-tannin sorghum silage (De Oliveira et al., 2007) or in condensed tannin extract from *Schinopsisquebrachocolorado* (Beauchemin et al., 2007) that seems not to be effective in cattle.

Saponins decrease protein degradation and favour -at the same time- microbial protein and biomass synthesis, two processes that result in reduced availability of hydrogen for CH4 production (Dijkstra et al., 2007). However, the mode of action of saponins seems to be mostly related to their anti-protozoal effect (Newbold and Rode, 2006; Martin et al., 2010).

Saponins have a detergent action which disrupts microbial cell membranes by formation of a complex with membrane sterols. Rumen protozoa are particularly sensitive to saponins which reduce their level in the rumen, resulting in the depression of methanogens associated with protozoa. Guo et al. (2007) have suggested that a decrease in methanogens associated with protozoa as exo- and endosymbionts could be the main mechanism by which saponin feeding reduces methanogenesis.

Nevertheless, the antiprotozoal effect of saponins may be transient and is not always accompanied by a decrease in CH_4 production, indicating that other modes of actions are also important.

The effectiveness of saponins seems to be closely connected with their chemical nature. Thus, effective preparations can reduce emissions by 15% to 40% depending on the dose and experimental setting (Hess et al., 2004).

For example, *Yucca schidigera* extract (with a steroidal nucleus) decreases methane production whereas Quillajasaponaria extract (with a triterpenoid nucleus) does not (Pen et al., 2006)

Yucca extract has been shown to decrease the number of rumen protozoa when fed to dairy cows or heifers (van Zijderveldet al., 2011a).

Some of the rumen methanogens live in close association with the protozoa (Newbold et al., 1995, Hegarty, 1999) and yucca extract has been demonstrated to lower methane production in vitro (Lila et al., 2003).

But the effectiveness of yucca products might differ depending on the different forms used. Thus, Lovett et al. (2006), using steers, observed a significant decrease in protozoa numbers in response to yucca extract supplementation (1.2 and 2.6 g/kg of DM), while van Zijderveld et al. (2011a) using higher concentration (3 g/kg of DM) did not observe any effect on methane production, in agreement with the observations of Holtshausen et al. (2009), who reported no differences in the number of protozoa when yucca powder was fed at 10 g/kg DM.

Van Zijderveld et al. (2011a) attribute these differences to saponin concentration of the supplement; higher in yucca extract than in yucca powder.

Finally, other phytochemicals that decrease methane production are the many biologically active molecules present in essential oils (Martin et al., 2010).

Among them, it has recently been shown that garlic oil and some of its components decreased CH4 production in vitro (Busquet et al., 2005a). This was attributed to the

toxicity of organosulphur compounds such as diallyl sulphide and allicin on methanogens.

European scientists have been collaborating in an exploration of plants that might be useful as alternatives to antibiotics for inhibition of methanogenesis in ruminant livestock (http://www.rowett.ac.uk/rumen_up/index.html). Seven products were selected, based on their ability to inhibit methane production by 15-27% without a detrimental effect on total VFA production or feed digestibility.

The plant species selected were the Italian plumeless thistle (*Carduuspycnocephalus*, 30% inhibition), the Chinese peony (*Paeonialactiflora*, 8-53%), the European aspen (*Populustremula*, 25%), the sweet cherry (*Prunusavium*, 20%), goat willow (*Salix caprea*, 30%), English oak (*Quercuspedunculata*, 25%) and Sikkimrhubarb (*Rheum nobile*, 25%).

From these species, 2 species (*Carduus* and *Rheum*) were evaluated in a RUSITEC analysis. On a high forage diet 16 and 22% inhibition of methanogenesis respectively was noted, while less inhibition (5 and 15% respectively) was observed on a high concentrate diet. In both cases, methane reduction was not accompanied by propionate enhancement or other favorable fermentation changes.

Therefore, details of the inhibition, such as the identity of the effective compound and its mechanism of action, remain to be clarified (see review of Kobayashi, 2010).

Recent research in Japan (Kobayashi, 2010) proposes natural materials for the reduction of rumen methanogenesis, such as plant-derived liquid (PDL)

PDL contains anacardic acid, a salicylic acid derivative with an alkyl group that inhibits Gram-positive bacteria including *bacilli* and *staphylococci*. Therefore, PDL is expected to selectively inhibit Grampositiverumen bacteria. Anacardic acid was suggested to be a propionate enhancer in early studies, although this fact has not been highlighted for a longtime.

This material induces a significant reduction in methane production in batch cultures (>95%) and in RUSITEC (>70%) without any adverse effect on feed digestibility or total VFA production. Furthermore, the extent of inhibition induced by PDL is much greater than that induced by monensin or by the materials proposed in the European project.

As occurs with YDS, PDL is believed to shift rumen fermentation toward more propionate and less methane production through selective anti-bacterial activities.

F. What about Increasing Animal Productivity?

In general, when animal productivity is improved through nutrition, management, reproduction or genetics, CH_4 production per unit of meat or milk is reduced. The amount of

feed energy associated with animal maintenance is about 70-75% in beef cattle and 50% in dairy cattle (Mathisonet al. 1998). The remaining feed energy is used for production.

> Thus as productivity increases, CH_4 emissions go up slightly, but CH_4 emissions per unit of product decreases (Johnson et al. 1996). This strategy is widely used in the dairy industry in Canada, where fewer animals are required to produce the same amount of milk, thereby reducing the number of animals maintained in production and the overall CH_4 produced (Boady et al., 2004).

First studies on this matter were showed by Kirchgessner et al. (1995); authors estimated that increasing milk production of dairy cows from 5000 to 10 000 L of milk annually in the EU, by using high grain rations or by improving the genetic merit of the dairy cow, would increase total CH_4 production per animal per year by 23%. However, CH_4 production per kg of milk produced would be reduced by 40%.

> Therefore, overall CH_4 emissions could be decreased by reducing animal numbers while maintaining milk production.

Improving productivity with the use of high grain diets must, however, be evaluated in terms of its cost of production and use of fertilizers and machinery, which will increase fossil fuel use and increase N_2O emissions (Boady et al., 2004).

> The cultivation and use of high-quality forages, which are cheaper than grain and do not involve increased use of fossil fuel through tillage, has proven to be a sustainable option for producers, and an efficient way to decrease N_2O emissions and increase soil carbon stores (Johnson et al. 1996).

Boady et al. (2004) conclude that increasing animal productivity is beneficial in reducing CH_4 production if animal numbers are reduced correspondingly. Continuous improvement of the efficiency of cattle production by nutritional and management strategies is profitable, and also reduces CH_4 production.

But increasing animal productivity to decrease the burden of methane at the cost of forcing animal physiologism seems to go against the new mentality that seeks sustainable livestock, where the animal produce according to their physiological rhythms.

> Until now, however, only few studies tried to determine the integrated environmental impact of conventional versus organic production. Changing from conventional to organic milk production, however, will increase CH_4production because of the, on-average, lower milk production level per cow and increased use of roughage (de Boer, 2003), but associated with intensive farming are other greenhouse gases derivative from combustion of fossil fuels at the farm, and during production and transport of concentrates and artificial fertilizer. Thus, although EU regulations on organic production do not address the use of non-renewable energy resources, organic production is expected to use less fossil fuel per tonne of milk than conventional production, due to the absence of artificial fertiliser and the relatively low use of concentrates in the cow/ steer´s diet.

Various researchers have addressed this important issue. Logically the first option available to organic farmers is to avoid low-quality pastures and forages, which are the feeds associated with higher methane emissions. An added benefit is that the use of these feeds will result in improved growth and milk production, which should lead to increase profits (Blair, 2011).

Use of improved pastures and improved forage quality is an obvious consideration as a means of reducing methane production, but the conclusions obtained in the different investigations, as pointed out by Blair, (2011) are influenced by how the results are interpreted. This will be an interesting matter to research in the fore coming years.

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Chapter 4

THE BARLEY HUSK: A POTENTIAL BARRIER TO FUTURE SUCCESS?

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ABSTRACT

In barley, the husk (hull) adheres to the pericarp, so is retained after threshing. Mutation in a gene on Chromosome 7H leads to non-production of the lipid responsible for adhesion and gives rise to grain that is hull-less (naked). This mutation occurred spontaneously during the domestication of barley and hull-less grain is traditionally preferred in some areas, e.g., in Tibet, where barley remains an important part of the human diet. In more recent times, hull-less barley has been developed in Canada as a feedstock for non-ruminants such as pigs and poultry and its use in malting brewing and distilling has also been investigated. Comparisons with hulled barley have proved difficult, particularly in the UK, where hull-less types, in a suitable genetic background, have not been readily available. There is also a lack of populations derived from crosses between hulled and hull-less types and, as a consequence, some data has been derived from malting barley that has been chemically de-husked. However, the production of some genetic variation in the UK hull-less variety Penthouse, by use of mutation, has permitted some lines with enhanced quality attributes to be identified. As the husk dilutes the contribution of endosperm constituents, hull-less barley provides a means of increasing components that may be of interest for human nutrition or industrial use. In this chapter, existing and potential uses of hull-less barley are reviewed and targets for future breeding programmes are suggested. The influence of environmental conditions on key traits and appropriate agronomic strategies are also discussed.

Keywords: Hull-less barley; genetics; β-glucan; malting; livestock feed; yield improvement

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ABBREVIATIONS

AGP	ADP glucose pyrophosphorylase
DH	Doubled haploid
DP	Diastatic power
EMS	Ethyl methane sulphinate
GI	Glycemic Index
GM	Genetically modified
HvCsl	*Hordeum vulgare* Cellulose synthase-like
LDL	Low-density lipoprotein
QTL	Quantitative trait locus
RS	Resistant starch
SCAR	Sequence characterised amplified region

INTRODUCTION

The developing barley grain is enclosed by two leaf-like structures, the lemma and palea [96], which comprise the husk (hull). The lemma covers the embryo and the dorsal part of the grain and generally extends into an awn at the distal end [96], while the palea covers the ventral part. The lemma and palea, which have a high content of insoluble dietary fibre and ash [106], overlap in most varieties, completely enclosing the developing caryopsis [18]. The husk comprises around 10 - 13% by weight of the dry, mature grain [18, 96]. In most barley varieties, unlike other cereals such as wheat, rice and rye, the husk adheres to the pericarp, so it is retained after threshing, but mutation in a gene on barley chromosome 7H gives rise to hull-less (naked) barley [71], in which the husk does not adhere.

This mutation appears to have arisen spontaneously at an early stage in barley domestication, as hull-less barley was first used for human consumption around 6,500 BC [143]. In some areas of the developing world, especially the more mountainous regions [141] it remains a staple part of the human diet. Historically, barley was an important food crop [94] and six-row hull-less types were cultivated on the European mainland, reaching the British Isles around 3,000 BC [34]. Hull-less barley was also introduced to Norway, along with einkorn, around 4,000 years ago and proved the more successful, probably due to its greater winter hardiness [94]. Barley remained an important food crop in some Northern European countries for many centuries [54, 70, 91], but hull-less types do not seem to have been preferred. That may have been indicative of a growing use of barley for other purposes. Introductions into the eastern part of North America, during the seventeenth century, for example, appear to have been largely for beer production [136], while the Spanish introduced barley into what are now Mexico [31] and the western USA mainly as animal feed, with some use for brewing [94].

The re-discovery of hull-less barley in North America resulted from investigations of the nutritional quality of barley germplasm in western Canada [21]. Initial interest and breeding efforts were concerned with feed for mongastric livestock, such as pigs and poultry, for which the husk is largely indigestible [19]. Hull-less barley also provides more metabolisable energy and better feed conversion rates than hulled barley [22, 106]. However, as removal of the

husk increases the contribution of endosperm components, hull-less barley offers potential advantages for a range of uses including human food and beverage production or bio-fuel [62]. These will be considered in this chapter. A major concern, though, is that removing the husk also reduces the weight of the grain and, consequently, the overall yield of the crop [102]. Increasing productivity will therefore be essential to any hull-less barley breeding programme and potential strategies will be outlined and discussed.

GENETICS OF HULL-LESS BARLEY

The hull-less trait was identified as resulting from a single recessive gene at the *nud* locus on chromosome 7H [48]. Gaines et al., [53] noted that lines carrying the dominant *Nud* allele produced a cementing substance, shortly after flowering, which causes the husk to adhere. Taketa et al., [122] identified this substance as a lipid, with the function of the *Nud* gene being regulation of the biosynthesis pathway. Early chromosome maps of barley e.g., those produced for the first Barley Genetics Newsletter [130] were largely based on morphological mutants and disease-resistance genes [57], so *nud* was incorporated into these. The later development of DNA-based markers greatly facilitated the development of chromosome maps in many species, with Graner et al. [57] noting that barley maps increased in marker number from 32 to 280 between 1951 and 2002. However, integration of molecular data from different populations and different marker systems initially proved problematic, until consensus maps were developed [75], [98]. Kleinhofs and Graner [74] subsequently developed a 'bin' map to able easier integration and molecular and morphological markers were gradually brought together.

Costa et al. [35] placed a number of morphological genes, including *Nud*, on to a well-established molecular map, while, using Sequence Characterised Amplified Region (SCAR) markers, Taketa et al. [123] were able to find one, designated sK17, tightly linked to the *Nud* locus. Three alleles at this locus were present in hulled barley, but only one in hull-less types, suggesting a monophyletic origin for hull-less barley. No hull-less wild forms of barley have been discovered [143], so Taketa et al. [123] postulated that hull-less barley derived from mutation in a domesticated hulled type. The absence of any hulled barley genotypes with the same sK17 allele as hull-less barley could be explained by the subsequent disappearance of this hulled type through human selection for the more palatable hull-less variant [123].

A large number of other traits have been associated with the hull-less phenotype, but these may simply relate to the dilution, by the husk, of components residing elsewhere in the grain. Alternatively, they may result from pleiotropic effects of the *nud* gene, or close linkage to it. An example of this is the content of β-glucan, the major component of barley endosperm cell walls [49, 52]. This is comprised of unbranched, linear polysaccharides of β-D-linked glucopyranosyl residues [139], with mixed linkages of β (1-4) and β (1-3) in a ratio of 70:30. The highly flexible β (1-3) linkages interrupt the regular cellulose-like structure in a random manner [27] resulting in large molecules that are readily soluble in water, creating highly viscous solutions, which are problematic in brewing, but may have beneficial effects in the human gut [26], as will be discussed later in this chapter.

Using whole genome association mapping, Mezaka et al. [86] identified the *nud* locus as affecting β-glucan content. This work was carried out with 89 Latvian barley accessions, of

which 22 were hull-less. Quantitative Trait Loci (QTLs) for β-glucan content have been associated with the same area of chromosome 7H in other studies. Li et al [78] noted a major QTL for β-glucan on chromosome 7 bin 7, where the *nud* gene had been mapped previously [123], while Molina-Cano et al. [88] found the QTL with the largest effect on β-glucan, in the same area. QTLs for β-glucan have been detected on a number of different chromosomes and, in some cases, candidate genes have been identified and confirmed. Burton et al. [29] discovered that cellulose synthase-like (*CslF*) genes were involved in the synthesis of β-glucan. Seven genes were identified and mapped, with four, *HvCslF3, 4, 8* and *10* occurring in a cluster on chromosome 2H [30]. The other three mapped to chromosomes 1H, 5H and 7H respectively, with *HvCslF 6* located very close to the QTL for β-glucan on chromosome 7H. The marker cdo673 was common to the maps produced by Costa et al. [35] and Burton et al. [30] so has been used as a reference point (Figure 1) to illustrate the close proximity of *nud* and *HvCslF6.* It is likely, therefore that this linkage will be difficult to break, and that effects on β-glucan associated with hull-less varieties result from linkage rather than pleiotropy.

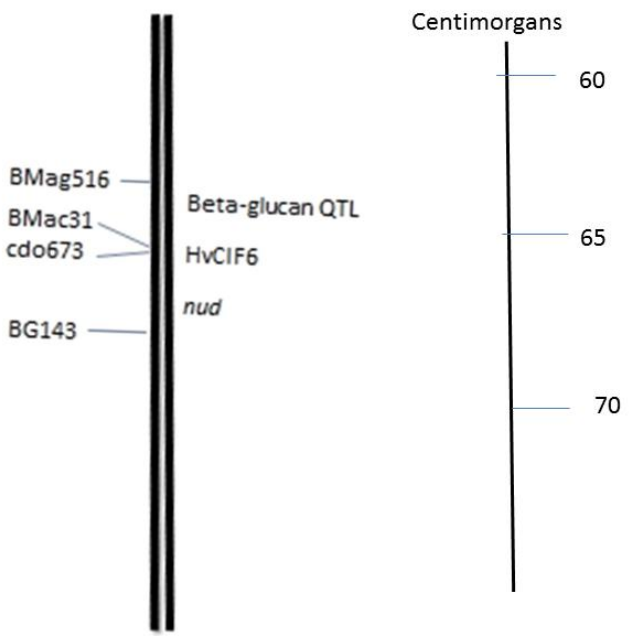

Figure 1. A section of Chromosome 7H of barley, showing the location of the hull-less (*nud*) locus in comparison to the *HvClF6* gene and a QTL for β-glucan detected by Molina-Cano et al. [88]. Markers on the left hand side are derived from linkage maps produced by Costa et al. [35] and Burton et al. [30].

SELECTING FOR LOW β-GLUCAN

Reducing the content of β-glucan has been a consistent target in selecting for improved malting quality in barley, as high levels have deleterious effects in both malting and brewing. They can reduce the rate of endosperm modification during malting [81] as they form the initial barrier to the enzymes responsible for protein solubilisation and breakdown of starch. Grain β-glucan exists in two portions, classed on the basis of their solubility in water [9] or

dilute acid [108] and enzymes synthesised and released during malting can solubilise and degrade around 80% of the total β-glucan content [135]. However, in addition to differences between cultivars, environmental conditions can influence the relative rates at which the soluble and insoluble portions are broken down [46]. High levels persisting into the final malt may be further solubilised during mashing, resulting in increased wort viscosity and reduced rates of filtration [16].

In the developing barley endosperm, the abundance of mRNA transcripts from the *HvCslF* genes can be determined, as an indication of the activity of the encoded enzymes. The highest levels are associated with *HvCslF6* and *HvCslF9*, the latter reaching a peak about 8 days after pollination [30] before declining. Much higher levels of mRNA, compared with those from *HvCslF9*, were detected from *HvCslF6* throughout grain filling, with a significant increase in the later stages. Comparison of a malting variety, Sloop, with the hull-less variety Himalaya, showed the latter to be higher in both β-glucan content and in abundance of mRNA associated with both genes [30]. Given the suggested monophyletic origin of hull-less barley [123] and the tight linkage between *nud* and *HvCslF6*, it would seem likely that many hull-less varieties will carry the same allele at the *HvCslF6* locus, so selecting for low β-glucan will require alternative strategies.

Mutations reducing β-glucan content have been induced and developed in cultivars such as Glenn and Minerva [2]. These have generally been identified by physical effects including thinner cell walls, but have not, to date, been associated with any of the *HvCslF* genes. By contrast an apparent absence of β-glucan has been observed in grain with a mutant allele at the *HvCslF6* locus [124]. This was also characterised by thinner cell walls and a softer grain texture. These mutations were induced in hulled genotypes, but a population of lines was also derived by treating the hull-less variety, Penthouse with the mutagen Ethyl-Methane Sulphinate (EMS) [119]. A number of these lines were included in trial over two seasons and the mean grain β-glucan contents measured in the first season are plotted against those from the same lines in the second season (Figure 2). There was a significant correlation between seasons and variation between genotypes, but although the ranking order of the lines varied slightly between years, no significant genotype x season interaction was detected [117].

None of these lines derived from Penthouse have, to date, been identified as mutants at a locus known to affect β-glucan content and there was no significant correlation between grain β-glucan content and the distilling quality parameters extract and alcohol yield, when the lines were malted, following a standard laboratory regime [119]. A number of the lines, differing in β-glucan content, were, therefore, selected and subsequently malted and assessed under several different steeping and germination regimes [117]. One line, with low grain β-glucan content gave alcohol yields significantly higher than those of both the parent variety, Penthouse and a well-established malting cultivar, Optic, after a slightly abbreviated steeping regime [117]. However, it was not possible to achieve high alcohol yields from any of the hull-less lines after a steep regime that included a single immersion. This may indicate that the hull-less lines derived from Penthouse were in an inferior genetic background to those tested by Agu et al. [4]. These authors were able to achieve good malting samples from two hull-less genotypes, following a steep regime that comprised a single six-hour immersion.

The lines tested by Agu et al. [4] had lower grain nitrogen contents than the Penthouse lines assessed by Swanston et al, [119]. Grain protein and, therefore, nitrogen contents were also high in the comparison made by Bhatty [19] between hulled and hull-less cultivars

during malting. All were steeped to a moisture content of 42% and, although this was achieved more quickly by the hull-less types, two nine-hour immersions, with a single air-rest were required. It is thus likely that increases in steeping time required during malting may result from environmental conditions, under which barley crops are cultivated, particularly those resulting in higher grain protein contents, in addition to inferiority in genetic potential.

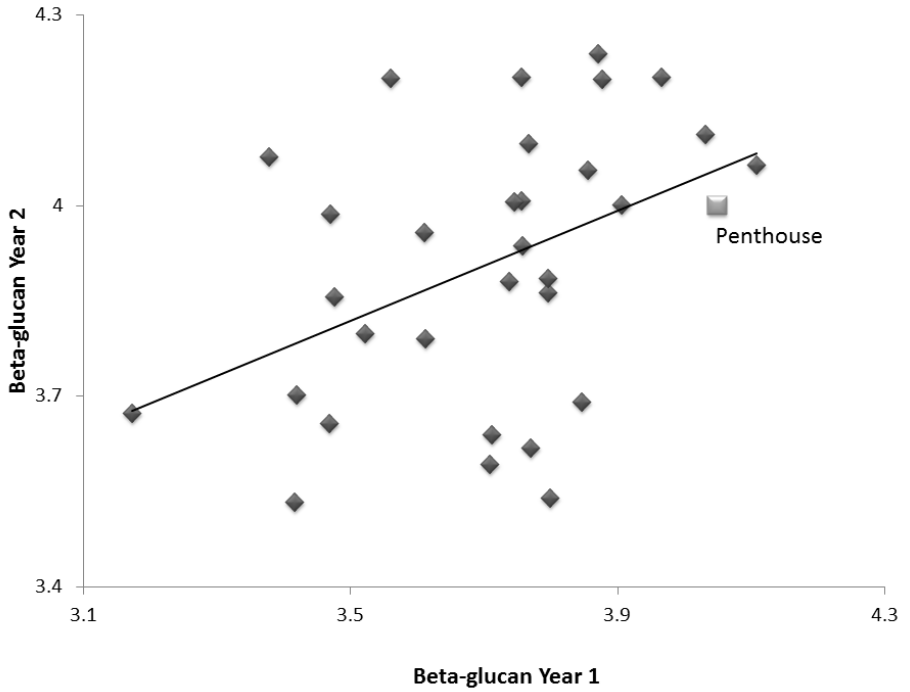

Figure 2. Mean grain β-glucan contents of mutant lines derived from the hull-less cultivar Penthouse grown in replicated trial plotted against mean values derived from the same lines, in the previous season. The correlation between the seasons was significant at the 5% level.

ENVIRONMENTAL EFFECTS ON β-GLUCAN CONTENT

Burton et al. [30] noted that mRNA from *HvCslF6* accumulated throughout grain filling in two varieties grown under Australian conditions. However, data from a range of cultivars, grown in the UK [108], showed synthesis of β-glucan content to be completed a week after maximum grain dry matter was reached, with the soluble portion reaching its final value one to two weeks earlier. This is likely to reflect a difference between the growing environments, as Swanston et al. [115], comparing Scottish and Spanish environments, noted similar patterns of β-glucan development in Scotland to those observed by Smith et al. [108] in the southern part of the UK. There was then a period of desiccation following completion of grain filling, before harvest ripeness was achieved. By contrast, in Spain, where grain development was accompanied by increasing temperature, β-glucan levels increased until harvest [115]. Results over two growing seasons showed Spanish conditions to result in higher total grain β-glucan content, compared to Scottish, with this difference resulting from increased levels of

the soluble portion [115]. This supported earlier data indicating that levels of soluble β-glucan increased considerably in hot, dry environments [89]. Smith et al. [108] suggested that this might be a mechanism to conserve water, by exploiting the capacity of matrix polysaccharides to form aqueous gels [50]. However, McNicol et al. [84] did not find any effect on β-glucan content, when drought stress was applied specifically at a late stage in the grain-filling period, although this was investigated under controlled environment conditions and was not accompanied by an increase in temperature.

MALTING OF HULL-LESS BARLEY

Use of hull-less barley for malting and brewing has been limited due to two main concerns, firstly the need for husk particles to form a filter bed when the wort is run off from the lauter or mash tun and, secondly, the potential for embryo damage during grain handling that might lead to poor or uneven germination during malting. Edney and Rossnagel [41] used a calcofluor test to demonstrate good cell wall modification in a Canadian hull-less genotype, showing that germination had not been a problem and also showed high levels of extract. However, friability scores were low, which Edney and Rossnagel [41] attributed to a more steely endosperm structure with unmodified areas, possibly exacerbated by higher grain protein contents from some locations.

Interest in the potentially higher extracts of hull-less barleys occurred with the development of new methods for wort separation, such as centrifugation or pressure mash filters, removing the need for husk particles [41]. However hull-less barley had been primarily developed for animal feed, in Canada [18], so poor malting traits, such as low friability would be likely in most cultivars. Edney and Langrell [39] noted, however, that adverse effects on friability could also result from the greater susceptibility of hull-less grain to high temperatures during kilning. Edney et al. [40] also observed lower levels of α-amylase in the malt of hull-less lines, from a cross between a hulled and a hull-less breeding line, which they suggested could result from kilning damage. However, it could also have resulted from poorer germination and modification as wort β-glucan levels were observed to be higher in the hull-less lines.

A population of doubled haploid (DH) lines was derived from a cross between a European malting variety, Proctor and the hull-less cultivar, Nudinka and used to demonstrate the presence of a gene for leaf stripe resistance closely linked to the *Nud* locus [97]. However this population does not appear to have been explored for malting quality parameters associated with the hull-less trait and there is a shortage of other populations, with well-documented chromosome maps, involving crosses between hulled and hull-less varieties. The absence of hull-less varieties that had been bred for malting also led to some experiments being carried out by de-husking part of a sample of an established malting variety, then making a comparison with the residual hulled sample [5], [39]. The effectiveness of this process, in giving rise to samples that performed in precisely the same way as hull-less barley, may be questionable, however, as chemical de-husking can remove other tissues [5], [6]. Acid treatment was also shown to influence the alcohol yield of hull-less barley [3].

Subsequently, hull-less varieties, better suited to malting and brewing began to be bred in Europe [110] and one such variety, grown to produce a low grain nitrogen content, gave a

considerably higher extract than the leading hulled malting variety. The hull-less line also had a high level of friability [110], so had not been adversely affected by kilning, but wort β-glucan and viscosity levels were also higher than those of the hulled malting variety. Agu et al. [3] noted a difference in the rate of filtration between an artificially de-husked malting variety and a poorer quality hull-less type and suggested that the extent of modification was a key factor in filtration rate. Achieving wort viscosity levels with hull-less barleys that are comparable with their hulled counterparts will, therefore be important, but that will require lower levels of β-glucan in the wort. Swanston et al. [119], however, did not find a good correlation between grain and malt β-glucan in the lines derived from Penthouse, indicating possible differences in β-glucanase activity, during malting, amongst the lines. Wang et al. [135] observed a similar situation in hulled barley, where malt β-glucan was more closely associated with malt β-glucanase activity than with grain β-glucan content.

The Penthouse lines also differed in the activity of β-amylase [118], one of the enzymes involved in the breakdown of starch and the only one to be synthesised during grain development and persist into the mature grain. During germination the other starch degrading enzymes are synthesised and all are collectively measured as diastatic power (DP). Comparison of several Penthouse lines differing in β-amylase activity showed them also to differ in the rate at which DP increased during malting and in its final level [118]. Edney and Rossnagel [41] also noticed effects of both the growing and malting environments on the DP level of a hull-less variety. Starch degrading activity is particularly important where large quantities of un-malted, starch-based adjuncts are added to a mash. This occurs in some North American brewing [28] and also in the production of Scotch grain whisky [17]. As enzyme production occurs in the endosperm, the husk dilutes activity, so hull-less barley would be of potential interest for such applications [4], [118]. Additionally, malt for grain distilling is generally kilned at lower temperature to preserve enzyme activity [17], [105], so the protection provided by the husk, during kilning [39] will not be required. The whole mash may also be pumped directly to the fermenter, in some Scotch grain distilleries [17], so there is no requirement for husk particles as a filtration aid.

Since filtration problems can largely be overcome, the main concern in the use of hull-less barley for malting is possible damage to the embryo, during grain handling. Although malt samples from hull-less barley, showing good and even modification have been made in both the laboratory [3, 4, 41, 119] and in commercial maltings [110], there still appears to be reluctance on the part of both growers and end-users to make widespread use of hull-less barley. As a consequence there is little incentive for plant breeders to develop hull-less lines with improved quality. Part of the reason may lie in the increased care required for handling the grain. Experience with hull-less barley in Australia has identified adjustments that can be made to combine-harvesters to permit gentler threshing of the grain. This has resulted in samples that show subsequent germination rates of 98-100% (Box, pers. commun), so where barley is grown under contract to supply specific niche markets, such careful handling may be possible [118].

HULL-LESS BARLEY FOR FOOD AND FEED

Although the wild progenitor of barley originated in the Fertile Crescent [73], the crop has been domesticated and cultivated over a wide, global area. Barley can be cultivated at greater latitude and higher altitude than other cereals [51], e.g., around 150,000 ha of barley were cultivated above 3,000m in Peru in 2007, with about 70% used for human consumption [56]. Barley accounted for 65% of total food production in Tibet in 2008, with only hull-less varieties grown [126]. Although frost-tolerant winter barley varieties have been introduced in lower areas, with milder temperatures, over 90% of Tibetan barley is spring sown and has a comparatively short growing season. There is however genetic variation, especially with regard to grain colour, which is exploited in differing uses, e.g., Chang, the main alcoholic beverage, is brewed from purple barley [126].

Although hull-less barley is made into a range of food and beverage products in Tibet, including meal, noodles and tea [126], its main product is *Tsangpa*. This is a roasted grain flour prepared by adding grains to pre-heated fine sand, to roast without burning, before sieving out the sand prior to milling [94], [125]. *Tsangpa* can be mixed with a range of other food products, added to tea and consumed as a beverage, or used as the basis for snack foods [126]. These authors also report that the main Tibetan cultivars are high in β-glucan content. As they represent the only major source, in the Tibetan diet, of dietary fibre, they are also responsible for the lower than expected levels of heart disease and colon cancers in a population that consumes large quantities of meat and dairy products [126].

In developed western countries, human food constitutes a very small part of barley use, e.g., in the USA, it represents around 1.5% of barley, compared to 30% for malting and brewing and 65% for animal feed [93]. However, there is considerable interest in promoting the food use of barley, due to a number of well-established health benefits. The advantages of soluble dietary fibre from cereals, in particular β-glucan, in lowering cholesterol were first established in oats [36]. Later work [83], [95] demonstrated barley to be as good, or better, as a source of β-glucan, so food from both sources can claim to potentially reduce the risk of coronary heart disease [47], so long as they contain at least 0.75g of soluble β-glucan per serving. In barley, β-glucans occur in the aleurone, sub-aleurone and endosperm tissues [140], so represent a higher proportion of hull-less, compared to hulled grain.

The highly viscous solutions created by soluble β-glucan, in the small intestine, slow the absorption of dietary lipids, while β-glucan can also bind bile acids and carry them to excretion, thus causing the body to break down cholesterol to replace them. These two modes of action result in a lowering of blood cholesterol, especially the low-density lipoprotein (LDL) component [69]. The viscosity in the gut, caused by soluble β-glucan, also slows the absorption of carbohydrates, avoiding the sharp peaks in post-prandial blood glucose levels [101]. The range in blood sugar responses is the basis of the Glycemic Index (GI) [68]. When sharp increases in blood glucose occur, the pancreas responds by rapidly releasing insulin. Regular consumption of foods with a high GI can result in insulin resistance and may also lead to type 2 diabetes [14].

Recent studies of the glycemic lowering effects of barley have focused on the ratio of amylose and amylopectin, within barley starch, in addition to β-glucan content [14]. Amylose molecules, when subjected to heat and moisture, may form gels or insoluble precipitates, as a result of linking together, through hydrogen bonding. This results in resistant starch (RS),

defined by Asp [12] as the fraction of starch that is resistant to digestive enzymes and enters the large bowel of healthy humans as an intact polymer of large molecular weight. Bjorck et al [23] noted an increase in RS and a decrease in *in vitro* digestibility of barley flours with increasing amylose content. Reduced blood sugar response was subsequently demonstrated in human subjects as RS increased from 2 – 10% in breads prepared from barley flours with increasing proportions of amylose [8]. An increased proportion of amylose, from around 25% in normal barley starch to 40% was first observed in a spontaneous mutation in the variety Glacier [85]. This was later shown to be a recessive allele in a gene on barley chromosome 1H [104]. Barley starch comprises both large (A-type) and small (B-type) granules and the high amylose character is associated with a reduction in size of the A-type granules [44], [82], which can then become more heavily embedded into the surrounding protein matrix [112]. A larger increase in the proportion of amylose, to around 70% of the starch content, was later observed in a mutant of the variety Himalaya [90]. This was suggested to result from modification of the starch synthase *IIa* gene and was located to chromosome 7H.

A gene mutation, with a different effect on starch composition, that has been mapped to the short arm of chromosome 7H, is waxy (*wx*). It reduces but, unlike the waxy mutation in maize, does not eliminate the activity of granule-bound starch synthase [45]. Consequently the proportion of amylose in barley starch is greatly reduced, to between 2 and 10% [128] and this will result in a reduction in RS. Both the waxy and high amylose mutations are associated with increased levels of β-glucan, but this may result from a reduction in starch content with alternative partitioning of a proportion of the photosynthate [108]. Swanston et al. [116] noted that combination of the two mutations had an additive effect in increasing β-glucan content, but reducing the content of starch which could be extracted from barley grain (Figure 3).

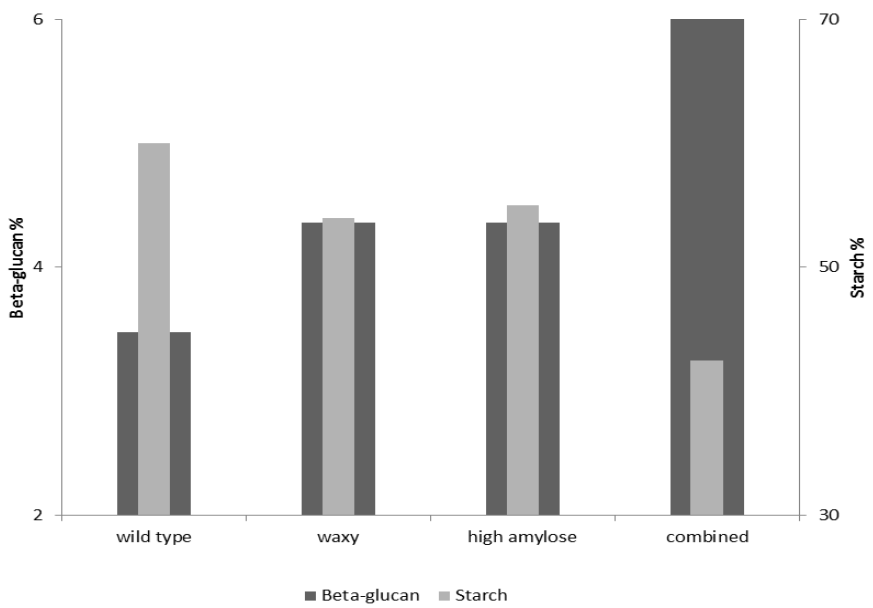

Figure 3. Mean starch and β-glucan contents in inbred lines from a cross between a high-amylose and a waxy barley variety. Data are taken from Swanston et al. [116].

Either mutation could also be readily combined with the *nud* gene, as the waxy gene is sufficiently distant on Chromosome 7H to permit ready recombination [88]. Box and Eglinton [25] reported that hull-less types with both waxy and high amylose starch have been bred in Australia and major health benefits were likely to result from increased β-glucan combined with the higher RS of the high-amylose type, although further agronomic development was required. However, Izydorczyk et al. [66] noted that β-glucan solubility tended to be lower in high amylose, compared to normal and waxy barleys. This would, potentially, reduce the benefits associated with soluble β-glucan, but solubility could be increased by the addition of protease or esterase during extraction. Whilst it is possible to readily combine high levels of soluble fibre and resistant starch in hull-less barley, the loss of hull and the reduction in starch content will lead to smaller grain size and an overall reduction in yield.

A similar situation occurred with the combination of hull-less and waxy with the short-awned mutation, *lk2*. Ullrich et al. [132] observed these genes to have additive effects on β-glucan content, but they also had an additive effect in reducing thousand kernel weight [113]. Strategies to address this yield loss will be considered later in this chapter, but the concentration of desirable traits will have advantages where barley is used as a food ingredient. The potential of barley as a partial replacement for wheat in bread-making has been assessed [94] and, to achieve an acceptable loaf volume, the upper limit for substitution is generally regarded as 30%. Using a barley with both high amylose and high β-glucan has been considered [80] although the line available was not an established commercial cultivar, so breeding efforts in this area would be required. Attention also has to be paid to processing, as the average molecular weights of β-glucans decrease with increased mixing and fermentation times [11]. Maintaining high molecular weights is important to retain the cholesterol-lowering effect, so modified baking processes have been developed [67].

Processing barley for food use normally includes some degree of pearling [14], in which outer tissues are progressively removed. Blocking or de-hulling is a form of abrasion designed to remove the husk but, due to differences in kernel size within a sample and the strength of adhesion between husk and pericarp [14], other tissues are frequently affected. By contrast hull-less barley can be directly rolled, flaked or milled although it is frequently pearled for specific markets or applications. Pearled barley is used mainly in soups, but can also be mixed with rice when the latter is in short supply, so is an important rice extender in parts of Asia [42]. Such mixtures can also give health benefits such as cholesterol lowering or glycemic control [63] as pearling increases the proportions of both β-glucan and starch [13].

Globally, the largest single use of barley is feeding to livestock, even in countries like Morocco, with relatively high levels of human food use. Here, approximately 80% of the barley grown is used to feed animals [10]. Desired attributes are, however, different from those required to improve human health e.g., high levels of β-glucan have been associated with problems when barley has been incorporated into poultry feed. Increased viscosity in the gut can reduce uptake of nutrients [138] and also result in ‘sticky’ faeces [55]. These problems have generally been addressed by the addition of enzymes, particularly β-glucanase, in feed preparations [32, 33]. Reduced β-glucan levels were also observed through the use of transgenic barley expressing both heat- and pH-tolerant β-glucanase [137], but the cultivation of genetically-modified (GM) crops remains a controversial issue in many parts of the world.

Although modifying the rate and timing of nitrogen fertiliser application can increase the quantity of barley grain protein, the nutritional quality remains poor due to the high horde in

content [37], with its low proportion of essential amino-acids. Some breeding, to improve protein quality, was attempted using high lysine or low hordein mutants such as Hiproly [92] and Riso 1508 [65], but pleiotropic effects of the high-lysine genes, particularly shrunken endosperms, resulting in lower kernel weight and reduced yield [38], remained problematic. No high-lysine varieties were released to farmers, despite the breeding effort in the 1970s and 1980s [131], and cereals are largely used, therefore, as a source of carbohydrate. The most important grain characteristics of barley for cattle feed are high starch content with low levels of protein and fibre [24]. This is similar to requirements for malting barley and associations between malting and feed quality have been observed [61], 87].

High levels of low-grade protein increase the amount of nitrogen excreted in manures [77], so lower protein content is also advantageous for barley fed to non-ruminants. In addition to nitrogen, some environmental pollution can occur through the secretion of phosphorus in the manures of monogastric animals. Non-ruminants are unable to degrade phytate [127], which is the main storage compound for seed phosphorus [79]. The production of barley mutants with low grain phytate content [100] have enabled breeding for low phytate barleys to be undertaken in Canada and the USA [131]. A total of six mutations reducing phytic acid have been detected and mapped to several barley chromosomes [142]. Marker-assisted selection has been possible as there is close linkage of the *lpa1-1* mutation to two molecular markers on chromosome 2H [76]. This mutation reduces phytic acid phosphorus by 50 -75% compared to the wild type [131] and has been crossed with both hulled and hull-less cultivars [99]. From comparison with a syntenic region of the rice genome, a gene encoding a sulphate transporter has been identified as a putative candidate gene for the *lpa1-1* locus [142].

NOVEL USES FOR HULL-LESS BARLEY

As indicated earlier in this chapter, barley is used extensively in the production of alcoholic beverages, but this usually occurs after the grain has been malted. Malted barley thus acts as a source of enzymes to degrade its own starch reserves or those of an adjunct. Un-malted barley has not been widely used as an adjunct in Scotch grain distilling as it has a considerably lower alcohol yield than either maize or wheat (Table 1).

Table 1. Typical husk percentages, starch contents and alcohol yields from three different cereals (starch and alcohol yield data from Walker, [134])

Cereal	Husk Content (%)	Starch Content (%)	Alcohol Yield (litres/tonne – dry wt.)
Maize	0	71	439
Wheat	0	63	430
Barley	10-13	55	347

This is largely due to its husk, which contributes nothing to alcohol production and which can also inhibit disintegration of the grain during the cooking process [134], impeding access to the starch. The husk and the soluble β-glucan component also create a number of problems

in processing [134]. Hull-less varieties therefore offer considerable advantages [105], particularly where extraneous beta-glucanases can be added to the mash. This is not permitted by the legal definition of Scotch whisky [43], but has been incorporated into assessing hull-less barley for fuel ethanol production [64].

Fuel ethanol has been produced in the USA for many years, using maize as the substrate [103], while more recent interest in parts of Europe has largely focussed on wheat [109]. In areas where maize is not cultivated, hull-less barley could be a potential alternative to wheat, especially where its earlier maturity would be advantageous [64]. The higher viscosity associated with barley mashes, compared to wheat, can be resolved by the addition of β-glucanases [64], even where high-gravity techniques are employed [129]. High-gravity mashes have a higher ratio of milled grain to hot water, reducing both water requirement and energy usage and Thomas et al. [129] were able to obtain an alcohol yield of 443 litres/tonne from a hull-less barley cultivar. This is broadly comparable with alcohol yields in wheat and the residual grain material provides a better feed for both ruminant and non-ruminant livestock [64] due to the higher levels of lysine in barley compared to wheat. Alcohol yields were not however increased by fermentation of glucose derived from β-glucan breakdown [64]. Thomas et al. [129] postulated that this could result from partial inhibition of β-glucanase by the glucose already in solution, so resolving this problem would make hull-less barley an even better feedstock for ethanol production.

Work in wheat [72] has shown alcohol yield to be dependent on both variety and environment, as there is a very strong negative correlation between alcohol yield and grain protein content. Similarly, breeding and agronomic approaches could optimise alcohol yield from hull-less barley, but enhancement of grain yield will also be required to optimise alcohol yield per hectare. Otherwise, the energy required for growing a crop and, in particular, for processing it into ethanol [121] could leave a negative energy balance, when the energy generated by the fuel ethanol is calculated.

The concept of using extraneous enzymes with a largely or completely un-malted cereal can also be extended to alcoholic beverages. The use of enzymes in brewing has been considered by Bamforth [15] as there are advantages from a significant reduction in resources, such as energy and water, required for malting in addition to less time and cost. Aastrup [1] described a beer made entirely from un-malted barley, using a commercially developed product to simulate the action of malt enzymes. Enzymes present in the un-malted grain e.g., the exo-proteases and β-amylase are not, however replaced and work in synergy with the extraneous enzymes [1]. If mash filters are deployed, husk particles are not required to form a filter bed, so hull-less barleys would appear to be ideally suited for this type of brewing [118], especially as removal of the husk will also increase the proportion of starch and the content of enzymes within the grain.

Mutation breeding has been used in an attempt to increase starch degrading enzyme activity [58] and higher levels of β-amylase were also observed in mutants derived from the variety Triumph [120]. A number of lines derived from the hull-less variety, Penthouse were also assessed for β-amylase activity over two seasons [118]. When data from the first year were plotted against those from the second (Figure 4), there was a significant correlation and a number of lines were shown to be considerably higher than the parental variety in both seasons. However, analysis of variance [118] showed a significant genotype x season interaction, in addition to highly significant effects of genotype and season. These lines also

showed considerable variation in quality parameters after malting, but they have not yet been assessed for brewing as un-malted grain samples.

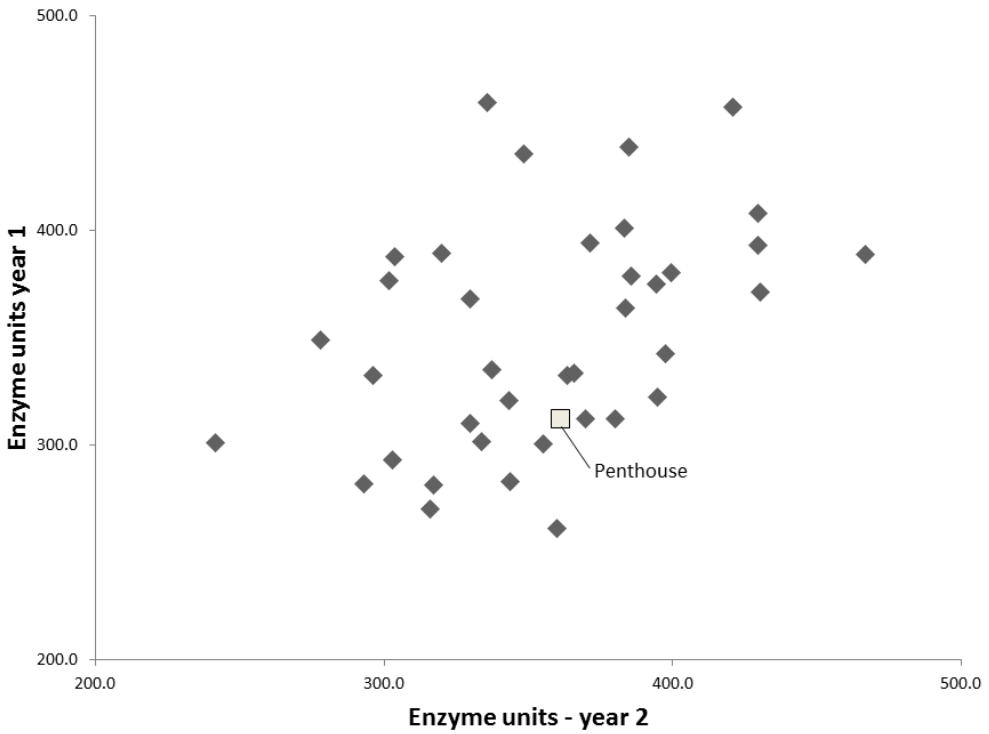

Figure 4. Grain β-amylase activities for 38 mutant lines in the hull-less variety Penthouse over 2 seasons. Values from year 1 plotted against those from year 2 have a correlation coefficient, r = 0.433, significant at the 1% level. The parent variety Penthouse is indicated by the open square symbol.

YIELD IMPROVEMENT IN HULL-LESS BARLEY

Although hull-less barley breeding has improved yields in Canada [20], Australia [25] and parts of Europe [133], it is still lower than that of hulled barley, as loss of the husk during threshing removes 10% of the grain yield. For human food and for feeding to non-ruminant livestock, the husk provides no useful function. Additionally, if barley is used for alcohol production, without prior malting, the only requirement for the husk is to provide a filter bed, in situations where that is necessary, although it will dilute the content of starch and, consequently, the yield of alcohol. In many respects, hull-less barley is a superior product to the husked alternative but, in the absence of premium payments to grain producers, the loss of yield is a significant concern. Spunar et al. [111] concluded that, in Europe, spring barley will remain the dominant crop for malting, due to its superior quality compared to winter barley, although the latter has a higher grain yield potential. As hull-less barley is unlikely to compete for the premium malting market, due to the risk of uneven germination and modification, one approach would be to incorporate the hull-less trait into the winter crop. This would offer the further advantage of an earlier harvest, enabling hull-less barley to be

harvested prior to the main malting barley crop. If hull-less winter barley became the dominant crop for food, feed and fuel use, it would also reduce the need for regular re-adjustment of the combine-harvester, to optimise threshing for hulled and hull-less barley.

A transgenic approach to increasing grain yield was proposed by Smidansky et al. [107], by enhancing ADP glucose pyrophosphorylase (AGP) activity. Wheat was modified with a gene from maize that coded for an altered large sub-unit of AGP and increased grain yields were observed, but the experiment was restricted to glasshouse conditions. In theory, a similar approach could be taken in barley, although successful transformation is more difficult in barley and appears to be very strongly influenced by variety [59]. In addition, the cultivation of GM crops and the use of GM products for human nutrition are not currently accepted in many countries, including those within the European Union (EU).

Heterosis represents a potential genetic mechanism to enhance yield, but because barley is a self-pollinating crop, developing systems for commercial-scale production of F1 hybrid seed has proved difficult until comparatively recently. However, a system employing cytoplasmic male sterility with a restorer gene, based on that first discovered by Ahokas [7], has been employed since the 1990s by a commercial barley breeding company in the UK. This has produced a succession of six-row winter feed varieties, which have given the highest grain yields in UK national trials [60]. F1 hybrids produce F2 seed, which thus segregate for genetic traits, so hybrid varieties would not be acceptable for purposes such as malting, where all the grain within a batch are required to modify in a homogeneous manner. However the hull-less trait is recessive, as are a number of mutant genes that have positive effects on food or feed quality, such as high amylose or low phytate. Additionally, the mutant gene, derived from the cultivar Chalky Glenn, which reduced β-glucan in waxy starch lines to the level of wild type barley [114], is recessive and could be introduced into hull-less lines intended for poultry feed or fuel ethanol production. If both parents, used to create the F1 hybrid, were, therefore, homozygous recessives at all the desired loci, it would be possible to incorporate high yield along with greatly increased utility for a number of applications.

CONCLUSION

For most applications, where grain does not require to be malted, hull-less barley would appear to be superior to the husked alternative. The increased starch content of the grain makes it a higher energy feed source for livestock and protein content is also increased by removal of the husk. For alcohol production from un-malted grain, whether in brewing or for vehicle fuel, hull-less barley offers a much higher yield of alcohol than covered barley, with a nutritionally superior by-product compared to wheat. However, the higher levels of β-glucan remain problematic and require the use of extraneous enzymes. A current challenge involves effectively converting the sugars produced from β-glucan breakdown into enhanced ethanol production for fuel use. Removing the husk increases the proportion of endosperm components within the grain, so hull-less barley is likely to become an increasingly important constituent of human food, particularly with the inclusion of high β-glucan and high levels of resistant starch. Although direct consumption of barley-based foods has declined, except within some of the more mountainous areas, barley is consumed, as described in this chapter, in combination with other cereals and this has considerable potential to increase. The loss of

around 10% of grain yield in threshing makes hull-less barley inherently lower yielding than covered barley, but methods for considerably improving grain yield, while maintaining the utility and versatility of the crop have been outlined in this chapter. Hulled, spring barley is likely to remain the dominant crop for malting, but the greater versatility of barley may be best expressed in hull-less genotypes.

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Chapter 5

GRAIN AND ENDOSPERM BIOPHYSICAL CHARACTERISTICS OF BARLEY MALTING

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ABSTRACT

Cultivated barley (*Hordeum vulgare*), derived from its wild progenitor Hordeum vulgare ssp. spontaneum, is among the world's earliest domesticated crop species, adapted to diverse environments and today represents the fourth most abundant cereal in both area and tonnage harvested. Malting quality is an economically important character of barley. Malsters require grains of known varieties with consistent quality and specify strict quality criteria for accepting new varieties. As starch is the major component of the barley grain changes in its biophysical or biochemical properties will have a direct effect on its malting properties. In order to achieve good malting quality, extensive endosperm breakdown, or modification, must accompany the germination phase of malting, with cell walls and protein being degraded and the starch granules exposed during the process. Hot water extract (HWE) of the resultant malt should yield a high of level of fermentable sugars from readily gelatinised starch granules that are depolymerised by malt amylases. Many characteristics or properties of the grain and endosperm drive these characteristics among them starch (amylose and amylopectin), protein (hordein composition), lipids content, fatty acid profile, hardness, particle and granule size. However, other biophysical properties or characteristics of the grain can also explain or modulate malting properties such as starch pasting properties (e.g., viscosity), water uptake, cell wall content, and interaction of chemical constituents (e.g., amylose and lipids). This chapter will provide an overview and discussion of some of the biophysical characteristics of barley grain and endosperm that have been reported to have an influence on malting quality. The main focus of this chapter is on starch characteristics, chemical interactions with the main grain chemical components and water uptake.

Keywords: Endosperm, water uptake, viscosity, malting

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ABBREVIATIONS

DH	double haploid
DMD	dry matter digestibility
FV	final viscosity
HWE	hot water extract
HPV	hot paste viscosity
OC	osmolyte concentration
PT	pasting temperature
RVA	Rapid Visco Analyser
SP	swelling power
MAS	marker-assisted selection
THR	trough
TTP	time to peak

INTRODUCTION

Cultivated barley (*Hordeum vulgare*), derived from its wild progenitor *Hordeum vulgare* ssp. spontaneum, is among the world's earliest domesticated crop species and today represents the fourth most abundant cereal in both area and tonnage harvested (http://faostat.fao.org). Approximately three-quarters of global production is used for animal feed, while 20% is malted for use in alcoholic and non-alcoholic beverages, and 5% as an ingredient in a range of food products (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011).

Barley is widely adapted to diverse environmental conditions and is more stress tolerant than its close relative wheat (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011). As a result, barley remains a major food source in poorer countries, maintaining harvestable yields in harsh and marginal environments (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011). In more developed societies it has recently been classified as a true functional food, mainly due to the fact that barley grain is particularly high in soluble dietary fibre, which significantly reduces the risk of serious human diseases including type II diabetes, cardiovascular disease and colorectal cancers that afflict hundreds of millions of people worldwide (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011).

Malting quality is an economically important character of barley and it is recognised to be a complex character controlled by many genes (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011; Evans et al., 2009; Wang et al., 2010). This makes malting quality difficult to manipulate genetically in breeding programs. Malsters require grains of known varieties with consistent quality and specify strict quality criteria for accepting new varieties (Emeberi et al., 2004). The principal requirement is for high malt extract potential, a general measure of soluble materials obtained from mashing malt into wort (Emeberi et al., 2004). During the brewing process, degradation products from starch are central in providing substrates for the fermentative phase; where the fermentative sugars contribute to malt extract production (Evans et al., 2009; Wang et al., 2010). Consequently the physicochemical properties of starch have a significant impact on the resultant malt quality and brew house

performance (Evans et al., 2009; Wang et al., 2010). As starch is the major component of the barley grain, changes in its biophysical or biochemical properties will have a direct effect on its malting properties. In order to achieve good malting quality, extensive endosperm breakdown, or modification, must accompany the germination phase of malting, with cell walls and protein being degraded and the starch granules exposed during the process. Therefore, the hot water extract (HWE) of the resultant malt should yield a high of level of fermentable sugars from readily gelatinised starch granules that are depolymerised by malt amylases (MacGregor et al., 1994; Swanston and Taylor, 1988 and 1990; Swanston et al., 1995, 2006; Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011). Many characteristics or properties of the grain and endosperm drive these characteristics among them starch (amylose and amylopectin), protein (hordein composition), lipid content, fatty acid profile, hardness, particle and granule size (Jadhav et al., 1998; Baik and Ullrich, 2008; Blake et al., 2011).

However, other biophysical properties or characteristics of the grain can also explain or modulate malting properties such as starch pasting properties (e.g., viscosity), water uptake, cell wall content and the interaction of chemical constituents (e.g., amylose and lipids) (MacGregor et al., 1994; Swanston and Taylor, 1988, 1990; Swanston et al., 1995 and 2006). Table 1 summarises the chemical and biophysical characteristics reported by several authors in barley grain and endosperm that influence malting.

This chapter will provide an overview and discuss some of the biophysical characteristics of barley grain and endosperm that have been reported to have an influence on malting quality. The main focus of this chapter is on starch characteristics, chemical interactions with the main grain chemical components and water uptake.

Table 1. Chemical and Biophysical characteristics of barley grain and endosperm that influence malting quality

Chemical characteristics	Property or trait
Starch properties	*Viscosity, pasting temperature, time to peak*
	Amylose and amylopectin ratios
	Starch granules (A and B)
Lipids	*Lipid content*
	Fatty acid profile
Protein	*Total protein*
	Hordeins profile, composition
	Soluble protein
	Amino acids
Interactions	*Amylose and lipids*
	Water and composition
Biophysical properties	*Hardness*
	Steeliness / mealiness
	Density
	Water uptake and distribution
	Particle size and distribution

Table 2. Published references relating chemical and biophysical characteristics with malting

Chemical or biophysical characteristics	Reference
Steeliness / mealiness	Darlington et al., 1996; 2000 Swanston and Taylor, 1990; Swanston et al., 2006 Chamberlain, 2000 Chandra et al., 1999
Protein/nitrogen	Agu, 2003; Agu et al 1998; Agu et al., 2001 Molina-Cano et al., 2002; Swanston et al., 1995 Leach et al., 2002
Pasting properties (viscosity, pasting temperature, time to peak)	Zhou et al., 2008 Wang et al., 2010 Cozzolino et al., 2012
Water uptake	Swanston and Taylor, 1990; Schillbach and Rath, 2004; Swanston et al., 2006; Mayolle et al., 2012; Cozzolino et al., 2013
Hardness	Gamalatha et al., 2008

CHARACTERISTICS OF THE STARCH GRANULE

Starch in the form of large (type A) and small (type B) granules are a main component of the endosperm of barley caryopses (DeHaas and Goering, 1983; Whittam et al., 1990; Oliveira et al., 1994; Evers et al., 1999; Schildbach and Rath, 1994; Chamberlain, 2000; Leach et al., 2002; Perez and Bertoft, 2010; Ferrari et al., 2010). According to different reports, the small starch granules generally make up approximately 90% of the total number of granules present, but only 10% of the total weight of the starch (Whittam et al., 1990; Schildbach and Rath, 1994; Chamberlain, 2000). The small starch granules tend to quickly become embedded in the protein matrix and inside the cell walls and might interfere with the production of malt of good quality (Schildbach and Rath, 1994; Chamberlain, 2000; Ferrari et al., 2010). Other authors have suggested that only approximately one-half of the small starch granules are degraded during the course of malting and kilning, determining that approximately 5% of the total starch content is not degraded to fermentable saccharides, which is important for subsequent conversion of sugars into alcohol (MacGregor, 1991; Evers et al., 1999; Schildbach and Rath, 1994; Chamberlain, 2000; Leach et al., 2002). The remaining small starch granules can form a starch haze and block filtration beds during processing and filtration (Evers et al., 1999; Schildbach and Rath, 1994; Chamberlain, 2000; Leach et al., 2002; Ferrari et al., 2010).

Release of starch granules from the protein matrix is also affected by the level of endosperm mealiness or steeliness (Schildbach and Rath, 1994; Chamberlain, 2000). Mealy grains release starch granules more easily than glassy granules (Ferrari et al., 2010). Starch granules in the endosperm of cereal grains are embedded to varying degrees in a protein matrix. Brennan and collaborators (1996) found that the starch-protein binding matrix is stronger in the poor malting quality varieties than good malting quality varieties.

It has also been reported that the physical characteristics of the barley endosperm significantly impacts on the processing behaviour of barley, where its compactness (structure of the endosperm is referred to as steeliness or mealiness) has been regarded as a useful indicator of malting quality in barley (Darlington et al., 1996 and 2000; Ferrari et al., 2010). Loosely packed endosperm gives soft (mealy) structure and facilitates better moisture and enzyme movements in the endosperm. Consequently, a mealy endosperm is more easily degraded by hydrolytic enzyme during malting (Swanston et al., 1990, 1995 and 2006). The endosperm of steely kernels is tougher and denser in structure and shows a slower rate of water uptake during steeping as well as poorer distribution of enzymes during malting (Chandra et al., 1999; Ferrari et al., 2010). Mealy kernels absorb water more rapidly than steely kernels and require less time to attain predetermined moisture content and tend to malt faster and modify better than steely kernels (Schildbach and Rath, 1994; Chamberlain, 2000; Ferrari et al., 2010).

Therefore, the steeliness appears to be a major factor restricting the uptake and distribution of water across the endosperm during steeping, where higher total protein content may not be an important factor in controlling water uptake of the kernel since high nitrogen content does not necessarily correlate with the steely areas which act as water barriers in the endosperm (Chandra et al., 1999; Ferrari et al., 2010).

ENDOSPERM HARDNESS

Even though barley varieties have not been reported as having extreme endosperm hardness values compared to that typical of hard and soft wheats, a small decrease in barley endosperm hardness through conventional breeding may translate to improved quality for the both the feed and malting industries with endosperm hardness variation (Srichuwong and Jane, 2007; Panozzo et al., 2007; Gamlatha et al., 2008).

It has been reported that differences in endosperm hardness between parents of a double haploid (DH) population (Arapiles x Franklin), were between 6.4 and 8.7 SKCS units collected from two localities of Victoria (Australia) from the same harvest (2001) (Esperance and Tarranyurk) (Panozzo et al., 2007). However, other studies using a barley DH population (Steptoe x Morex) did not significantly diverge from each other in endosperm hardness (Beecher et al., 2002). In another report, a population derived from Steptoe and Morex were not different in endosperm hardness and their progeny varied by 39.5 SKCS units. Small but significant negative correlations ($r = 0.37$) were reported between SKCS hardness and dry matter digestibility (DMD) using data from two harvests (Beecher et al., 2002). On average, this translated to an increase of 1% in DMD for every decrease in endosperm hardness of 5 SKCS units within the DH progeny (Beecher et al., 2002). Negative genetic correlations were also reported between SKCS hardness of barley and malt friability (0.60 to 0.23) (Osborne et al., 2007).

The involvement of the functionality of the *hordoindoline* has been suggested to be involved in the barley hardness as reported by various authors (Panozzo et al., 2007). For example in the Arapiles x Franklin population; both parents are highly selected commercial malting varieties, and this is most likely why there is a lack of variation in the nucleotide sequence for the three genes at the *Ha* locus (Wilkinson et al., 2008). The variation in

hardness values, as measured by the SKCS, could be a result of a combination of other traits in barley, such as husk thickness and husk adherence to the endosperm, β-glucan content, arabinoxylan content, protein composition and content, or grain diameter (Panozzo et al., 2007). Any of these grain components may have a greater influence on the SKCS hardness value than the functionality of the *hordoindolines* (Beecher et al., 2002; Fox et al., 2007a and b; Panozzo et al., 2007; Edney and Mather, 2004; Emebiri et al., 2004; Laidò et al., 2009).

Grain hardness has been also associated with other characteristics such as milling energy, grain splitting, grain weight, size,shape and β-glucan (Han et al. 1995, 1997 and 2004; Gamlatha et al., 2008; Burton et al., 2008; Emebiri, 2009). Grain size, 1,000 grain weight or percent screenings, and grain protein percent have been reported in several studies influencing endosperm hardness in both barley and wheat (Coventry et al., 2003; Walker et al., 2008; Fox et al., 2007; Psota et al., 2007; Beecher et al., 2002).

STARCH PASTING PROPERTIES

Starch pasting properties are influenced primarily by amylose and amylopectin contents (Batey, 2007; Booth and Bason, 2007). Several authors have reported the use of the Rapid Visco Analyser (RVA) instrument as a method for determining barley and malt quality in routine analysis or as selection tool in breeding programs (Batey, 2007; Bason and Blakeney, 2007; Booth and Bason, 2007; Zhou et al., 1998; Zhou et al 2008; Zhou and Mendham, 2005). Recent studies have reported relationships between starch pasting properties measured using RVA and malting quality parameters such as hot water extract (HWE) in barley (Batey, 2007; Bason and Blakeney, 2007; Booth and Bason, 2007; Zhou et al., 1998; Zhou et al., 2008; Zhou and Mendham, 2005; Cozzolino et al., 2012).

Starches of low amylose content exhibit lower pasting temperature (PT), and greater hot paste viscosity values (HPV) and swelling power, than starches with higher amylose contents (Zheng and Sosulski, 1998; Zheng et al., 1998; Cozzolino et al., 2012). Lower PT of starch would be expected to be an advantage during processing as sufficient gelatinization would occur during the short processing times used commercially (Zhou et al., 1998; Wang et al., 2010). In recent years, the use of RVA has been proposed as a tool in breeding by various authors and in particular it has been demonstrated that the RVA method can be used for measuring the pasting properties of starch or malt quality (Bason et al., 1993; Booth and Bason, 2007; Zhou and Mendham, 2005; Bason and Blakeney, 2007; Glennie-Holmes, 1995a, 1995b and 1995c; Zhou et al., 2008; Wang et al., 2010; Cozzolino et al., 2012). It has been reported that the functional properties of starch are affected strongly by genetic factors (Bason et al., 1993; Booth and Bason, 2007; Zhou and Mendham, 2005; Bason and Blakeney, 2007; Glennie-Holmes 1995a, 1995b and 1995c; Zhou et al., 2008; Wang et al., 2010; Cozzolino et al., 2012). Much of the published data on starch properties has been obtained on isolated starch and is mainly related to issues regarding the separation of the effects associated with starch, β-glucans and other components of the grain (Zhou et al., 1998; Wang et al., 2010). However, starch isolation requires a difficult and time-consuming procedure even when employed on a laboratory scale. Therefore, the use of flour in predicting starch properties would be more practical in a breeding program (Zhou and Mendham, 2005; Wang et al., 2010).

Overall, in terms of genetic progress, improvements in malt extract have been moderate over the years compared with gains made for grain yield (Emeberi et al., 2004). A number of genes that encode malting quality traits have been cloned and mapped, showing a continuous pattern of phenotypic distribution, indicating that although malting quality is controlled by genetic factors, it is also influenced by environmental conditions (Emeberi et al., 2004). In relation to specific RVA parameters, previous studies showed that pasting properties of barley flour were closely related to malting quality and PT. The QTLs associated with flour pasting properties on barley grain not only provide a better understanding of the genetic factors influencing these traits but also help to find linked markers for molecular marker-assisted selection (MAS). To date, most of the studies have focused on QTLs associated with malting quality parameters such as hot water extract, diastatic power, protein content and β-glucan content (Han et al., 1995 and 1997; Mather et al., 1997; Gao et al., 2004; Emebiri et al., 2004; Hayes et al., 1993; Marquez-Cedillo et al., 2000; Panozzo et al., 2007). Overall, the QTLs identified by the previous authors for malting quality cover nearly all the different chromosomes. Only two reports on QTLs controlling pasting properties of barley flour measured by the RVA method and its relationship with malt extract were found in the literature (Zhou and Mendham, 2005; Wang et al., 2010).

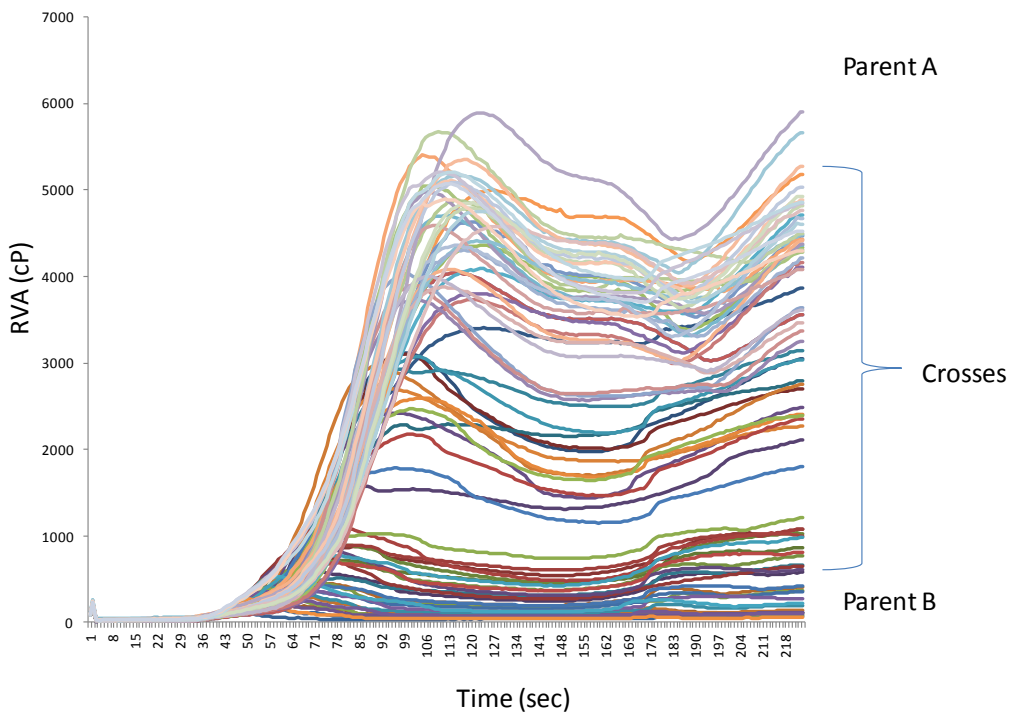

Figure 1. Example of rapid visco analyser (RVA) profiles indicating the spread in pasting properties between parent lines and crosses.

An example of the phenotypic variation encountered in the RVA traces derived from the parents and DH lines from a mapping population is shown in Figure 1. A great variation among DH lines for all the RVA measurements was observed. This can also be associated with the proportion of small starch granules (positively correlated with PV and FV and

negatively with initial pasting time), amylose to amylopectin ratio and the relative diameter of large starch granules. It has also been suggested that lipid content and the interrelationship between amylose and lipid might influence the pasting properties of cereal grains (Goode et al., 2005a and 2005b; Goode and Arendt, 2006; Batey, 2007; Wang et al., 2010; Cozzolino et al., 2012).

In a number of studies, different environmental conditions encountered in each season (mainly related to rainfall) showed greater effects on flour pasting properties, especially in PV, THR, FV and BRD on samples sourced from different seasons (Zhou et al., 2008; Zhou and Mendham, 2005; Wang et al., 2010) with low PT being related to high malt extract. Glennie-Holmes (1995a, b and c) also found that good malting quality was associated with low TTP viscosity and FV, but not necessarily with low PV or peak area. These authors also suggested that the highly significant relationship between PT and malt extract might be due to PT being an indicator of the relative ease of starch solubilisation and hydrolysis (Goode et al., 2005a and b; Goode and Arendt, 2006; Batey, 2007). Therefore, barley grain with starch having lower PT might be more accessible for malting, consequently the granules might swell more easily under mashing conditions and are thus more susceptible to enzyme hydrolysis. Lower PT of starch is also favourable for processing as sufficient gelatinization would occur during the short processing times used commercially (Zhou et al., 1998 and 2008; Wang et al., 2010; Zhou and Mendham, 2005; MacGregor and Fincher, 1993).

AMYLOSE-LIPID INTERACTIONS

Most cereal grains, including barley, are low in lipid and fatty acid content (less than 6% w/w). However its composition influences lipid stability, their functionality properties during processing and storage as well as other biophysical characteristics (e.g., water uptake) (Debet and Gidley, 2006 and 2007; Liu, 2011; Kaukovirta-Norja et al., 1997; Morrison, 1995; Morrison ad Gadan, 1987; Morrison et al., 1993; Patindol et al., 2012; Seefeldt et al., 2011). It has been suggested that cereal lipids associated with starch, influence the gelatinization temperature, leaching of soluble polysaccharides, and contribute to the swelling of starch granules (Tester and Morrison, 1990; Tester et al., 1991 and 1995; Morrison, 1995; Morrison et al., 1993). Starch can also form complex added lipids under some conditions, and in particular amylose-lipid complexes have been studied with reference to the behaviour of starch in the baking industry and dough making, as well as determining important functional interactions with a range of molecules in food based cereals (Tester & Morrison, 1990; Tester et al., 1991 and 1995; Morrison, 1995; Blazek et al., 2011; Vasanthan and Bhatty, 1996; Tang et al., 2001; Tang and Copeland, 2007).

Several chemical and physical factors might influence the changes in beer aroma, taste and foam during processing and storage (Bravi et al., 2009 and 2012). Recently, it has been suggested that lipids can adversely affect beer quality by influencing flavour and foam stability, where the level and the quality of the lipids in beer depends on their content and composition in the raw materials (barley and malt) and on the brewing process (Bravi et al., 2009 and 2012). Although starch-lipid complexes have been analysed by various methods, relatively few studies have been concerned with the influence or the concentration of lipids on starch pasting properties in cereal grains (Tang and Copeland, 2007) and in particular on the

basic data concerning barley varieties for malting and beer production (Tester and Morrison 1990; Tester et al., 1991, 1995 and 1997; Morrison, 1995). Therefore, considering that starch gelatinization and hydrolysis are key factors for efficient mashing and brewing (beer production), understanding starch functional properties such as gelatinization, pasting and retrogradation, and the interactions between amylose and lipids, is of vital importance for effective use of barley for industrial applications (e.g., beer and whisky production), as well as for use for screening and selection of new and improved varieties. It has been reported that during the RVA analysis, amylose might form complexes with lipids, producing gels with increasing spacing between junction zones, giving aggregates that are less compact and yielding high viscosities (Srichuwong and Jane, 2007; Tang and Copeland, 2007). Differences in the pasting properties or RVA profiles of different starches might be attributed to differences in phospholipids content, as well as the smalland fractured structure of starch granules. It has been reported that an increase in PV correlates significantly with a decrease in lipid content, although the apparent amylose content might also increase (Dithal et al., 2011). Dithal and collaborators (2011) also suggested that PV might be affected more by the lipid content than by the apparent amylose content. The higher the lipid content associated with the amylose-lipid complexes (and the higher amount of surface-associated lipid and protein) might reduce granule swelling and lower the PV to a greater extent in smaller granules compared to larger granules (Srichuwong and Jane, 2007; Dithal et al., 2011). It has also been reported that the lipids are connected in some way with metabolism in the amyloplast and with the synthesis of starch. This association might indicate a possible relationship between lipids and amylose synthesis (Morrison, 1995; Tester and Morrison, 1990, 1997; Tester et al., 1991 and 1995; Jane et al., 1992, 1999 and 2003). The interactions between amylose and lipids can also affect the water solubility of some of the macromolecules and this might have an effect on the pasting properties of the sample.

WATER UPTAKE BY THE ENDOSPERM

The uptake of water by seeds is an essential and initial step towards germination. For malting barley, this is represented by the first stage of the malting process, often referred to as steeping (Brookes et al., 1976; Bryce et al., 2010; Mayole et al., 2012). Steeping is the first process in malting and its purpose is to increase the water content of the grain up to 43–46%, however such a simple step encompasses several and different metabolic processes that affect germination and the final malt quality (Molina-Cano et al., 1995, 2002 and 2004). The initial hours of barley steeping are critical to the development of components such as enzyme activity, hormonal development and release that will determine the quality of the malt (McEntyre et al., 1998). Kernel morphology, chemical composition (e.g., protein, starch, lipids, cell wall composition), genetics and environment factors (e.g., temperature), influence the physiology of the kernel during these initial hours (Brookes et al., 1976; Molina-Cano et al., 2002; McEntyre et al., 1998; Agu, 2003 and 2005; Agu and Palmer, 1995 and 2001; Agu et al, 2007; Mayole et al., 2012; Cozzolino et al., 2013). Variations in water imbibition rates may also lead to inefficient hydration and therefore to over or under modification of the endosperm for industrial use (e.g., malting) (Brookes et al., 1976; Molina-Cano et al., 1995 and 2002; McEntyre et al., 1998). However, a general comprehensive understanding of the

biophysical, physiological and biochemical determinants of seed germination and their interactions is still lacking (Becker, 1963; Bewley, 1997). More information regarding the pathway(s) and dynamics of water penetration in barley and the corresponding initiation of metabolism is required in order to produce barley varieties in a sustainable manner (Bewley, 1997; Gamlatha et al., 2008).

Overall, little and/or contradictory studies have been found on the factors that influence water uptake in barley, in particular the effect of protein content (Brookes et al., 1976; Gamlatha et al., 2008). It has been reported that protein content and β-hordein content of the endosperm of barley can impede water movement in the endosperm (Brookes et al., 1976; MacGregor, 1991 and 1993; Molina-Cano et al., 2002). In a recent study, Gamlatha and collaborators (2008) found no relationship between total protein content of the endosperm and water uptake of several barley kernels during steeping. Similar results were reported by Leach and co-workers (2002).

Other factors such as endosperm physical characteristics and chemical composition were found to be responsible for water uptake (Chandra et al., 1999; Agu et al., 2007; Agu, 2003 and 2005; Agu and Palmer, 1998 and 2001). For example, steeliness appears to be a major factor restricting the uptake and distribution of water across the endosperm during steeping, where higher total protein content may not be an important factor in controlling water uptake of the kernel since high nitrogen content does not necessarily correlate with the steely areas (Chandra et al., 1999). The endosperm cell walls of barley kernel tissues and their components also have an important impact on malt quality. Hardness of the endosperm or water uptake during steeping may be influenced by cell wall organization and the composition of the endosperm (Chandra et al., 1999; Gamlatha et al., 2008). Overall, water uptake by the barley grain affects the malting process and the quality of the malt obtained and genetic and environmental factors have been reported to have an influence in water uptake by the grain (Mayolle et al., 2012; Swanston et al., 2006). Differences in water uptake between varieties or lines have been reported by various authors, where protein content, starch, endosperm structure and composition, cell wall components and β-glucans are considered to be responsible for the water uptake (Barber et al., 1994; Brookes et al., 1976; Ferrari et al., 2010; Molina-Cano et al., 1995, 2002 and 2004; Ogushi et al., 2002). It is well known that during the steeping of barley, the grains must imbibe sufficient water for adequate modification of the endosperm during malting and this step has an important impact on the final quality of the malt (Mayolle et al., 2012; Cozzolino et al., 2013). Therefore, the monitoring and measurement of water uptake during steeping will allow a reduction in the amount of water usage and effluents with a decrease in the cost of production and time saving (optimization) in addition to allowing a better control of the steeping process (Mayolle et al., 2012; Cozzolino et al., 2013).

SWELLING AND WATER SOLUBILITY PROPERTIES

The swelling power or property (SP) of starch is measured as the ratio of the wet weight of the sediment gel to its dry weight (Sasaki and Matsuki, 1998; Lai and Cheng, 2006; Xie et al., 2005). In a number of studies, swelling is considered a property of the amylopectin and its value decreased linearly with amylose content (Tester and Morrison, 1992; Tester and

Karkalas, 1996 and 2006; Xie et al., 2005). It has also been reported in several cereal starches that a high proportion of long chain molecules in amylopectin contributed to the increase in swelling properties of the sample. Water solubility index (WSI) is a property that depends on the starch modifications or characteristics as well as changes in particle size and can be calculated as the percent of dry matter in the supernatant (Sasaki and Matsuki, 1998; Lai and Cheng, 2006; Li and Yeh, 2001; Xie et al., 2005). Both water solubility index and water absorption are functional properties of starches when dispersed in water and are related with the gelatinization properties of the starch (Steeneken, 1989; Sasaki and Matsuki, 1998; Lai and Cheng, 2006; Xie et al., 2005; Mariotti et al., 2006).

OSMOLYTE CONCENTRATION

Recent studies demonstrated that finished malt osmolyte concentration (OC) correlates well with HWE and other malt quality parameters and may be used as an early indicator of malt quality at later stages of the malting process (Duke and Henson, 2011 and 2009). HWE depends on the total mass of material in solution in wort and finished malt, whereas OC depends on the molar concentration of all solutes (osmolality) in the malt (Duke and Henson, 2011 and 2009). Seed OC is also an indicator of pre-harvest sprouting in barley and has been used in the determination of dextrose (glucose) equivalents in processes such as α-amylase degradation of starch (Duke and Henson, 2011 and 2009). Like HWE, finished malt OC depends on the degradation of seed storage compounds, such as starch, proteins, lipids, and cell wall components, such as β-glucans, that provide substrates, for example glucose, amino acids, and other metabolites necessary for growth and development during germination. Each product of storage compound and cell wall degradation and synthesis of proteins and metabolites could reflect one or more indicators of malt quality, such as DP, α-amylase activity, and β-glucan concentration (Duke and Henson, 2011 and 2009). In addition, Eeach product of storage compound and cell wall degradation and each soluble protein or metabolite produced during germination increases the molar solute concentration within the seed and contributes to an increase in the OC within the seed (Duke and Henson, 2011 and 2009). Since there are numerous different osmolytes, from numerous metabolic pathways active in germinating seeds that contribute to the OC, it is a logical assumption that OC is a complex quantitative trait, such as is HWE (Duke and Henson, 2011 and 2009).

CONCLUSION

As we described, malting quality is an economically important character of barley and it is recognised to be a complex character that is modulated by a combination of biophysical characteristics and properties of the grain. These characteristics make malting quality difficult to manipulate genetically in breeding programs. Malsters require grains of known varieties with consistent quality, and specify strict quality criteria for accepting new varieties. Many characteristics or properties of the grain and endosperm drive these biophysical characteristics (amylose, amylopectin, hordein composition, lipids content, fatty acid profile, particle and granule size, among other characteristics). Other biophysical properties or characteristics of

the grain can also explain or modulate malting properties such as starch pasting properties, grain water uptake, cell wall content and interactions between amylose and lipids, among others. Consequently such bio-physicochemical properties of starch have a significant impact on the resultant malt quality and brew house performance.

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Chapter 6

VACUOLAR H^+-PPASE (*HVP*) GENES IN BARLEY: CHROMOSOME LOCATION, SEQUENCE AND GENE EXPRESSION RELATING TO Na^+ EXCLUSION AND SALINITY TOLERANCE

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ABSTRACT

Three vacuolar H^+-pyrophosphatase (*HVP*) genes have been identified in barley (*HVP10*, *HVP1* and *HVP3*). The first gene, *HVP10*, was recently localised to chromosome 7HS in a mapping population developed from the cross Barque-73 x CPI-71284. *HVP10* showed increased gene expression after 24 hours of salt stress in roots of the variety Kashima, but not until the third day of salt stress in roots of both Barque-73 and CPI-71284. *HVP10* was also more highly expressed following salt stress in shoots of one of the barley genotypes (CPI-71284). It is hypothesised that *HVP10* plays tissue-specific roles in both Na^+ exclusion (roots), and in compartmentalisation of toxic Na^+ into vacuoles (shoots) in salinity tolerant barley genotypes. In the current study, a second gene, *HVP1*, was mapped to the long arm of chromosome 7H in barley. Different expression profiles for *HVP1* across different barley genotypes were observed in this study in response to salt stress. *HVP1* appears to be involved in two reactions of barley plants to salt stress: (1) adjustment to osmotic stress following initial NaCl application in both roots and shoots (cv. Kashima); and (2) management of toxic Na^+ concentrations in shoots during the ionic phase of salt stress (cv. Barque-73). In this study, *HVP1* gene sequence has eight exons and seven introns. The predicted amino acid sequences of HVP1 in the three cultivars used in this study were identical, and were also the same as sequences found in databases for barley cultivar Morex although there were a number of SNPs within the coding regions between genotypes. These results are similar to those previously published about HVP10. This suggests structures of both HVP10 and HVP1

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are very conserved among barleys, and it supports a hypothesis that promoter regions of both genes are involved in the regulation of gene expression. A third gene, *HVP3*, has been previously described but it was mapped in chromosome 1H in current study, and deduced that the gene contains four exons and three introns. In our experiment *HVP3* was expressed neither in roots nor in shoots of any of the barley genotypes tested, either under control conditions or in response to salt stress. Based on published EST data, it is hypothesised that *HVP3* plays an important role for proton pump only during seed development and is not linked to abiotic stress responses. Nucleotide sequence analysis indicated that during evolution there may have been insertions/deletions occurring within two highly conserved domains shared by *HVP1/HVP10* and *HVP3* genes.

Keywords: BAC library analysis, barley, chromosome location, gene expression, gene mapping, *HVP* genes, intron/exon structure, Na^+ exclusion, proton pump, q-RT-PCR, QTL analysis, salinity tolerance, vacuolar H^+-PPase

ABBREVIATIONS

BAC	bacterial artificial chromosome
EST	expressed sequence tag
HVP	*Hordeum* vacuolar pyrophosphatase
q-RT-PCR	quantitative reverse-transcriptase polymerase chain reaction
V-PPase	vacuolar pyrophosphatase

INTRODUCTION

The proton pump is one component of millions of biochemical processes that are occurring in all living plant cells. But how important is it for a plant to maintain a gradient of protons in the cytoplasm or vacuole? The vacuolar proton pump is an extremely important process, which many other processes depend upon. In favourable, non-stressed conditions, the maintenance of protons in vacuoles generates osmolarity for homeostasis during cell expansion and the continuous growth and development of plant organs. At the same time, proton pumps are directly involved in internal pH regulation in both cytoplasm and vacuole. A spectacular visual example of this is the observed colour change in flower petals of petunia from pink to blue, where proton pumps increase vacuolar pH (Quattrocchio et al., 2006; Verweij et al., 2008). Significant pH differences have also been found between upper and lower layers of petal cells, that are directly related to flower colour in *Anagallis monelli* L., and anthocyanidin pigments in the vacuoles (Quintana et al., 2007). Anthocyanidin biosynthesis involves transport of proanthocyanidin into the vacuole. In *Arabidopsis* and *Medicago trancatula*, genes *TT2* (Transparent Testa 2) and *MATE* (Multidrug and Toxic Compound Extrusion), respectively, encode antiporters controlling proanthocyanidin transport into the vacuole. A higher proton gradient in the vacuole is necessary for transporting proanthocyanidin back out (Zhao and Dixon, 2009). Rubber biosynthesis in latex of *Hevea brasiliensis* also requires optimum cytoplasmic pH and it has been shown that vacuolar proton pumping is essential for supports this process (Rizhong et al., 2009).

During fruit development, vacuolar proton pumps are important for the regulation of osmolarity and acidity in vacuoles to support the accumulation of sugars and organic acids. The accumulation of sugars and organic acid into vacuoles of fruit cells is controlling by different enzymes, such as sucrose synthase and vacuolar invertase (Tanase et al., 2002; Yamada et al., 2007), and protein density of vacuolar membrane is changed during fruit development (Shiratake et al., 1998). All of these processes are directly related to proton pumps into vacuoles of fruit cells (Reviewed by Silva and Gerós, 2009). Recent reports show that the proton pump plays a crucial role in early stages of fruit development and enlargement in pear (Suzuki et al., 1999), grape (Venter et al., 2006), lemon (Aprile et al., 2011) and tomato (Mohammed et al., 2012).

A second important role of the vacuolar proton pump is in relation to abiotic stress responses, such as salinity and drought, where a gradient of protons into the vacuole is required for antiporter activity involved in stress adaptation. For example, in conditions of salinity stress the Na^+/H^+ antiporter (NHX) acts to re-locate toxic ions, primarily Na^+, into the vacuole, but it can only work together with the driving force of a proton gradient in the opposite direction (Reviewed by Silva and Gerós, 2009; Kronzucker and Britto, 2011). The volume of the central vacuole under salt stress increases dramatically to act as a reservoir for Na^+, osmolytes and water, as has clearly been seen in the halophyte ice plant, *Mesembryathemum crystallinum* (Jou et al., 2007). The functioning of the proton pump is a very important component of reactions of plants to abiotic stresses, primarily high salinity and drought.

Therefore, there are two major stages in plant development where vacuolar proton pumping is important: (1) during the vegetative stage of seedlings and young plants, proton pumps regulate cell osmolarity. This is important for cell expansion in non-stressed conditions, and for compartmentalisation of toxic ions of Na^+ into the vacuole (salt stress), and water balance and osmo-homeostasis (drought stress); (2) during the reproductive stage, vacuolar proton pumping plays important roles in acidification of vacuoles and controlling flower colour, and in the accumulation of sugars and organic acids in vacuoles of cells regulating fruits size and development.

PYROPHOSPHATASE VS. ATPASE

There are two known classes of enzymes catalysing proton pumping in plant cells, distinguished by the substrate used as an energy source. Pyrophosphatase (PPase) enzymes use pyrophosphate (PPi), a biochemical ‘waste’ and, therefore, PPases are efficient, low-energy cost vacuolar pumps (Reviewed by Maeshima, 2000; Silva and Gerós, 2009). Additionally, high concentrations of PPi in the cytoplasm can inhibit some important biochemical processes, such as gluconeogenesis as observed in young developing *Arabidopsis* plants (Ferjani et al., 2011), and in latex of the rubber tree, *Hevea brasiliensis* (Rizhong et al., 2009). Therefore, PPases perform a ‘double function’: removing excessive PPi from the cytoplasm and pumping protons into the vacuole. Only one type of pyrophosphatase (V-PPase) has been found to be associated with the vacuolar membrane with a trans-membrane proton transporting activity. Two other types of pyrophosphatase (soluble and membrane-associated) are also present in the tonoplast, but have other functions

(Maeshima, 2000). V-PPase is a unique enzyme and present only in microbes and members of the plant kingdom (Luoto et al., 2011) but has not been found in animal cells. V-PPase consists of a single large subunit with ranging in size of between 600-771 amino-acid residues (Maeshima, 2000).

The second class of proton pump, ATPases, use ATP as an energy substrate, and are widely found among all living organisms, including microorganisms, plants and animals (Reviewed by Ratajczak, 2000; Silva and Gerós, 2009). ATPases are associated with both vacuolar and plasma membranes, but are structurally variable between 8 and 14 subunits. Several subunits of barley V-ATPase have been studied and shown to be needed for the involvement of this enzyme in the sequestration of Na^+ ions into vacuoles (Berkelman et al., 1994; Tavakoli et al., 1999). It is hypothesised that plant cells achieve a balance between pyrophosphatase and ATPase activities depending on substrate supply, and energy needs of the plant during development and in response to abiotic stress (Maeshima, 2000; Krebs et al., 2010). For example, it was shown that severe disruption of male gametophyte development in Golgi membranes of *Arabidopsis* V-ATPase mutant is unable to be prevented by V-PPase activity alone (Dettmer et al., 2005). Additionally, heavy metals, such as zinc and nickel, can differentially regulate the expression of both H^+-ATPase and H^+-PPase in cucumber (Kabala and Janicka-Russak, 2011), but this crop perhaps has another mechanism for secretion of Na^+ ions into the apoplast rather than into the vacuole (Kabala and Klobus, 2008).

PYROPHOSPHATASE GENES

The first gene encoding a pyrophosphatase was discovered in the model plant *Arabidopsis thaliana* (Sarafian et al., 1992) and was named *AVP1* (*Arabidopsis* vacuolar pyrophophatase). However, a second gene *AVP2* was later described, indicating the existence of a *V-PPase* gene family (Drozdowicz et al., 2000). Homeologous genes from other plant species have since been discovered and published, including in red beet (Kim et al., 1994), rice (Sakakibara et al., 1996), mung bean (Nakanishi and Maeshima, 1998), pear (Suzuki et al., 1999), *Vigna unguiculata* (Otoch et al., 2001), durum wheat (Brini et al., 2005), grape (Venter et al., 2006) and rubber tree, *Hevea brasiliensis* (Rizhong et al., 2009).

In barley, three H^+-pyrophophatase genes have been described. The first is *HVP1*, which is responsive to abiotic stresses, such as high salinity. *HVP1* showed higher levels of gene expression in roots of barley (cv. Kashima) after 5 hours of salt stress (Fukuda et al., 2004). However, NaCl application in the experiment was done in a single step, and it is likely that strong osmotic stress or even osmotic shock was imposed (Shavrukov, 2013). Therefore, *HVP1* may be responsive to the osmotic component of salt stress rather than the ionic component.

A second *V-PPase* gene in barley was named *PP10* (= *HVP10*) (Tanaka et al., 1993), and is also responsive to abiotic stresses. *HVP10* showed higher expression in roots of barley (cv. Kashima) following salt stress, but the gene expression profile was different from *HVP1* (Fukuda et al., 2004). Recently, we found that *HVP10* is a likely candidate for the *HvNax3* locus in barley, controlling better Na^+ exclusion from shoots and larger biomass production under salt stress. The tolerance allele originated from a wild barley parent, *Hordeum vulgare* ssp. *spontaneum*, accession CPI-71284 (Shavrukov et al., 2010a and 2013).

Recently, a third V-PPase gene, *HVP3*, was described in barley (Wang et al., 2009). *HVP3* was found in a barley EST library using *in silico* computer analysis to search for barley homeologs of wheat genes. Very little information was published regarding *HVP3*, with only a partial EST sequence available (Wang et al., 2009).

MAPPING OF *HVP* GENES IN BARLEY

Until recently, the genetic location of barley *HVP* genes was unknown. We mapped *HVP10* following the identification of a single QTL for Na^+ exclusion in an advanced-backcross QTL (AB-QTL) population originating from the cross between cultivated barley (*Hordeum vulgare* ssp. *vulgare*) Barque, selection 73 and wild barley (*H. vulgare* ssp. *spontaneum*) accession CPI-71284 (Shavrukov et al. 2010a and 2010b). The identified *HvNax3* locus was mapped to a genetic interval of 1.3 cM, located 6.3 cM from the distal end of chromosome 7HS, relatively close to the centromeric region (Shavrukov et al., 2010a). *HVP10* is the most likely candidate gene for *HvNax3*. Recently, we identified *HVP10* as a single gene in BAC clone HVVMRXALLeA-0262H05 from a barley (cv. Morex) BAC library (Shavrukov et al., 2013). *HVP10* was mapped on the corresponding region of the physical map, at a distance of 47 – 161 Kbp from the distal end of chromosome 7HS, using a consensus physical barley map (http://mips.helmholtz-muenchen.de/plant/barley/index.jsp).

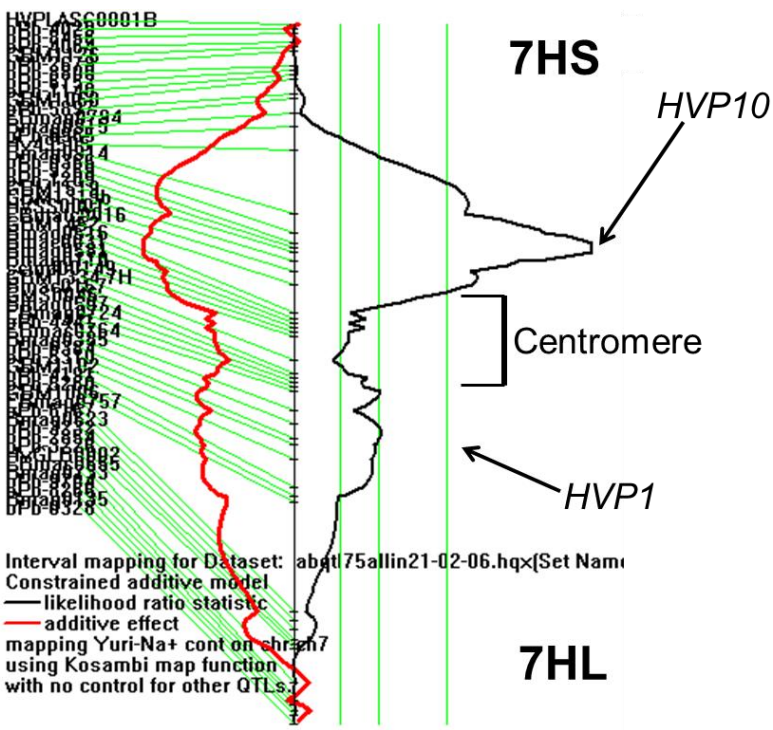

Figure 1. Mapping of two *HVP* genes (*HVP10* and *HVP1)* in a barley AB-QTL population originating from the cross between barley cv. Barque, selection 73 and *H. spontaneum*, accession CPI-71284 (from Shavrukov et al., 2010a and 2010b with modifications).

We also identified the genetic location of *HVP1*, using the Barque-73 x CPI-71284 AB-QTL population and *in silico* bioinformatics analysis. *HVP1* has been maped to the long arm of chromosome 7H and is very close to the centromere (Figure 1).

We identified *HVP1* in Morex contig 137615 (CAJW010137615 carma=7HL) on the Assembly WGS Morex database (http://webblast.ipk-gatersleben.de/barley), confirming the genetic location of *HVP1*. This Morex contig corresponds to Fingerprinted contig 320 (http://mips.helmholtz-muenchen.de/plant/barley/index.jsp), but it has not yet been introgressed into the consensus physical map. Therefore, we are currently unable to identify the exact position of *HVP1* within contig 320 on the barley physical map of chromosome 7HL.

Using the same approach, we determined that *HVP3* is located on chromosome 1H. However, no QTL was found in this location in the barley AB-QTL population under salt stress. We identified this gene in Morex contig 2552365 (CAJW012552365 carma=1H) on the Assembly WGS Morex database, corresponding to Fingerprinted contig 44244. As with *HVP1*, the exact position of the contig containing *HVP3* remains unknown.

EXPRESSION AND FUNCTIONAL ANALYSIS OF *HVP* GENES

The three *HVP* genes identified in barley were analysed for expression in both roots and shoots of young plants of barley Barque-73 and wild barley CPI-71284 during exposure to 150 mM NaCl, using methods previously described (Shavrukov et al., 2010a and 2013). Seedlings were grown in supported hydroponics (Shavrukov et al., 2012) for 10 days until the third leaf started to emerge. Salt was then added as twice-daily increments of 25 mM NaCl to avoid osmotic shock (Shavrukov, 2013), until 150 mM was reached. Three plants as independent biological replicates were used for each of the two barley genotypes (Barque-73 and CPI-71284), with two treatments (salt stress and control), six time-points and two tissues (roots and shoots). Samples were immediately frozen in liquid nitrogen for RNA extraction and cDNA synthesis as previously described (Shavrukov et al., 2013). Primers used for q-RT-PCR are listed in Table 1.

Expression of the three *HVP* genes was analysed using q-RT-PCR and normalised with reference to the house-keeping gene *HvGAP* (Shavrukov et al., 2013). Results are presented in Figure 2.

The three *HVP* genes showed different expression profiles. *HVP10* was significantly ($p>0.95$) more highly expressed on the third day of salt stress in roots of CPI-72184 compared to Barque-73, although *HVP10* expression was increased in roots of both barley genotypes on this day in salt-stressed compared to control plants. *HVP10* expression in shoots on the third day of salt stress was similar but with much greater expression of the gene in salt-stressed CPI-71284 relative to Barque-73 (2.1-fold increase; $p>0.99$).

However, *HVP10* expression in roots was about 10-fold higher than levels of expression in shoots. There was clearly differential expression of *HVP10* in roots and shoots of barley in response to salt stress (Shavrukov et al., 2013). Similar results were previously reported (Fukuda and Tanaka, 2006), where *HVP10* was more highly expressed in roots than in shoots of barley cv. Kashima. The expressions of two other H^+-pyrophosphatase genes, *HVP1* and *HVP3*, were also analysed in Barque-73 and CPI-71284.

Table 1. List of primers for *HVP* genes used for q-RT-PCR

Gene name	Primer sequence (5' – 3')	Annotated accession number
HVP10	(F): GGTCTGTGGGCTGGTCTGATTATTG (R): GCTGACGTAGATGCTGACAGCAATAG	D13472
HVP1	(F): AAAGAGCCTGGGCCCGAAAGGC (R): TCTTGAAGAGGATTCCTCCATAG	AB032839
HVP3	(F): GGGTCCCAAGGGCTCGGAGGCG (R): ATATTTGTATCAGTTGATTATGAGG	AK362588

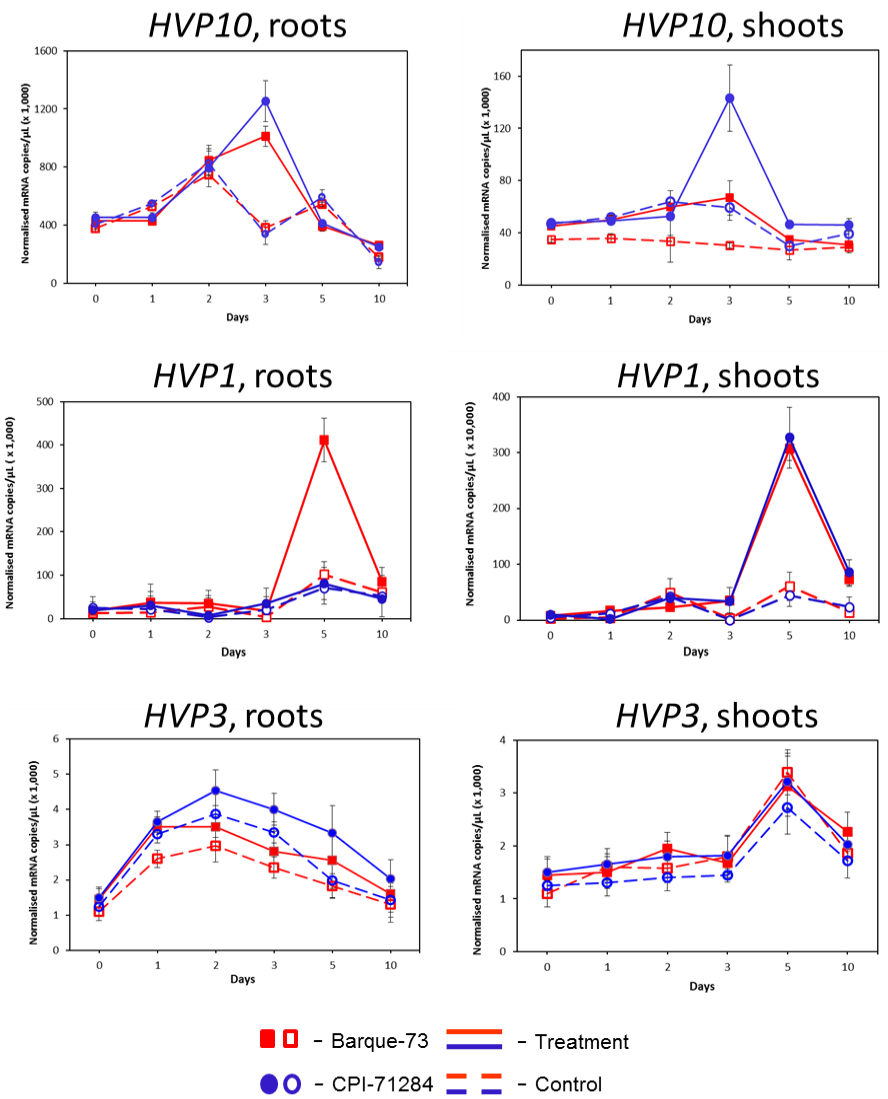

Figure 2. Expression of *HVP10*, *HVP1* and *HVP3* in both roots and shoots of barleys Barque-73 and CPI-71284 in response to 150 mM salt stress. The first gene was described in Shavrukov et al. (2013).

There was a dramatic increase in *HVP1* expression in roots of Barque-73 on the fifth day after first salt application (5.1-fold compared to non-stressed roots; p>0.999), while the *HVP1* expression in roots of CPI-71284 did not change with salt stress, and was similar to non-stressed Barque-73 (Figure 2).

In shoots of both Barque-73 and CPI-71284, *HVP1* mRNA levels were similarly higher (approximately 10-fold; p>0.999) on the fifth day of salt application compared to non-stressed seedlings.

These results indicate that *HVP1* expression showed a delayed response to salt stress compared to *HVP10*. Tissue-specific trends in expression of *HVP1* were also very different from *HVP10*, where both barley genotypes had high expression of *HVP1* in shoots but only in roots of Barque-73.

Based on our results, we hypothesize that *HVP10* and *HVP1* have complementary expression profiles and both may have a function in barley plants in response to salt stress: when the expression of *HVP10* was reduced following maximum levels on the third day of salt stress, the expression of *HVP1* then peaked. More experiments are required accurately follow *HVP10* and *HVP1* gene expression in the time periods between 2-3, 3-5, and 5-10 days (Figure 2). Our results are similar to previously published findings, where Ueda et al. (2006) showed increased expression of both *HVP10* and *HVP1* in roots of barley cv Haruna Nijo, but only after one day of salt stress.

This may be because high salinity (200 mM NaCl) was applied in a single step, which perhaps caused osmotic shock (Shavrukov, 2013), and no samples were collected after the first day of salt application (Ueda et al., 2006).

HVP3 RNA levels were below detection limits in both roots and shoots of both barley genotypes, and were not responsive to salt stress (Figure 2). There is very little published information about *HVP3*, a single report suggesting that this gene is expressed only in developing seeds (Wang et al., 2009).

Our results indicate that *HVP3* is not expressed in roots or shoots in response to salt stress, at least during the vegetative stage of plant development. More experiments with plant tissues sampled during reproductive stages of development are needed to determine expression patterns of *HVP3* in barley.

SEQUENCE ANALYSIS OF HVP POLYPEPTIDES

Analysis of the predicted amino-acid sequences of the three HVP proteins confirmed that first two polypeptides (HVP10 and HVP1) are very similar to each other while the third protein (HVP3) is more divergent. Alignment of the three HVP polypeptides from annotated accessions is presented in Figure 3.

Quantification of the identities and similarities of the three known HVP proteins in barley (Figure 4) confirms that HVP3 is more genetically distant from HVP10 and HVP1, which are also similar to those published (Brini et al., 2005; Venter et al., 2006). The identity/similarity relationships between the three HVP proteins appear to be linked to their functions: *HVP10* and *HVP1* are strongly responsive to salt stress, while *HVP3* is unresponsive and is probably expressed in developing seeds.

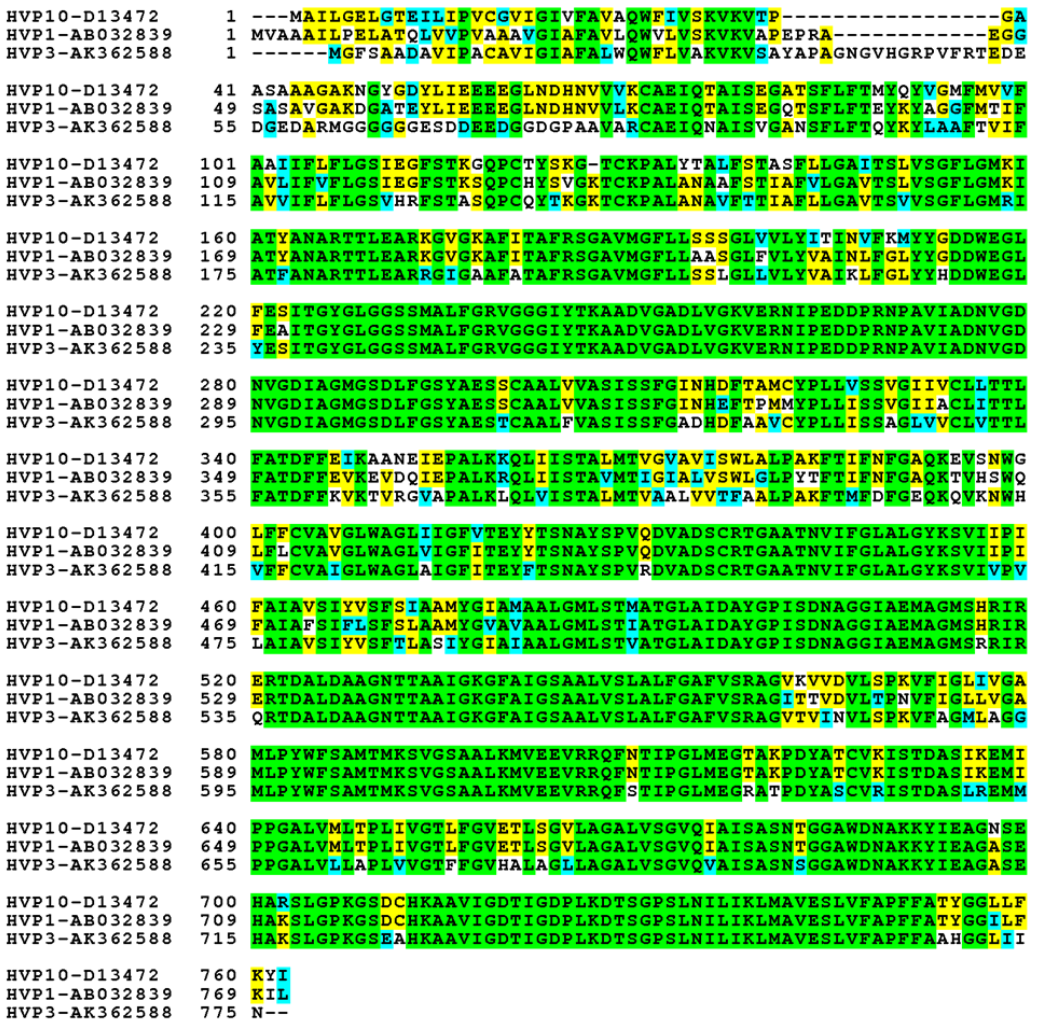

Figure 3. Alignment of predicted amino-acid sequences for HVP10 (Acc. No. D13472), HVP1 (Acc. No. AB032839) and HVP3 (Acc. No. AK362588) from barley. Consensus sequences are highlighted in green.

HVP GENE SEQUENCES

We previously reported that the coding regions of *HVP10* amongst Barque-73, CPI-71284, Morex and Kashima (Accession No. D13472) had ten SNPs, but that none of these altered the predicted amino-acid sequence (Shavrukov et al., 2013). In the current study we found six SNPs in the coding regions of *HVP1* between the same four barley genotypes (cv. Kashima, Accession No. AB032839) but, similarly, amino-acid sequences of HVP1 were identical (data not shown). Our study of polymorphism in *HVP3* is not yet complete. The lack of sequence diversity in both *HVP10* and *HVP1* indicates that *V-PPase* genes in barley are very conserved.

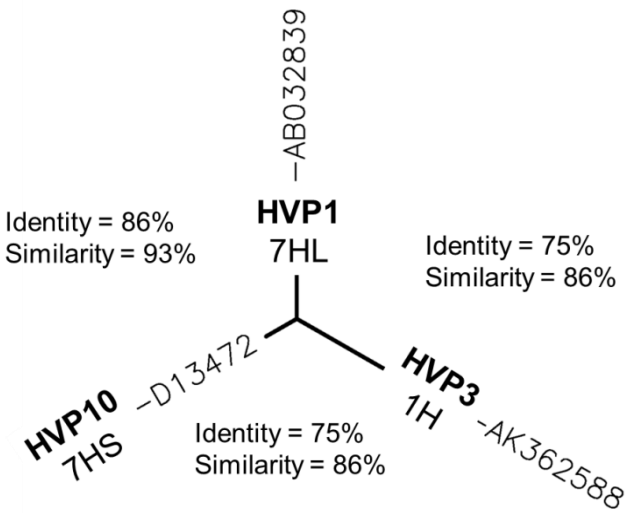

Figure 4. Dendrogram of identity/similarity between three HVP proteins in barley.

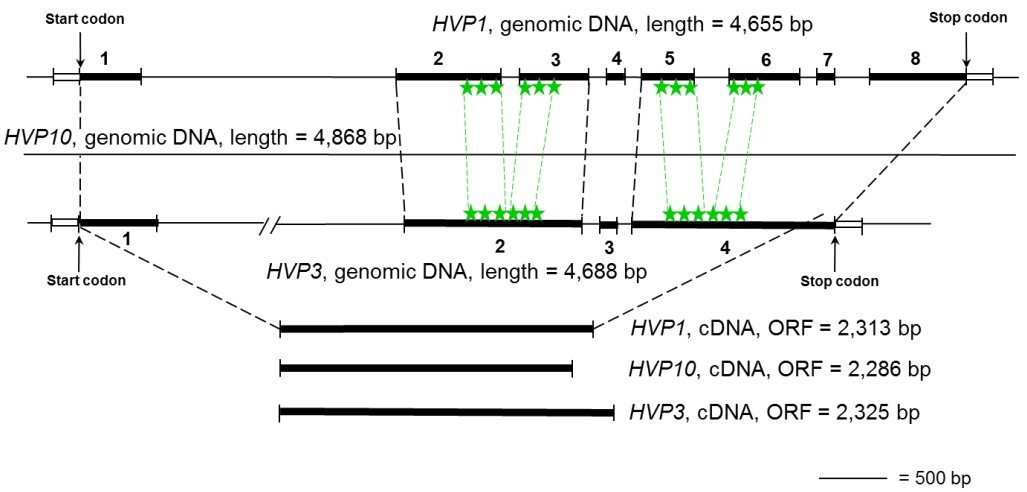

Figure 5. Comparative exon/intron structures of three *HVP* genes in barley cv. Morex and corresponding cDNAs. Exon/intron structure for *HVP10* is unknown and not shown in the upper part of the figure but we expected it should be similar to *HVP1*. Broken lines show corresponding regions across the genes. Green asterisks show strongly conserved domains across all three *HVP* genes, corresponding to blocks of residues highlighted in green in the alignment in Figure 3: 232-309 and 499-570 for HVP1-AB032839.

We have identified the full sequence of the *HVP1* and *HVP3* genes in cv. Morex, based on the sequence of CAJW010137615 and CAJW012552365 clones, respectively, while the sequence of *HVP10* is still undergoing (Figure 5). *HVP1* contains eight exons and seven introns, accounting for 4,655 bp in total length between start- and stop-codons in Morex genome. *HVP10* is 4,868 bp in size, and we can expect similar gene structure according

strong similarity between HVP1 and HVP10 polypeptides. *HVP3*, however, has only four exons and three introns and, therefore, appears to be significantly different from *HVP1*.

HVP1 and *HVP10* have very similar gene and protein sequences, which are very likely related to their similar responses to salt stress during vegetative stages of plant development. However, small differences in sequences in the coding regions between *HVP1* and *HVP10* may be responsible for different tissue-specific responses of the two genes to salinity across time. The third gene, *HVP3*, is very different in sequence and intron/exon structure from the other two HVP genes, which may relate to its different function.

V-PPASE GENES IN PLANTS

With respect to gene structure and exon/intron formula, barley *HVP* genes can be classified in two groups: (1) eight exons and seven introns; and (2) four exons and three introns. The first group of *V-PPase* genes contains the majority of H^+-pyrophospatase genes in plants. It includes: *AVP1* in *Arabidipsis*, *TVP1* in durum wheat (Mullan et al., 2007), four genes in rice, *OVP1 - OVP4* (Choura and Rebaï, 2005), two genes in bread wheat, *TaVP1* and *TaVP2*, (Wang et al., 2009), *HVP1* (current study) and possibly *HVP10* in barley. The second group of *V-PPase* genes is relatively small and does not contain any genes from *Arabidopsis* but includes *OVP5* from rice (Choura and Rebaï, 2005), *TaVP3* from bread wheat (Wang et al., 2009) and *HVP3* (current study).

There is also a third group of *V-PPase* genes, containing 14 exons and 13 introns, which includes *AVP2* from *Arabidipsis*, one non-identified gene in rice (accession AK070310) and five cDNA clones isolated from wheat, which possibly represent a splice variants of a single gene (Mullan et al., 2007). We were unable to identify any *HVP* genes in barley belonging to this group.

Observed similarity in gene structure also corresponds to chromosomal location of the orthologous genes. For example, the barley genes from group 1, *HVP10* and *HVP1* were mapped in barley chromosome 7H, short and long arms, respectively (Shavrukov et al., 2010a, 2013, and current study). *AVP1*-orthologous genes in bread wheat was localised to chromosome group 7 (Mullan et al., 2007), and *OVP1* and *OVP2* were identified in syntenous regions of rice chromosome 6 (Sakakibara et al., 1996; Choura and Rebaï, 2005). A similar situation is evident for genes from group 2: *HVP3* was localised to barley chromosome 1H (current study), while the orthologous gene *OVP5* was identified in the syntenous region of rice chromosome 5 (Choura and Rebaï, 2005). These observations confirm a similarity in orthologous gene structure and their corresponding genetic locations. However, many other *V-PPase* genes in other plants have no identified sequences, gene structure and genome localisation and, therefore, more research is required to make a wider conclusion about similarity between H^+-pyrophospatase genes.

EVOLUTION OF INTRONS/EXONS IN *HVP* GENES

Consider the following question: why does *HVP3* have such a unique arrangement of exons/introns when compared to *HVP1*, if *V-PPase* genes represent a single gene family in

barley? A reduction in numbers of exons from eight to four in *HVP3* reflects evolutionary diversity of *HVP* family genes. Interestingly, coding regions of the *HVP* genes are not so different, despite large re-organisations in exon/intron structure among the genes.

Two highly conserved domains were identified in the amino-acid residue alignment of the three HVP proteins, corresponding to two regions: residues 232-309 and 499-570 for HVP1-AB032839 (Figure 3). We indicate these regions on Morex genes *HVP1* and *HVP3* in Figure 5 with green asterisks. It has been found that two introns are present in the gene *HVP1*, one in each of the two identified conserved domains, which are absent in the gene *HVP3* (Figure 3).

Our comparison of nucleotide sequences of these two fragments in the *HVP* genes and amino-acid sequences of the corresponding regions in HVP proteins indicates the precise locations of intron splicing in *HVP1* during transcription from DNA to mRNA. We are confident that two of the introns are present in the highly conserved domains, while two other introns are located in semi-conserved domains in *HVP1*, which are not present in *HVP3*.

The presence of an additional four introns in *HVP1* may be a result of long-term evolution of the *HVP* family gene in barley. But which process, insertion or deletion, happened during evolution? If insertions of the introns took place in *HVP1*, this gene originated from the more ancient *HVP3*. By contrast, the introns in *HVP1* may have been deleted over time to give rise to *HVP3*. Resolving this question will help to identify evolutionary hierarchy between *HVP* genes and will be carried out in future.

PRACTICAL APPLICATIONS OF *HVP* GENES FOR BARLEY BREEDING

Three vacuolar H^+-pyrophosphatase (*HVP*) genes play important roles in the growth and development of barley. These genes control the process of proton pumping into the vacuole and are key genes in important cellular reactions.

Two of the pyrophosphatase genes, *HVP1* and *HVP10*, are very important in the response of plants to abiotic stresses, primarily salinity and osmotic regulation. We have shown that *HVP10* is associated with Na^+ exclusion from shoots and greater biomass production under salinized conditions (Shavrukov et al., 2010a and 2013). *HVP1* is also involved in plant responses to salt and osmotic stress (Fukuda et al., 2004 and current study). Both genes are highly expressed during the vegetative stage of barley development, and are thought to contribute to plants' ability to cope with elevated levels of salinity in soils, by regulating cellular homeostasis. If barley plants are growing in favourable conditions (without salinity or drought), *HVP1* and *HVP10* are not likely to be highly expressed (Figure 2; Fukuda et al., 2004; Shavrukov et al., 2013). This suggests that these genes are mostly important under conditions of stresses.

By contrast, *HVP3* is not responsive to abiotic stresses (Figure 2), but seems to be expressed specifically in reproductive tissues, i.e., during seed development in barley (Wang et al., 2009). We hypothesise that this gene is very similar to *V-PPase* genes previously identified in tomato, pear and grape fruits during development of the fruiting organ. *HVP3* probably plays a role in barley in controlling pH regulation and accumulation of pigments, and is expressed during seed development in non-stressed conditions.

V-PPase genes play an important role in improving plant growth under abiotic stresses, as evidenced by studies utilising gene transformation technology. For example, genetic transformation with *AVP1* improved tolerance to both salt and drought stresses in cotton plants, increasing fibre yield by at least 20% compared to wild type (Pasapula et al., 2011). Transgenic creeping bentgrass (*Agrostis stolonifera* L.) with the same *AVP1* showed significantly higher fresh and dry weights than wild type under salt stress (Li et al., 2010). It has been reported also that transgenic tobacco plants with *TsVP* from the halophyte plant, *Thellungiella halophila*, accumulated more solutes in control conditions and more Na^+ under salt stress then wild type, and showed 60% greater dry biomass in the presence of salinity (Gao et al., 2006). Co-expression of two transgenes in one cassette from wheat, *TVP1* (Vacuolar H^+-pyrophosphatase) and *TNHXS1* (Na^+/H^+ antiporter), improved significantly salinity tolerance of the transgenic tobacco plants compared to controls (Gouiaa et al., 2012). Chilling is another abiotic stress and it was reported that V-PPase activity is dramatically reduced due to chilling, resulting in cytoplasmic acidification and inactivation of essential metabolic reactions in mung bean (Kawamura, 2008). However, over-expression of *OVP1* significantly enhanced cold tolerance in transgenic rice, increasing plant growth under these conditions (Zhang et al., 2011). Such studies using transgenic plants demonstrate the important role of *V-PPase* genes in stress responses, and are positive pilot studies for the improvement of barley production using genetic transformation. But even natural polymorphism in *V-PPase* genes amongst barley germplasm should be harnessed for barley improvement. We hope barley breeders can accommodate our knowledge about *V-PPase* genes and work towards producing new barley cultivars with better growth and tolerance to abiotic stresses.

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Chapter 7

APPLICATION OF BARLEY IN CEREAL TECHNOLOGY

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ABSTRACT

Barley (*Hordeum vulgare*) is one of the oldest agricultural crops worldwide and it is one of the economically most important plants. Barley grain is widely used in production of malt, beer and whiskey. Small portion of barley grain is processed into various size of pearls, flour or flakes. Barley flour was used to enrich wheat bread and pasta replacing 30-40 % of wheat flour. Barley flour affected changes of dough properties, deteriorates its machinability, and decreases the volume of bread and bakery products. Despite technological disadvantages, consumption of cereal barley products is nutritionally beneficial, due to the significant content of non-starch polysaccharides (fiber), e.g., pentosans (arabinoxylans), (1→3)(1→4)-β-D-glucans (β-glucans), fructans, and also soluble proteins with essential amino acids and bioactive ingredients. The distribution of the nutrients and the functional constituents in the barley grain was not uniform. The single milling streams of Czech hulled and hull-less barley varied in polysaccharides content and physico-chemical properties. It was possible to select the attractive barley flour and meal streams with desired composition according to their yield, and technological and nutritional quality. The results of chemical analyses were supported with FT-IR spectra and multivariate analyses. The potential of barley grain was also used to prepare barley sourdough with specific natural antimicrobial properties. The additions of barley sourdough affected sensorial and nutritional properties of bread, inhibited growth of molds, and prolongate shelf life of the product. Barley flour and bran were confirmed as a suitable medium for the growth of lactic acid and propionic acid bacteria. In recent years barley bread and dairy products with added barley pearls appeared on the Czech market as well as a barley juice prepared from a young barley plant.

Keywords: Barley β-glucans; Barley flour; New barley functional food; Barley sourdough; Shelf-life

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ABBREVIATIONS

F	single flour barley streams
M	single meal barley streams
SRC	solvent retention capacity
FT-IR	Fourier transform-infrared spectra
PCA	principal components analysis
SDF	soluble dietary fiber
IDF	insoluble dietary fiber
TDF	total dietary fiber
FOS	fructo-oligosaccharides
LAB	lactic acid bacteria
PAB	propionic acid bacteria

INTRODUCTION

Barley is a resilient plant, tolerant of a range of conditions, which may have been cultivated since 15 000 BC. Cultivated barley, *Hordeum vulgare*, is mainly grown for animal feed, especially for pigs, for malting and brewing in the manufacture of beer and for distilling in whisky manufacture. Barley is consumed in Tibet and Morocco in large quantities. A small amount of barley is used for foodstuffs in Western world. Pearled barley is eaten in soups and stews in the United Kingdom, in the Central and Eastern Europe and in the Far and Middle East. Ground barley is also used in bread and porridge in some countries (McKevith, 2004).

The barley meal and flour were the dominant bakery milled cereals in Bohemia and Moravia (today Czech Republic, the Central Europe) in last centuries (18th and 19th century). There were the traditional bakery products barley pancake and scone in the Czech Republic (Figure 1).

The differences of barley grain components limits possibility of its use of traditional bread. Barley bread prepared only from barley flour showed low specific volume. Lower content of high molecular barley hordeins and hordenins with their distinct properties (elasticity and extensibility) were not able to make typical gluten network in compared to wheat proteins (gluten). The colour and flavour of barley based bakery products are also different from wheat or rye bakery products. Some of the consumers often describe barley bread and barley bakery products as products with bitter taste, stiffer consistency and more compact crumb and darker colour (Prihoda et al., 2003).

The Czech Republic was incorporated in the European Union (EU) programme BARLEYBREAD in 2009 (BARLEYBREAD-a project financed by EU within 6th Framework Programme).

The aim of the study was to investigate consumers' acceptance for barley bread with different barley/wheat ratios and different contents of salt. Effects of giving health information about barley and salt reduction on consumer acceptance were also studied. A sensorial descriptive test of breads by a trained panel and preference mapping were performed based on consumer tests in six European countries (UK, Spain, Turkey, Estonia, Czech Republic and Norway) (Granli et al., 2009; http://www.esn-network.com/888.html).

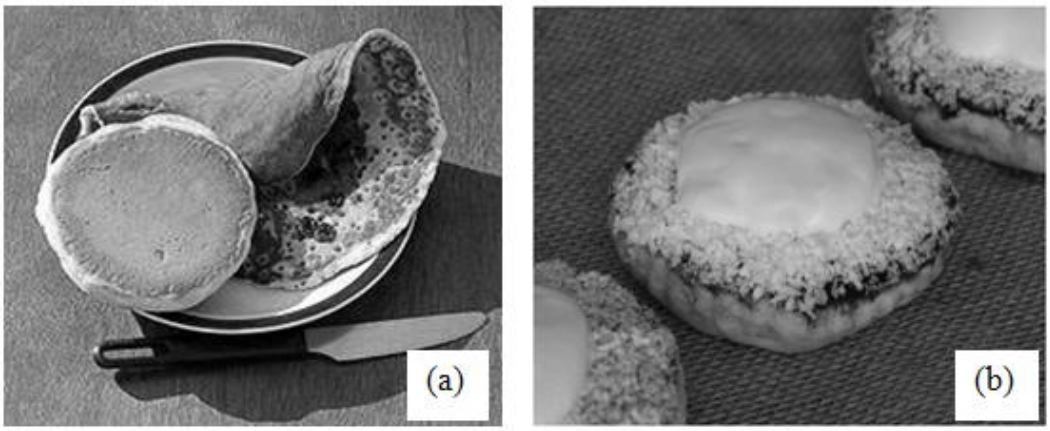

Figure 1. Typical barley flour based bakery products in the Czech Republic: (a) pancakes and (b) scones (with permission from Zeelandia Company, Czech Republic).

General, the aim of this framework programme was to propose production of health beneficial fiber-rich bread from European barley cultivars. A single result of renew a barley consumption was to decrease of imported soya for feeding consumption and to increase of soluble fiber intake in human diet. Fiber has an important effect on health improvement and anti-aging effect of not only European population.

USE OF BARLEY IN FOOD TECHNOLOGY

The incorporation of barley preparations to foods provides physiological benefits and also alters processing and handling of foods as well as their texture, colour, flavour and taste. Incorporation of barley into wheat based foods results in alterations in food properties due to the combined effects of dilution of gluten and the physico-chemical properties of barley components. Barley and barley components influence dough viscoelastic properties by a dramatic increase in water absorption of dough (Izydorczyk et al., 2005 and 2008; Jacobs et al., 2008).

Andersson and Åman (2011) discussed the research on some different functional barley products made in the Western world and they described the latest use of barley for cereal foods (e.g., muffins, yeast-leavened bread, pasta, noodles, cookies and biscuits) and use of barley for other foods (e.g., bars, extruded crisp bread, puddings and tortillas etc.).

Thondre and Henry (2009) tested the effect of lowering glycemic response in unleavened flat bread fortified with barley β-glucan. The behaviour and degradation of barley β-glucan in white wheat bread was investigated during baking (Cleary et al., 2007). The incorporation of barley β-glucan resulted in sticky dough, lowered specific volume and lowered height of bread compared to the control wheat bread. Barley β-glucan improved the nutritional quality of white wheat bread by reducing of starch digestibility.

New functional barley based products were developed in partnership from Institute of Chemical Technology (Prague, Czech Republic) and Zeelandia Company (Malsice, Czech Republic). In cereal technology of recent years, barley was included in light and dark bread, baguette, breakfast cereals, flakes, snack stick, pasta, and various sweet bakery products (e. g.

buns, muffin, biscuits, cookies, scones, panettone etc). The presented barley based bread and bakery products contained a different ratio of barley flour (Figure 2). The part of wheat flour was replaced by barley flour in breadmaking. The incorporation of barley flour in case of pie recipe was 26%, in baguette recipe 35%, in barley bread 60% while muffins were prepared with 100% of barley flour. Portion of barley in pannetone recipe was 25% and in biscuits 40%.

Barley flour can also be used to pasta. Oriental Asian noodles were successfully enriched with fiber-rich barley fractions (Izydorczyk et al., 2005). Barley flour replaced part of the wheat flour or semolina in pasta with good results (Slukova, internal research, unpublished data). The optimal addition of barley flour was 30% in pasta recipe. The pasta was evaluated in dry state and after cooking preparation and it showed acceptable sensorial properties. Moreover, the barley based pasta contained slightly higher content of total dietary fiber and β-glucans and significantly higher content of resistant starch. The addition of 50% of barley flour to the recipe of pasta showed unpleasant, more cereal and bitter taste of prepared pasta, and also unattractive shape and appearance of pasta after its cooking.

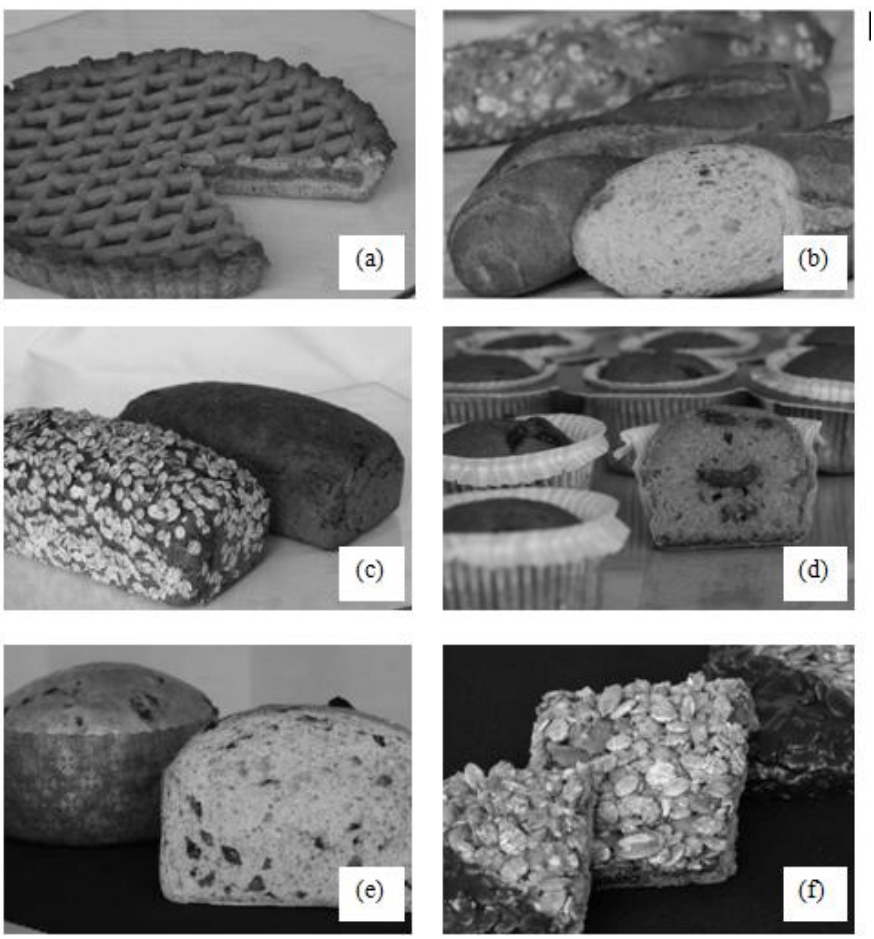

Figure 2. Samples of the barley based products: (a) pie, (b) baguette, (c) barley bread, (d) muffin, (e) pannetone, (f) biscuits (with permission from Zeelandia Company, Czech Republic).

There is up-date the interesting use of barley in dairy-farming, meat industry and gastronomy in Czech Republic. Processed low-fat cheese with barley pearls (brand name was GLADIATOR) were successfully presented in Eco-Trophelia competition 2012 (a student competition, a novelty fair trade in France, West Europe) (EcoTrophelia. EU, 2012; http://www.ecotrophelia.eu/?s=Gladiator&search=). Processed cheese is a very favourite dairy product in some parts of Europe; this product is an important source of calcium and animal proteins in human nutrition. The application of barley fibrous material due to the beneficial role of dietary fiber in human nutrition is a way of new functional food development. There was the content of total fiber (2.9%) and β-glucans (0.3%) in the processed cheese, and the energy value of the cheese was only 705 kJ (168 kcal) (per 100 g of the product). The rheological and sensorial properties of the processed cheese with barley pearls were agreed with the demands and acceptability of consumers and legislation.

Moreover, barley pearls with whey medium was tested as effective a prebiotic substance in spread and yogurt and other dairy products (Slukova, internal research, unpublished data).

Roasted ground barley can serve as no caffeine coffee substitute. Extract from young barley plant can be used for production of soft drinks such as green barley juice (EcoTrophelia.EU, 2011; http://www.ecotrophelia.eu/wp-uploads/2011CZPRODUCT.pdf), and tea etc.

Barley grain, pearl or flour also could be processed by extrusion. Extruded barley sticks, crisp barley bread and pasta also (with the best addition of barley flour 30%) showed very good and palatable textural and sensorial characteristics (Slukova, internal research, unpublished data).

CULTIVATION OF BARLEY

The fifty-seven two-rowed spring barley varieties, seventeen two-rowed winter barley varieties and thirty-three multi-rowed winter barley varieties are registered in database of Czech Republic (valid for year 2012). Hull-less spring barley (label *AF Lucius*®, Agrotest fyto, Czech Republic) is only determined for food and feed application in the Czech Republic. The rest of barley varieties (all hulled) are included in malt production and in special processing. Any one cultivated winter barley variety for food production is registered in Czech Republic. The cultivation of several varieties of hull-less spring barley (*Taiga*, and *Lawina* etc.) and winter barley (*Hiberna*) are licensed in European Union. (http://www.vukrom.cz/about-us?set_language=en)

Renewed interest about barley for health impact was initiated in research institute of Canada and USA, then Australia, Japan, United Kingdom and other states. This interest lead to new barley breeding for direct food consumption and for production of barley products with increased content of dietary fiber, especially soluble fiber (β-glucans) and other nutrients (vitamin E etc.). The numbers of spring barley varieties are registered in above mentioned countries: barley with higher content of β-glucans, higher content of proteins, preferential proportion of amino acids, waxy starch type and with other nutritional benefits. The disadvantages of breeding of the new barley varieties are lower yield, smaller grain size and impaired agricultural characteristics. The new food hull-less barley variety betaBARLEY® with very high content of β-glucans (more than 6%) was registered in Germany (Western

Europe) in 2011 and this variety finds large use in not only bread production (Dieckmann, 2011).

To develop a food barley industry and make verifiable health claims, hull-less barley cultivars that give a consistent level of β-glucan must be developed, and best-practise guidelines developed for agronomic management, harvesting and post-harvest management. Testing of β-glucan concentration across sites and seasons would need to be included as part of cultivar evaluation. Realisation of these benefits requires understanding of genotype and environment effects on β-glucan concentration and how this variation affects biological activity of barley foods. The role of β-glucan as an assimilate buffer adds complexity to interpreting the effects of environment during grain filling. Autumn sowing and fungicide increased the duration of grain filling, decreased β–glucan concentration but increased environmental stability; possibly due to lower demand for assimilate buffering (Dickin et al., 2011).

There are three types of barley varieties based on starchy component. The portion of amylopectin and amylose is about 74-79% and 25-30% in normal (standard) barley starch, respectively. Waxy barley shows low content of amylose (from 10% to 0%) and this barley grain contains higher content of total and soluble β-glucans and β-glucans with high molecular weight in compared to normal barley type. However, high-amylose barley shows higher content of amylose (40-45%). Single type of barley with unusual amylose and amylopectin content showed different physico-chemical properties (thermal and pasting properties, glass transition, water sorption, recrystallization etc.) (Song and Jane, 2000).

NUTRITIONAL COMPOSITION OF BARLEY

Barley is a source of carbohydrates (starch and non-starch polysaccharides, and reducing sugars), proteins, B vitamins, minerals and lipids. As well as barley contains a range of bioactive ingredients such as tocols, flavonoids and phenolic compounds. These compounds may provide some of health benefits. Barley also contains a number of anti-nutrients (e. g. phytic acid). Barley varieties have been classified as hull-less (known as naked) and hulled (known as covered). Hull-less barley varieties have better nutritional value than hulled ones as they contain more proteins, lipids and soluble dietary fiber (Soares et al., 2007; Zhang et al., 2002).

Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plants substances. Dietary fibers promote beneficial physiological effects including laxation, and/or blood cholesterol and blood glucose attenuation (AACC Report, 2001). It is known that fiber compounds are considered as multifunctional substances positively affecting the activity of human body. Dietary fiber can decrease of risk of cardiovascular diseases, prevention of colon and rectum cancer, water balance, satiety, prevention of overweight and obesity, effect of gut microbiota and immunity system, prevention of constipation and type-2 diabetes (Gorecka et al., 2000). For this reason the recommended intake of dietary fiber is more than 25 g/day in the diet for adults in European Union (EFSA Journal, 2010).

Table 1. Permitted health claims and conditions of use of the claim relating to barley grain fiber, β-glucans and β-glucans from oats and barley (Commission Regulation EU No 432/2012)

Nutrient, substance, food or food category	Claim	Conditions of use of the claim
Barley grain fiber	Barley grain fiber contributes to an increase in faecal bulk	The claim may be used only for food which is high in that fiber as referred to in the claim HIGH FIBER as listed in the Annex to Regulation (EC) No 1924/2006.
Beta-glucans	Beta-glucans contribute to the maintenance of normal blood cholesterol levels.	The claim may be used only for food which contains at least 1 g of beta-glucans from oats, oat bran, barley, barley bran, or from mixtures of these sources per quantified portion. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 3 g of beta-glucans from oats, oat bran, barley, barley bran, or from mixtures of these beta-glucans.
Beta-glucans from oats and barley	Consumption of beta-glucans from oats or barley as part of a meal contributes to the reduction of the blood glucose rise after that meal.	The claim may be used only for food which contains at least 4 g of beta-glucans from oats or barley for each 30 g available carbohydrates in a quantified portion as part of the meal. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained by consuming the beta-glucans from oats or barley as part of the meal.

In Europe, the European Food Safety Authority (EFSA) has approved nutrition and health claims (valid from 14th of December 2012) (Commission Regulation EU No 432/2012). Permitted health claims relating to barley grain fiber, β-glucans and β-glucans from oats and barley are shown in Table 1.

The optimized formulation for bread based on 100% hull-less barley flour was developed for nutrition purposes considering to the health claim requirements for β-glucan (Kinner et al., 2011). This barley bread complies with the permitted health claims in 2012 (Commission Regulation EU No 432/2012).

The positive effects of β-glucans are mainly attributed to its high viscosity in aqueous solution and thus increasing the viscosity of the contents within the intestinal tract (Jalili et al., 2000). Viscosity in the small intestine is determined by the concentration, molecular weight and solubility of β-glucan. Viscosity of β-glucan is indirectly decreased due to degradation of the molecular weight during food processing (Åman et al., 2004; Andersson et al., 2004 and 2008).

Dietary fiber can be classified into water-soluble (SDF) and water-insoluble dietary fiber (IDF). The terms soluble and insoluble dietary fiber have been used in the literature to differentiate between viscous, soluble types of fiber (e.g., pectins) and insoluble components such as cellulose. Therefore, FAO/WHO proposed the distinction between soluble and insoluble fiber should be phased out (FAO/WHO, 1998). Soluble cereal fiber such as β-glucans, arabinoxylans, galactomannans and arabinomannans is effective in reducing total blood cholesterol, reducing the heart attack and promoting satiety. Insoluble cereal fiber consists of cellulose, insoluble hemicelluloses and lignin, and it helps in treating constipation and reduces the risk of colon cancer and diverticular disease (Salovaara et al., 2007).

The dominating barley soluble fiber components are (1→3)(1→4)- β-D-glucans (β-glucans) and arabinoxylanes. There is no particular concentration of β-glucans in the aleurone layer in barley and the distribution appears to be even across endosperm (Collins et al., 2010). The endosperm cell walls are built up mainly of β-glucans and contain smaller amount of arabinoxylanes, while aleurone cell walls are composed primarily of arabinoxylanes (about 70%), with smaller amounts of β-glucans (26%). The other valuable dietary fiber components such as fructans (fructo-oligosaccharides, FOS), glucomannans and arabinogalactans are present in the endosperm cell wall especially. The structure, content and chain length (degree of polymerization) of fructans were studied in grain, flour or bread of wheat, barley and rye (Verspreet et al., 2012; Jenkins et al., 2011; Andersson et al., 2009). Galactomannans and arabinogalactans have not yet been thoroughly investigated.

CHARACTERIZATION OF BARLEY MILLING STREAMS

A milling flow of barley grain is considerably different from that of wheat and to some part also from rye mill flow. Barley fraction rich in β-glucan were obtained by various combinations of pearling, grinding, and sieving and air classification. The milling performance, chemical compositions, rheological and physicochemical properties of hull-less barley fiber-rich streams were investigated (Andersson et al., 2000 and 2003; Holtekjølen et al., 2008).

Izydorczyk et al. (2003) reported a simplified roller milling procedure that gives a high yield of a fiber-rich stream from hull-less barley. According to a milling study by Zheng et al. (2000), the relative distribution of β-glucans within the endosperm of barley genotypes grown in Canada varied significantly, and was dependent on the content of β-glucans in the grain. In barleys with low of content β-glucans, levels were relatively higher in the subaleurone layer than the endosperm, whereas barleys with average to high content of β-glucans contained more β-glucans in the endosperm than the subaleurone layer. Milling streams of hull-less barley, and dough and bread with hull-less barley flour were analyzed with regard to properties of β-glucans (their viscosity and molecular weight distribution), dough formation and bread making (Andersson et al., 2004). The chemical composition (ash, protein, starch, β-glucan and arabinoxylan) of the milling streams varied depending on barley type (normal, waxy and high-amylose barley). Streams with higher contents of β-glucans could be obtained by air classification (Andersson et al., 2000). Barley flour showed variations in water absorption capacity and baking performance with different polysaccharide content and

composition (Holtekjølen, 2008). Hull-less barley flour was incorporated into white and wholegrain wheat bread and this bread showed acceptable and desirable nutritional and sensorial properties (Skrbic et al., 2009).

The distribution and sorption retention capacities of polysaccharides in single milling streams from two-rowed, spring, malting, a hulled (*Bojos*) and a hull-less (*Malz*) normal starch Czech barley variety were determined. The aim of this study was to assess the milling results with regard to the yield of single streams in connection with their chemical composition, especially β-glucans content. The contents of β-glucans, arabinoxylans, fructans, total dietary fiber, total starch and damaged starch were compared in single flour barley streams (F) and single meal barley streams (M). The flour and meal barley milling streams differed in their particle sizes (granulation), ash content and colour, yield and chemical composition. Single flour milling streams were obtained from single part of barley grain. Designed flours and meals 1 to 9 and 13 to 15 were from stage of breaking barley grain while designed flours and meals 10 to 12 and 16 to 18 were from stage of reduction.

Knowledge about physicochemical properties (sorption retention capacity) of single barley milling streams and their possible effects on the baking performance (water addition, dough manipulation, and crumb characteristics) are very important during breadmaking.

The single milling streams of hull-less and hulled barley varied in polysaccharides content and sorption properties. Most of hull-less milling streams showed higher content of β-glucan (Figure 3), total dietary fiber, damaged starch and arabinoxylans but lower content of fructans than hulled milling streams. The content of total dietary fiber varied from 5.1% (F1) to 22.7% (F16) and in selected meal streams from 15.0% (M12) to 25.5% (M7) in hull-less barley. The insoluble dietary fiber constituted a predominant part of total fiber (except for a stream F7). Single hulled barley streams had much lower total fiber content (as much as threefold) than single hull-less streams.

The individual polysaccharides were localized in various barley grain tissues. β-glucans were found in the endosperm, subaleurone and aleurone layer. Arabinoxylans were observed in pericarp and aleurone layer mainly. Fructans were localized in subaleurone and aleurone layer, and damaged starch was determined especially in meal streams on the basis of the flow milling diagram.

Solvent retention capacity (SRC) of milling streams of hull-less and hulled barley is shown in Figure 4 and Figure 5, respectively. Solvent retention capacity is the weight of solvent held by the flour or meal after centrifugation; it is expressed as percent of solvent in flour or meal weight on a 14% moisture basis. The highest values of water absorption (SRC 1) were caused by the presence of water-soluble components (soluble β-glucans, fructans and arabinoxylans), water-soluble proteins and damaged starch. Generally, meal streams showed higher hydration capacity than flour streams, and milling streams of hulled barley showed lower water retention capacity than milling streams of hull-less barley. The highest content of arabinoxylans (SRC 2) was observed in meals M7, M9, M10, M11, M12, M17 and M18 mainly, and a significant content of arabinoxylans was found in a flour stream F16 of hull-less barley. The milling streams of hulled barley showed lower contents of arabinoxylans compared with milling streams of hull-less barley. The distribution of arabinoxylans in hulled streams appear to be similar to that of arabinoxylans in hull-less, but the slight differences were observed in streams F9, F17, M4 and M11. The retention capacity of sodium carbonate (SRC 3), which is related to the content of damaged starch, it is shown more important in

meal streams. SRC 3 profiles of hull-less and hulled streams are very similar except the streams F16, F17, F18, M4 and M11 (Figure 4 and Figure 5).

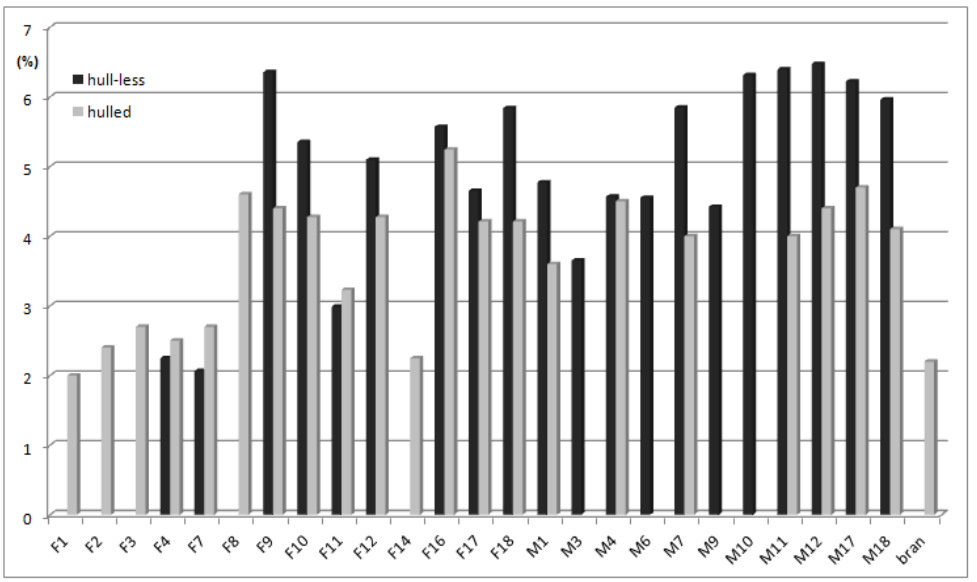

Figure 3. Comparison of the content of β-glucans (% in dry matter) in barley streams (F-flour milling streams, M-meal milling streams) (Velebna et al., 2012).

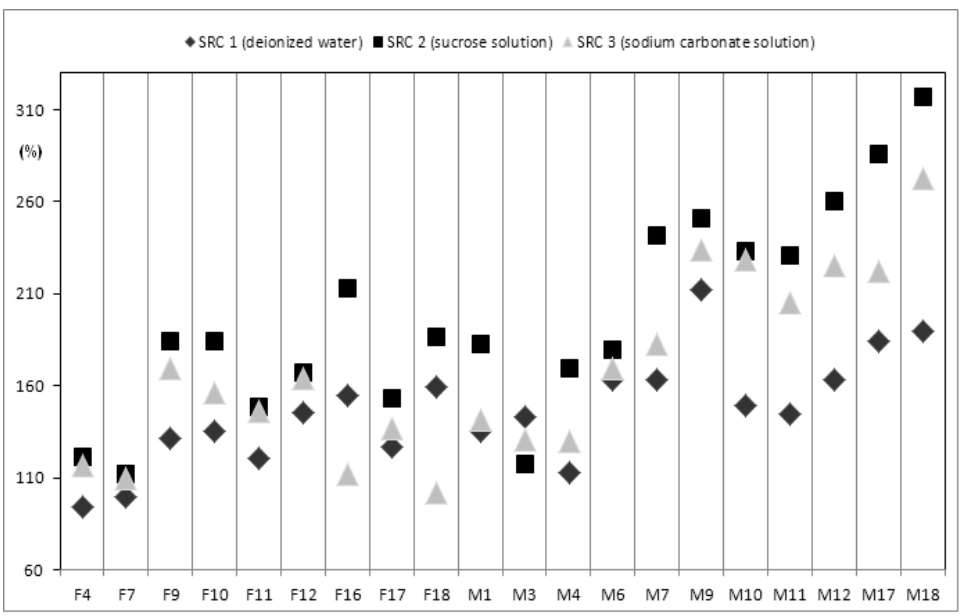

Figure 4. Solvent retention capacity of hull-less barley milling streams (Velebna et al., 2012).

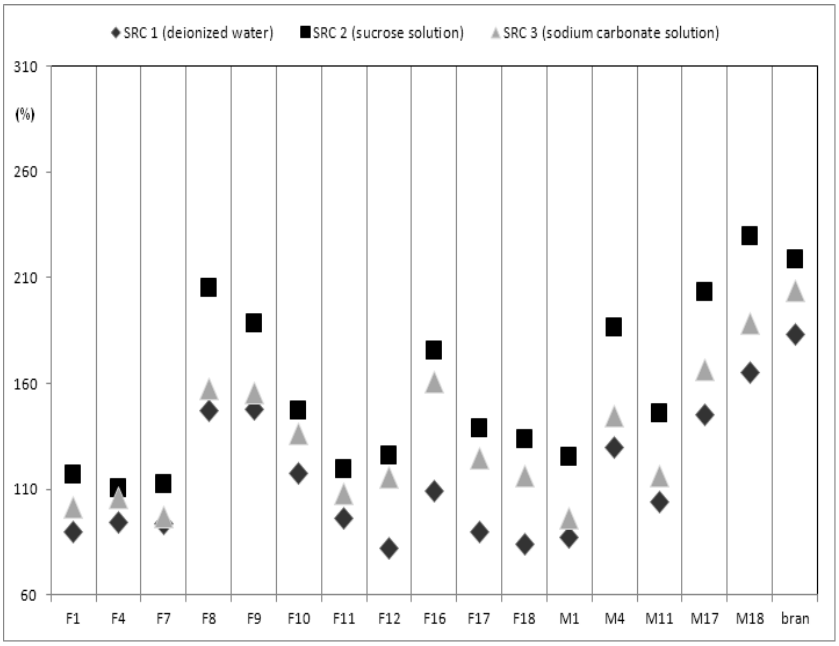

Figure 5. Solvent retention capacity of hulled barley milling streams (Velebna et al., 2012).

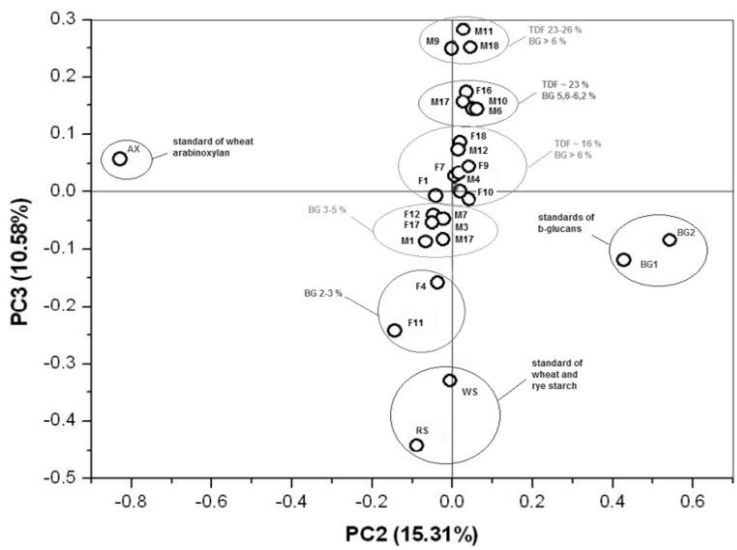

Figure 6. PCA score scatter plot of the FT-IR spectra of hull-less barley flours (F) and meals (M) in the 1,200-800 cm^{-1} wavenumber region. The clusters included the separate single milling streams on the basis of starch and non-starch polysaccharides content (Velebna et al., 2012).

The results of chemical analysis were supported by FT-IR study and multivariate analysis.

Fourier transform-infrared spectra (FT-IR) spectra and principal components analysis (PCA) analysis could be used to a fast screening of the barley streams with regard to different polysaccharides composition (Figure 6). It was possible to select the attractive flour and meal streams with their desired yield, technological and nutritional quality (Velebna et al., 2012).

EFFECTS OF FOOD PROCESSING ON BARLEY COMPONENTS

During barley milling and pearling, there was a dramatic increase in insoluble barley dietary fiber content, while the content of β-glucans was slightly decreased (Sikorski, 1997).

Table 2. Dietary fiber content of barley materials before and after extrusion (Slukova et al., 2011)

Sample characterization	Dietary fiber content in barley		
	IDF	SDF	TDF
	(% in dry matter)		
untreated barley pearl	5.8	4.2	13.5
extruded barley pearl	6.8	5.5	13.6
extruded barley grain	4.6	4.5	9.6

IDF-insoluble dietary fiber, SDF-soluble dietary fiber, TDF-total dietary fiber.

The effects of barley processing technology on nutritional value of the barley products were investigated. Thermo mechanical processing (extrusion) and thermal (baking) could influence the content and composition of total dietary fiber. Extruded barley grain and barley pearl showed the slight increase of insoluble dietary fiber, soluble dietary fiber and also β-glucans during barley pearl extrusion (Table 2). The content of the barley components were compared to untreated barley materials (Slukova et al., 2011).

Vasanthan et al. (2002) described the possibilities of change of fiber fraction content in plant materials during extrusion. The redistribution of part of the insoluble fraction to soluble fraction can lead to the increase of soluble dietary fiber content. Or the insoluble dietary fiber can be fragmented to lower molecular weight fragments and these fragments may not be detected as fiber fraction by the enzymatic assay methods which are used for determination of dietary fiber and β-glucans content in barley materials. The increase in total dietary fiber in barley flour could be due to the formation of retrograded amylose (resistant starch). Maillard reaction products or enzyme-resistant indigestible glucans were formed by transglycosidation during extrusion.

The bread making process consists of three major stages: mixing, fermentation and baking (Dewettinck et al., 2008). During baking, starch undergoes a series of changes known as gelatinization which is important to the susceptibility of starch to enzymatic breakdown. Processing conditions and ingredients may also influence the formation of resistant starch in barley based products. Resistant starch is a fraction of starch which is not digested in the small intestine, and resistant starch is fermented like soluble fiber by the colonic microflora producing short chain fatty acids. Increase in both baking temperature and baking time was found to increase the content of resistant starch in barley bread (with 70% of barley flour addition).

During bread making the average molecular weight of barley β-glucan has decreased with longer time of dough mixing and fermentation (from 2 000 kDa to 140 kDa). The β-glucan was degraded by endogenous β-glucanases in the flour probably. It is necessary to keep the important content of high molecular weight β-glucans because of low molecular

weight β-glucan (about 80 kDa) is less effective in increasing excretion of bile acids and cholesterol-lowering effect. The effect of baking on the change of molecular weight and content of β-glucan was not significant (Coles et al., 2007). However, Salovaara et al. (2007) found the depolymerisation and rapid decrease of β-glucan viscosity during baking of bread. The β-glucan content in the bread was lower as compared to that in flour.

Table 3. The comparison of β-glucans content in barley raw materials and barley based bread and bakery products (Slukova et al., 2011)

	Portion of barley in flour blends in recipe	β-glucans content in flour blends	Theoretical β-glucans content in final products	Real β-glucans content in final products
	(%)	(% in dry matter)		
Bread	60.0	3.6	3.2	3.1
Cookies	40.0	1.8	1.1	0.9
Baguette	35.0	1.7	1.2	1.1
Panettone	25.0	1.1	0.7	0.6
Scone	20.0	1.3	0.7	0.6

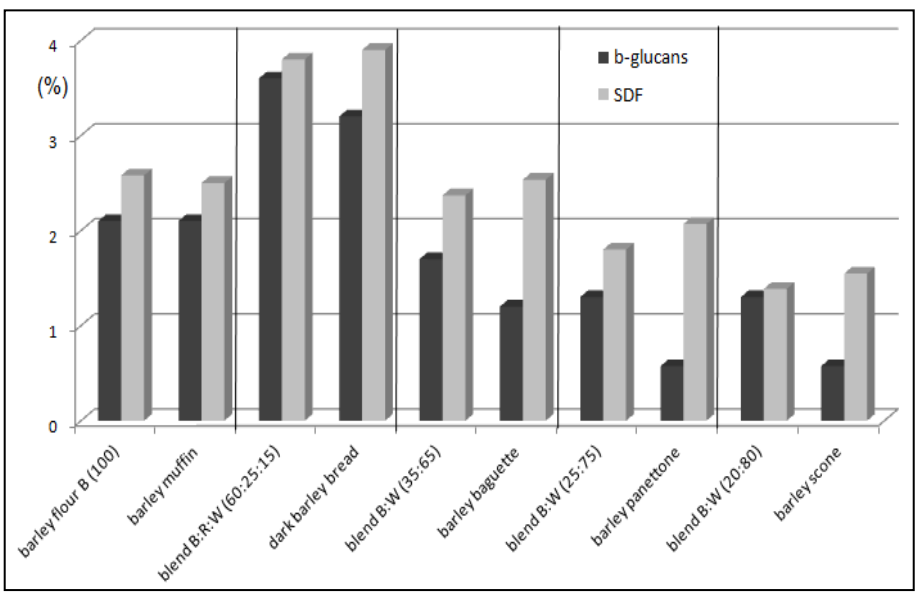

Figure 7. Content of β-glucans and soluble dietary fiber (SDF) in flours and barley based products (B-barley flour, W-wheat flour, R-rye flour) (Slukova et al., 2011).

The changes in cereal fiber of barley bread and bakery products were observed during bread making. Some of barley based products were shown in Figure 2. However, the single valued results were not obtained. The unique tendency was not observed in change of insoluble, soluble and total dietary fiber content in flours and final bread and bakery products. The comparison of β-glucan content in raw-materials and barley based bread and bakery products is shown in Table 3 (values are related to dry matter of material). The content of β-

glucans was 3.6% in blend flour (barley:rye:wheat, 60:25:15) and 3.1% in dark barley bread. The decrease of β-glucans content was evident during bread making. The lower β-glucans content (in comparison with the basic flours) was observed for barley based products, excepting muffin (Table 3). The comparison of β-glucan content and soluble dietary fiber in flours and products is shown in Figure 7.

The dietary fiber contents in flours and of them prepared barley based products are compared in Figure 8.

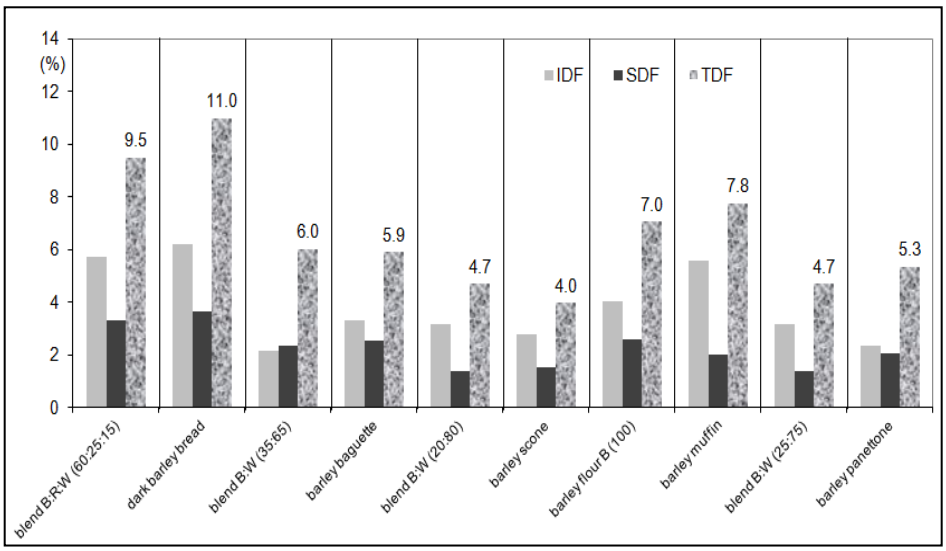

Figure 8. Dietary fiber content in flours and barley based products (B-barley flour, W-wheat flour, R-rye flour) (Slukova et al., 2011).

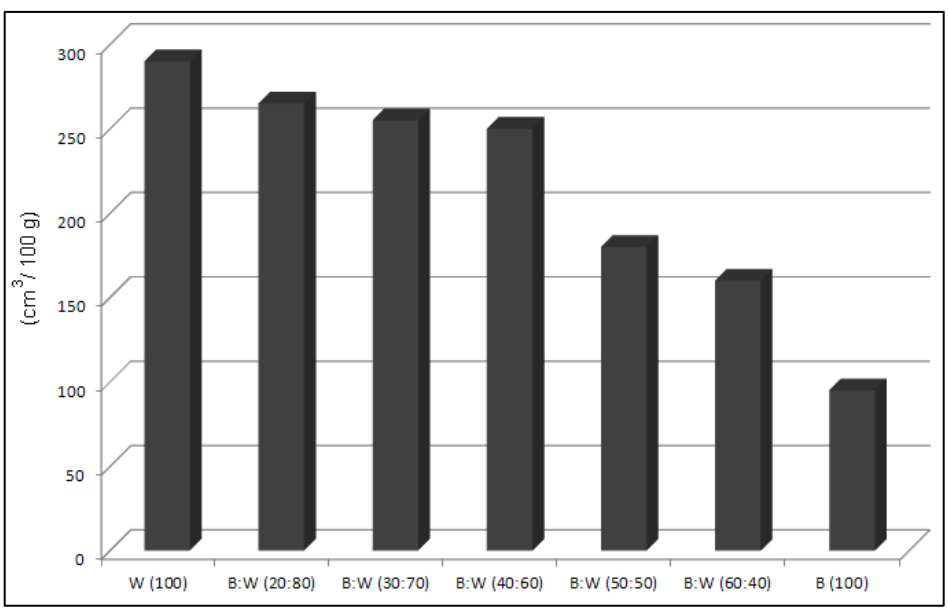

Figure 9. Specific volume of bread with different portion of barley flour (W-wheat flour, B-barley flour) (Slukova et al., 2011).

The highest content of fiber was found in dark barley bread by reason of barley flour, rye flour and barley pearl incorporation. As fiber content of flour and product is compared, the single valued results are not obtained. However, the small change in dietary fiber profile was observed during bread making. The dark barley bread, muffin and panettone had higher total dietary fiber content, while total dietary fiber of baguette was like as that in flour blend, and total dietary fiber of scone was lower. The content of all fiber components was decreased in barley scone, soluble dietary fiber content was decreased in muffin and insoluble dietary fiber content was decreased in panetonne. Proportion of IDF/SDF did not change (Slukova et al., 2011).

The addition of barley flour (up to 40%) and rye flour to wheat flour lead to decrease of specific volume of prepared bread and bakery. The specific volume of barley bread with different ratio of barley flour is shown in Figure 9. The sensorial properties of bread with the addition of 40% of barley flour were unpalatable. The similar results were confirmed by Bhatty (1986), Holtekjølen et al. (2008) and Baik and Ullrich (2008).

EFFECTS OF CEREAL FERMENTATION ON BARLEY COMPONENTS

A use of different fermented products of barley milling provides another possibility of utilization of barley in human nutrition. The use of cereal fermentation by means of yeast and lactic acid bacteria on barley flour or barley bran showed interesting results with regard to improve the baking (flavour, structure and stability of bread) and nutritional quality of bread. In addition, sourdough can also retard starch digestibility leading to low glycemic responses and improve bioavailability of minerals and bioactive compounds (Poutanen et al., 2009). Cereal fermentation may produce non-digestible polysaccharides (dietary fiber) or new bioactive compounds (such as prebiotic oligosaccharides). Also cereal fermentation may degrade (reduce) gluten to develop gluten-free products.

Sourdough is represented by yeast and lactic acid bacteria, especially. Investigation of the microbial composition of sourdough is relevant in order to determine the potential activities of sourdough microorganisms. The most important group of sourdough fermenting bacteria is the *Lactobacillus* genus (Corsetti and Settanni, 2007; Ryan et al., 2008; Gerez et al., 2009; Dalie et al., 2010). Marklinder et al. (1996) observed effects of flour from different barley varieties on properties of barley sourdough and on quality of barley sourdough bread. The results showed that barley sourdough improved the taste of baked bread especially.

Cofermentation with *Lactobacillus buchneri* and *Lactobacillus diolivorans* represented a process to produce propionic acid in sourdough and to increase antifungal capacity of bread (Zhang et al., 2010). *Lactobacillus plantarum* (SM39) was cultured in combination with *Propionibacterium freudenreichii* (DF13) and co-culture process in a whey permeate medium was optimized to produce high levels of folate and vitamin B12 for nutrition, organic acids for antimicrobial effects and exopolysaccharides for improved texture and shelf life of bread (Hugenschmidt et al., 2011).

In order to provide the barley sourdough, barley flour or bran were fermented using both *Lactobacillus plantarum* (JM-57V-7A) and *Lactobacillus sanfranciscensis* (CCDM 451) and moreover Propionibacterium freudenreichii subsp. freudenreichii under the different processing conditions. Whey medium was used as a source of substrate for selected

microorganisms. Fermented products were exposed either to fluid drying (temperature 20-40 °C, time 7 h) or drum drying (temperature 160 °C, time 30 s), extrusion (temperature 50°C and low pressure). Thermal processing conditions were set to concentrate sourdough but not destroy natural produced organic acids. The barley sourdough was added to wheat toast bread formula. The addition of barley sourdough influenced bread quality and prolongation of bread shelf life especially.

Table 4. Composition and properties of barley sourdoughs (Slukova et al., 2012a)

Type and specification of sourdough (S)	Proteins	Total fiber	BG	LA	PA	AA	TTA	pH
	(%)						(°)	
S1-barley bran, whey medium, addition of synthetic PA and LA, fluid drying	15.2	10.0	4.5	0.8	1.9	0.5	40.5	4.5
S2-barley flour, water, LAB, addition of synthetic PA, fluid drying	11.9	5.9	5.8	1.7	1.2	0.6	101.5	4.2
S3-barley flour, whey medium, PAB, fluid drying	14.2	8.5	6.5	4.8	0.4	0.6	33.0	4.8
S4-barley bran, whey medium, PAB, fluid drying	12.8	8.1	4.5	0.6	0.6	0.4	12.5	6.3
S5-barley bran, whey medium, PAB, LAB, fluid drying	11.6	7.5	3.2	2.3	0.4	0.4	16.8	5.8
S6-barley bran, whey medium, PAB, extrusion	14.2	7.9	5.2	8.1	15.7	9.6	23.0	4.8
S7-barley flour, water, addition of synthetic PA, drum drying	9.2	2.7	1.5	8.3	0.6	1.4	137.5	2.6

S-sourdough, BG-β-glucans, LA-lactic acid, PA-propionic acid, AA-acetic acid, TTA-total titratable acids.

Also sourdough from barley flour or barley bran with the amount (2%, v/w) of chemically prepared lactic acid and propionic acid was added to wheat toast bread formula. Inhibition ability of chemically prepared organic acids was compared to the ability of natural organic acids formed during fermentation of the same types of sourdough. Lactic acid bacteria (LAB) and propionic acid bacteria (PAB) were used for their complementary carbohydrate metabolism. LAB mainly produces lactate and PAB can metabolize lactate to acetate and propionate. These natural organic acids could serve in food as preservatives owing to their antimicrobial effect. Inhibition activity of natural barley sourdough and synergic effect of barley sourdough with added acids were determined by means of growth of molds *Penicillium expansum* and *Fusarium culmorum* artificially inoculated on toast bread crumb.

Moreover, fermented types of barley sourdough affected sensorial and nutritional properties of toast bread; they inhibited growth of molds, and prolonged shelf life of the product.

The results of chemical analysis of barley sourdough are shown in Table 4. During fermentation the increase of total and soluble dietary fiber and proteins (especially soluble low molecular proteins) content was observed in barley sourdough compared with that in original barley flours and bran.

Table 5. Organic acids content (%), TTA (°), mold grow, sporulation and shelf life (days) of white wheat toast breads (Slukova et al., 2012a)

Bread (B)-sourdough (S) (addition of sourdough, w/w)	LA	PA	AA	TTA	Mold growth	Sporulation	Shelf life
B1-S1 (2%)	0.26	0.01	0.06	39.0	++	++	9
B2-S2 (2%)	0.28	0.02	0.08	39.5	+	+	10
B3-S3 (2%)	0.36	0.02	0.08	38.0	+	++	11
B4-S4 (10%)	5.70	0.80	1.67	37.5	+	++	12
B5-S5 (10%)	7.46	0.73	1.62	38.5	+	+/++	10
B6-S6 (10%)	5.00	0.95	1.71	38.0	+	++	14
B7-S7 (10%)	5.40	0.20	0.50	48.0	++	++	10

S-sourdough, B-bread, mold growth and sporulation: (+) weak, (++) medium, (+++) strong.

The addition of barley sourdough to bread also improved the consistency and bread making quality of yeast-leavened dough. The doughs were extensible and not sticky. Barley sourdoughs showed very good retention and binding capacity of water and usual aqueous solutions. The uniform water distribution and tight water binding was provided in dough structure during bread making. This effect also influenced staling rate of the prepared toast bread. The results of this investigation were presented on *Symposium on Cereal Fermentation for Future Foods 2012* (Slukova et al., 2012a) and they were published only in Czech research paper in Czech language so far (Slukova et al., 2012b).

The longer shelf life showed breads with the barley sourdough containing natural formed organic acids (breads B3, B4 and B6, see Table 5). These breads were acceptable, tasty, with soft crumb and slow staling rate. It was observed that addition of fermented barley bran in whey medium was very effective in inhibition activity in bread.

The continued investigation will require a large number of other analysis and clear verification antimicrobial properties of barley sourdough and its possibilities of other uses in bread and bakery production.

Moreover, dried barley sourdough could be used as thickening agent, as a part of instant food (e.g., soup, sauce and dressing) etc. It was observed that hordein and hordeinin protein fraction (gluten) was reduced during fermentation. So fermented barley products were not so bitter (due to the lower content of hordein protein fraction) and they would be acceptable for gluten-free food production. This is the challenge for future.

CONCLUSION

Barley is a desirable food component with health benefits provided by dietary fiber, especially partly soluble β-glucan fractions. Barley can also be a valuable component in low glycemic foods and new functional foods. Fermented barley products could be used to produce new gluten-free future foods. It is necessary to submit the breeding and developing of high-yielding hull-less and high-amylose cultivars of barley with high content of soluble fiber. The recent permitted health claims in European Union could increase the interest from food processors to develop, produce and offer new tasty and healthy commercial barley based foods. The greater interest, knowledge and higher consumption of barley products could be expected in consumers in Western countries.

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Chapter 8

ABIOTIC STRESS RESPONSE IN BARLEY AND THE EMERGENT ROLES OF MICRORNAS

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ABSTRACT

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops, typically yielding more than 150 million tons of grain per annum internationally. It is used extensively in a number of food and beverage products as well as in animal feed. The growth, survival and yield of barley are affected severely in different parts of the world due to abiotic stresses such as soil salinity, drought and boron toxicity. Research is underway worldwide to investigate genetic factors that may enable crop plants to adapt to abiotic stresses, to allow varietal selection and/or transgenic developments of stress tolerant plants for food security. Among the regulators of stress response are non-coding small RNA species called microRNAs (miRNAs). These are endogenous to animals and plants, and regulate gene activity by silencing the target mRNAs in various ways. In plants, they have been shown to play direct roles in regulation of a number of normal physiological processes as well as stress response. Over 125 conserved and 130 putative miRNAs are currently reported in barley, and many of these may have roles in responses to salinity, drought, oxidative and other stresses. This work focuses on barley miRNAs and their involvement in abiotic stress response.

Keywords: Barley, abiotic stress, salinity, drought, gene regulation, microRNAs

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ABBREVIATIONS

AGO	Argonaute
DCL1	Dicer Like-1
CSD	Cu/Zn superoxide dismutase
HEN1	Hua Enhancer 1
miRNA	microRNA
MUT68	a terminal nucleotidyltransferase
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
RISC	RNA-induced silencing complex
ROS	reactive oxygen species
siRNAs	small interfering RNAs
qPCR	quantitative PCR

1. INTRODUCTION

Barley (*Hordeum vulgare L.*) is one of the most important crops worldwide, ranking fourth amongst cereal crops based on production. It is typically cultivated over 56 million hectares of arable land and produces >157 million tons annually (Food and Agriculture Organization, 2012). Barley is used in staple foods such as porridge and bread and is a key component in beer and other beverages. It has been in human use since 8,000 BCE throughout North Africa, Southern Europe, East Asia, and North and South America. Barley belongs to the grass family Triticeae and has a 5.1 Gbp genome (The International Barley Genome Sequencing Consortium, 2012). The barley grain has a highly nutritive composition of carbohydrates (80%), protein (7-25%), lipids (3%), vitamins, minerals and phytochemicals (Newman and Newman, 2008; p58). Although it is now used mainly for animal feed, malt and seed, it is still a major food in parts of Asia and North Africa. It is also a functional food and is reported to reduce blood cholesterol, assisting in prevention of cardiovascular disease, hypertension, stroke as well as diabetes (Newman and Newman, 2008; p208). Barley can withstand a wide range of climates such as deserts or high mountains, and grow under marginal conditions such as drought (Lopez-Castaneda and Richards, 1994), alkaline soil (Murata et al., 2006) or frost (Plotnikov et al., 2012). However, environmental challenges such as soil salinity and prolonged drought cycles affect its growth significantly. These issues and the expected environmental changes necessitate improvements of the barley germplasm for future food security.

2. EFFECTS OF ABIOTIC STRESSES ON BARLEY

Soil salinity, drought, nutrient inadequacy, and toxicity of certain elements are amongst the most common abiotic stresses that affect barley as well as other crops. Salinity is a global issue, currently affecting over 100 countries and about 800 million ha (>6% of total land) (Munns and Tester, 2008). Drought stress also severely impedes growth of barley and other

crops. The effects of water deficit stresses include cell dehydration, production of reactive oxygen species (ROS) such as superoxide (O_2-), and hydroxyl radicals (OH-) and hydrogen peroxide (H_2O_2), causing oxidative damage to enzymes and cellular structures, stomatal closure leading to reduced CO_2 uptake, severely reduced plant growth rates and grain yield (Gill and Tuteja, 2010). Plants also require optimum temperature for normal growth and development, and extreme conditions cause severe stress effects in unadapted plants. Chilling prevents water absorption while freezing increases cell dehydration, leading to osmotic stress and hyper-accumulation of ROS. Exposure to high temperatures also results in significantly reduced yields of barley (Bavei et al., 2011). Barley, together with wheat, is also susceptible to boron and aluminium toxicity. Boron is an essential micronutrient, important for the structures of cell membranes and walls, and its deficiency results in stunted growth (Dell and Huang, 1997). However, it is toxic to barley at higher levels, and areas such as about 5 million ha of arable land in South Australia contain toxic levels of boron (Schnurbusch et al., 2010). Aluminium toxicity is prevalent in acidic soils and about 50% of the world's arable land is estimated to be acidic (Wang et al., 2006). Aluminium stress in barley was shown to affect the uptake and translocation of a number of minerals including phosphorus, calcium, magnesium and copper (Ali et al., 2011).

Considering the above-summarised physiological effects of the diverse abiotic stresses, it is essential to develop an understanding of the innate mechanisms of stress tolerance in plants, identify any genetic diversity in these, and use gene technologies if appropriate, for ensuring crop productivity. Plant responses to abiotic stresses involve a number of genes, regulatory pathways and signaling mechanisms that function in diverse ways such as salt uptake and transport, osmotic balance, redox balance, chaperone activities and other protective roles (reviewed in Munns and Tester, 2008; Hirayama and Shinozaki, 2010; Atkinson and Urwin, 2012). Among the recently discovered regulators of gene expression are the small non-coding RNAs. These fall into three main groups: microRNAs (miRNAs), small interfering RNAs (siRNAs) and piwi-interacting RNAs (piRNA), the latter being restricted to animal cells. Of these, all three can result in degradation of target coding mRNAs, miRNAs and siRNAs can cause gene silencing by prevention of translation, while siRNs and piRNAs can cause gene silencing by chromatin modifications. The area of gene regulation in plants by small RNAs has been reviewed extensively (Chen, 2009; Naqui et al., 2012; Arikit et al., 2013), and is outside the scope of the present work. This article focusses on a specific aspect, i.e., of the roles of plant miRNAs in abiotic stress response. It provides a summary of the structure and modes of actions of miRNAs, followed by an overview of miRNAs involved in abiotic stress responses in plants including barley, and ends with a perspective on how they may be used for crop improvement.

3. miRNAs: Post-Transcriptional Regulators of Gene Expression

3.1. Discovery of miRNAs

miRNAs are endogenous, typically 19-25 nucleotides long, single-stranded, non-coding RNAs found in both animal and plant transcriptomes, and have roles in post-transcriptional

regulation of gene expression through regulation of target mRNAs. The first miRNA, *lin-4*, was discovered by Lee et al. (1993) in *Caenorhabditis elegan* as a small, non-coding RNA with two transcripts of approximately 22 and 61 nucleotides. It was found to play an important role in timing control of larval development by regulating a target gene, *lin-14*, reducing the transcript levels of the latter by sequence complementarity between *lin-4* and the 3'UTR of *lin-14* mRNA. This discovery opened up a new concept about post-transcriptional regulation of gene activity by small RNAs. Reinhart et al. (2002) cloned small RNAs in *Arabidopsis thaliana*, and 16 of these matched the features of miRNA. They had different expression levels in seedlings, leaves, stems, flowers and siliques. Later on, numerous miRNAs were identified in diverse algae, e.g., 19 miRNAs in the green alga *Chlamydomonas reinhardtii* (Zhao et al., 2007), 26 in the brown alga Ectocarpus (Cock et al., 2010), and 231 in the red alga *Porphyra yezoensis* (Liang et al., 2010), suggesting they may be involved in gene regulation events in plants as well, considering the algal origin of plant chloroplast. To date, miRNAs have been identified in diverse fungi, plants and animals (miRNA Database; http://www.mirbase.org/). MiRNAs appear to have evolved into family members from ancestral to related species of organisms. Cloned miRNAs from *C. elegans* and *C. briggsae*, or human and mouse, have shown miRNA conservation between them (reviewed in Bartel, 2004). A majority of miRNA families were also found to be conversed between *A. thaliana* and rice (Jones-Rhoades et al., 2006), and many also amongst barley, wheat, maize, sorghum, Brachypodium and Populus (Schreiber et al, 2011; Yao et al, 2007). Many appear to be encoded by multiple genes (Table 1).

3.2. Some Terminology and Definitions Related to miRNAs

3.2.1. Terms Related to MiRNA Structure

The term 'primary miRNA' (pri-miRNA**)** refers to the long stem-loop (hairpin) structure, i.e., two sequences that complement each other within the same strand, the paired area forming the stem and the unpaired bases forming the loop. The 'precursor miRNA' (pre-miRNA) is a short stem-loop structure of about 64-303 nucleotides that is cut from pri-miRNAs, with the mature miRNA sequence usually located in the stem (Bartel and Bartel, 2003; Xie et al., 2005). The miRNA genes have their own promoter and typical TATA-box motif. In plants, they are usually monocistronic, located in intergenic regions or unannotated loci (Naqvi et al., 2012). The term miRtron refers to a group of introns encoding mature miRNAs. These introns are spliced out, forming hairpin structures and passing the dicing process (Ruby et al., 2007) (explained later). The term 'seed sequence' refers to the six nucleotides at positions 2-7 in a miRNA. It plays an important role in miRNA function, as miRNAs regulate their target mRNAs by recognizing and binding to them via complementarity of the seed sequence to mRNA (Lewis et al., 2005). The strand complementary to miRNA in the duplex (Figure 1), or the antisense miR strand**,** is often called miRNA* (i.e., star sequence). The miRNA and miRNA* strands are also called miRNA5p and miRNA3p, respectively, to indicate the 5' and 3' strands of the encoding DNA. The miRNA* strand was initially thought to be discarded or degraded when the miRNA-miRNA* duplexes were loaded into RISC complexes (Mourelatos et al., 2002) (described below). However, Czech et al. (2009) showed that it can be also incorporated with AGO2, and take active roles in gene silencing pathways.

Table 1. Conserved miRNA families in plants and the number of identified genes in sequenced genomes

miRNA family	Sequence (5'-3')	*Oryza*	*Hordeum*	*Triticum*	*Brachypodium*	*Arabidopsis*	*Populus*	Maize	Sorghum	Reference
miR156	UGACAGAAGAGAGUGAGCAC	12	2	4	10	12	11	3	3	(2,4,6)
miR159/319	UUUGGAUUGAAGGGAGCUCUG	8	2	11	12	6	15	3	3	(2,4,6)
miR160	UGCCUGGCUCCCUGUAUGCCA	6	2	2	5	3	8	1	1	(2,4,6)
miR162	UCGAUAAACCUCUGCAUCCAG	2	0	0	0	2	3	0	0	(2,6)
miR164	UGGAGAAGCAGGGCACGUGCA	6	3	3	3	3	6	1	1	(2,6)
miR165	UCGGACCAGGCUUCAUCCCCC	0	0	1	0	1	0	0	0	(6)
miR166	UCGGACCAGGCUUCAUUCCCC	12	4	2	8	9	17	2	2	(2,4,6)
miR167	UGAAGCUGCCAGCAUGAUCUA	10	4	5	6	4	8	3	3	(2,4,6)
miR168	UCGCUUGGUGCAGAUCGGGAC	2	6	3	3	2	2	2	2	(2,4,6)
miR169	CAGCCAAGGAUGACUUGCCGA	17	5	10	9	14	32	5	5	(2,4,6)
miR171	UGAUUGAGCCGUGCCAAUAUC	9	2	8	5	4	10	3	3	(2,4,6)
miR172	AGAAUCUUGAUGAUGCUGCAU	4	0	6	6	5	9	3	3	(2,4,6)
miR390	AAGCUCAGGAGGGAUAGCGCC	1	1	1	1	3	4	0	0	(2,4,6)
miR393	UCCAAAGGGAUCGCAUUGAUC	2	1	3	3	2	4	1	1	(2,4,6)
miR394	UUGGCAUUCUGUCCACCUCC	1	0	0	1	2	2	0	0	(1,2,4)
miR395	CUGAAGUGUUUGGGGGAACUC	23	1	2	3	6	10	0	0	(1,2,4)
miR396	UUCCACAGCUUUCUUGAACUG	5	3	6	6	2	7	1	1	(2,4,6)
miR397	UUGAGUGCAGCGUUGAUGAA	2	2	3	4	2	3	0	0	(2,4,6)
miR398	UGUGUUCUCAGGUCGCCCCUG	2	0	2	2	3	3	0	0	(1,2,4)
miR399	UGCCAAAGGAGAAUUGCCC	11	4	2	5	6	12	1	1	(2,4,6)
miR408	CUGCACUGCCUCUUCCCUGGC	1	0	1	2	1	1	1	0	(2,4,6)
miR437	AAAGUUAGAGAAGUUUGACUU	1	0	1	0	0	0	1	0	(5,6)
miR444	UUGCUGCCUCAAGCUUGCUGC	1	2	1	0	0	0	0	0	(2,4,6)
miR827	UUAGAUGACCAUCACGCAAACA	1	1	0	0	0	0	0	0	(3,4)

Underline indicates seed sequence. References: (1): Gao et al., 2012; (2): Jones-Rhoades et al., 2006; (3): Lacombe et al., 2008; (4): Schreiber et al., 2011; (5): Sunkar et al., 2005; (6): Yao et al., 2007.

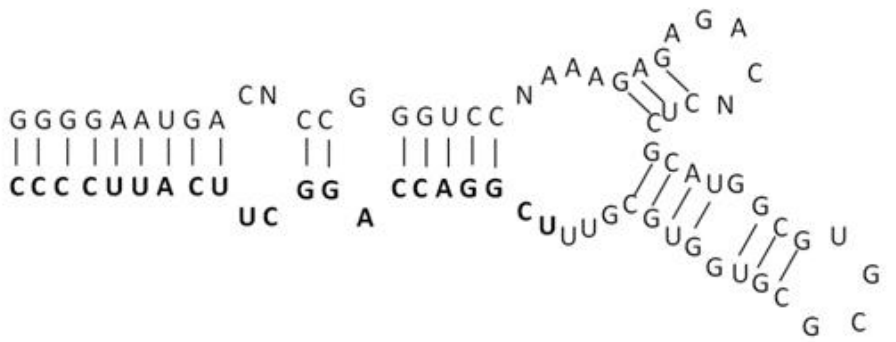

Figure 1. The stem-loop structure of barley pre-miR166m.The stem-loop structures are indicated. The miR166m sequence is shown in bold (figure based on Kantar et al., 2010).

3.2.2. miRNA Families

miRNAs are generally considered to belong to the same family when there is significant similarity in the sequences of the mature miRNAs, and often identity in their seed sequence. The names of miRNAs ending with suffixes a, b, etc. generally indicate they belong to the same family. However, in some cases they may not belong to a family, yet share sequences due to being transcribed by polycistronic genes or miRNA clusters, with different mature miRNAs being processed from one transcript. The suffixes may also indicate miRNAs expressed from opposite strands of pre-miRNA, or sense-antisense pair. These may thus have similarity in their respective 5' to 3' sequences; however, they cannot be processed from the same transcript and do not function together (Kruszka et al., 2013).

3.2.3. IsomiR

The term IsomiR refers to a group of miRNAs that vary at 5' and/or 3' ends. This concept came from Morin et al. (2008), who applied Illumina deep sequencing to identify miRNAs in human embryonic stem cells and found that several miRNAs exhibited abundant variants. IsomiRs are produced by imprecise excision of Drosha and Dicer within the pre-miRNA (Morin et al., 2008) (mechanism of miRNA biogenesis described later). Based on the variations, they are classified into 5' isomiRs, 3' isomiRs and polymorphic isomiRs. The 3' isomers are found commonly in animals and plants, while the 5' isomer and polymorphic classes are rarely observed but hold significant proportion in the miRNA populations that they are present in (Neilsen et al., 2012). The 5' and 3' isomer classes each appear to be generated by exoribonucleases (which shorten the miRNA sequences), and nucleotidyltransferases (which extend the miRNAs) (Martin and Keller, 2007). Polymorphic isomiRs are likely produced by RNA editing, which can occur either in pri-miRNAs or pre-miRNAs. The editing typically involves substitution of adenosine by inosine (A-I) in double-stranded RNAs by 'adenosine deaminase acting on RNA' (ADAR) enzymes (Nishikura, 2010). Further studies (Cloonan et al., 2011; Lee et al., 2010) suggest isomiRs can regulate mRNAs based on their successful association with both the RISC complex (see later) and polysomes in the translation process. However, the properties of isomiR such as stability, incorporation efficiency and target modulating capacity are unclear (Neilsen et al., 2012).

3.3. Biogenesis of miRNAs

3.3.1. Transcription of Precursor mRNA

In plants, mature miRNAs are encoded by their own genes. MiRNA genes are transcribed by RNA polymerase II into primary-miRNAs (pri-miRNA) (Figure 2), which then undergo 5' capping and 3' polyadenylation (Xie et al., 2005). Genes of some miRNAs overlap with protein-encoding genes, wherein pri-miRNAs usually reside in the introns. In this case, pri-miRNA is produced by splicing of the intron, followed by maturation of miRNA from the spliced-out intron (reviewed in Brown et al., 2008).

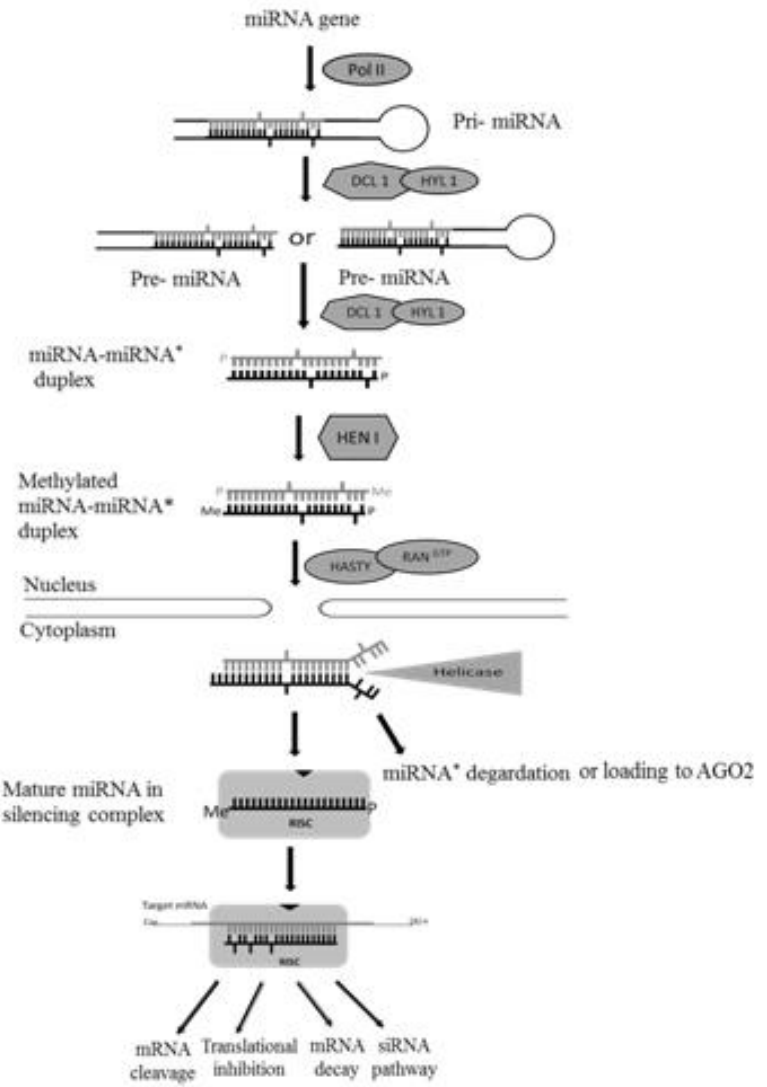

Figure 2. miRNA biogenesis and mechanisms of action.The binding of miRNA-RISC complex to target mRNA results in cleavage, translational prevention or degradation of mRNA (figure based on Bartel, 2004; Chen, 2009; Czech et al., 2009; Jones-Rhoades et al., 2006).

3.3.2. miRNA Processing and Export to Cytoplasm

A pri-miRNA may contain one or more miRNA sequences within pre-miRNA molecules (Figure 2). In Arabidopsis, the processing of pri-miRNA to pre-miRNA(s) occurs in the nucleus, with the help of a 'microprocessor', an enzyme complex that includes Dicer Like-1 (DCL1), an RNAse type III enzyme, the Hyponastic Leaves 1 (HYL1), a dsRNA-binding protein, and enzymes such as C2H2 zinc-finger protein SERRATE (SE) and nuclear CBC, assisting to make the activity of DCL1 more efficient (reviewed in Bologna et al., 2012) (Figure 2). DCL1 then makes another cut, now in pre-miRNA, to liberate the miRNA together with its reverse complement, forming the miRNA-miRNA* (or miRNA5p-miRNA3p) complex (Bologna et al., 2012).

The Arabidopsis genome contains four Dicer-like enzymes, of which only DCL1 is responsible for miRNA maturation while the others generate siRNAs (Unver et al., 2009). The miRNA-miRNA* duplex has 3' overhangs of two nucleotides on each strand. The plant miRNAs (but not animal miRNAs) then undergo methylation on 2'OH on each 3' terminal nucleotide by Hua Enhancer 1 (HEN1) (Li et al., 2005). The methylated duplex leaves the nucleus to enter cytoplasm with the assistance of the exportin family of transporters (Park et al., 2005) (Figure 2). The methylation is suggested to protect the miRNAs from degradation by 3' exonucleases, from the 3' uridylation activity (which adds poly-U on their 3' ends, which presumable increases the rates of 5' decay, e.g., that of mRNA degradation products; Shen and Goodman, 2004), and from the miRNA from being used as primer for RNA polymerase for transcription of other genes (reviewed in Guleria et al., 2011). Unlike uridylation, adenylation has been suggested recently to stabilize the duplex in both animal and plant miRNAs (reviewed in Bologna et al., 2012).

3.3.3. Loading of miRNA Into the Silencing Complex

After export to cytoplasm, the miRNA-miRNA* duplex is unwound by a helicase to release the mature miRNA (Figure 2), (Guleria et al., 2011). The miRNA strand is then incorporated into the RNA-induced silencing complex (RISC), which plays an important role in miRNA function. The RISC has a protein called Argonaute (AGO), which is a key component of the complex. The AGO proteins are highly conserved among eukaryotes and have three important domains, the PAZ, MID and PIWI, that are responsible for the function of target cleavage (Thieme et al., 2012). PAZ is an RNA-binding domain that binds to 3' end of miRNA, while MID binds to the 5' end of the target mRNA and PIWI functions as RNaseH enzyme, cleaving the target mRNA by exonuclease activity (Peters and Meister, 2007). The number of members in the AGO family differs among different species. Arabidopsis and rice have contain 10 and 18 AGOs, respectively (Yu and Wang, 2010). Interestingly, studies in Arabidopsis showed that AGO1 has a role in mRNA cleavage, AGO7 cleaves miR390 specifically, while AGO4 and AGO6 take part in siRNA-mediated regulation of DNA methylation associated with gene silencing (Yu and Wang, 2010). Deep sequencing also uncovered that the process of miRNA binding to RISC was based on its 5' nucleotide; AGO1 of Arabidopsis binds to miRNA with 5' uridine; AGO2, AGO4, AGO6 and AGO9 prefer 5' adenine, while AGO5 associates with 5'cytosine (Thieme et al., 2012). It also appeared initially that only the miRNA strand of the duplex is loaded into RISC, the miRNA* strand being degraded (Mourelatos et al., 2002). However, Czech et al. (2009) showed that miRNA* is not always degraded, and can be incorporated with AGO2 as siRNA in the silencing pathway. The siRNAs are outside the scope of this work; however, in summary,

siRNA-mediated DNA methylation in Arabidopsis results in gene repression and has significant roles in regulating plant development as well as response to biotic and abiotic stresses (Kanno and Habu, 2011).

3.3.4. Turn-Over of miRNA

The miRNAs were thought to be stable sequences. However, recent studies show mechanisms that accelerate the decay of miRNAs. In *Chlamydomonas reinhardtii*, MUT68 (a terminal nucleotidyltransferase) and RRP6 (ribosomal RNA processing protein 6 in 3'-5' exosome RNase complex) were found to take such a role, wherein MUT68 adds polyU to the 3' end of miRNAs, followed by cleavage by RRP6 (Ibrahim et al., 2010). In Arabidopsis, a family of small RNA degrading nucleases (SDNs), exoribonucleases that can specifically cleave single-stranded mature miRNAs was reported, with their activity affected but not prevented by the 2'O methylation (see above), and miRNA turn-over was shown to be important for regulation of developmental patterning (Ramachandran and Chen, 2008). The SDN gene family seems to be conserved in eukaryotes (Ramachandran and Chen, 2008). The researchers also later showed Arabidopsis to encode HEN1 SUPPRESSOR1 (HESO1), a terminal nucleotidyltransferase which promotes 3' uridylation of miRNAs (Zhao et al., 2012). Targets of miRNA are also proposed to induce miRNA degradation by SDNs (reviewed in Ruegger and GroBhans, 2012).

3.4. Mechanisms of Regulation of Gene Expression by miRNAs

The target mRNAs show almost perfect or imperfect complementarity with the miRNA. When the miRNA-loaded RISC binds to the target mRNA at the binding sites, AGO from the complex acts as an endonuclease on the mRNA by two main mechanisms: mRNA cleavage, or miRNA-direct translational inhibition (translational repression).

These mechanisms are well-reported in plants (Bartel, 2004; Rhoades et al., 2002), while a further mechanism, mRNA decay, has been reported in animal systems (reviewed in Guleria et al., 2011).

3.4.1. mRNA Cleavage

This process is suggested to occur when the miRNAs complement their target mRNAs almost perfectly (Rhoades et al., 2002).

The AGO of RISC cleaves the phosphodiester bonds in the mRNA, releasing its fragments. RISC is then liberated, to be incorporated into another miRNA and cleave further transcripts (Jones-Rhoades et al., 2006). For example, miR398 in Arabidopsis directly cleaves the mRNAs of CSD1 and CSD2 which encode Cu/Zn superoxide dismutases, enzymes in the redox regulation pathways. Interestingly, the miR398 expression is decreased under oxidative stress, which allows CSD1 and CSD2 accumulation, resulting in stress tolerance (Sunkar et al., 2006).

Table 2. Barley miRNAs from mirBase*

miRNA	miRNA family	Sequence	Length	Putative target(s) and reference(s)
hvu-MIR156	MIR156	UGACAGAAGAGAGUGAGCACA	21	Squamosa promoter-binding-like protein (1)
				Teosinte glume architecture 1 (2)
				Promoter binding protein (2)
				SBP transcription factor (1)
				F-box domain containing protein (2)
				Protein kinase (2)
				Resistance protein (2)
hvu-MIR159a	MIR159	UUUGGAUUGAAGGGAGCUCUG	21	Zinc finger AN1 and C2H2 domain-containing stress-associated protein (3)
hvu-MIR159b		UUUGGAUUGAAGGGAGCUCUG	21	MYB transcription factor (1)
				Triosephosphate isomerase 2)
				60S ribosomal protein L30 (2)
hvu-MIR166a	MIR166	UCGGACCAGGCUUCAUUCCCC	21	HD-ZIPIII transcription factor (2)
hvu-MIR166b		UCGGACCAGGCUUCAUUCCCC	21	
hvu-MIR166c		UCGGACCAGGCUUCAUUCCCC	21	
hvu-MIR168-5p	MIR168	UCGCUUGGUGCAGAUCGGGAC	21	Argonaute 1(1)
				Flame chlorosis virus-like agent (2)
				Fowl adenovirus D (2)
hvu-MIR168-3p		GAUCCCGCCUUGCACCAAGUGAAU	24	G-box-binding factor (3)
hvu-MIR169	MIR169	AAGCCAAGGAUGAGUUGCCUG	21	CCAAT-binding transcription factor (1)
				SRPK4 (2)
hvu-MIR171-5p	MIR171	UGUUGGCUCGACUCACUCAGA	21	GRAS family transcription factor (1)
				Scl1 protein (3)
hvu-MIR171-3p		UGAUUGAGCCGUGCCAAUAUC	21	6,7-dimethyl-8-ribityllumazine synthase (3)
hvu-MIR397	MIR397	CCGUUGAGUGCAGCGUUGAUG	21	Laccase-precursor (3)
hvu-MIR399	MIR399	UGCCAAAGGAGAUUUGCCCCG	21	ATUBC24/PHO2/UBC24 (PHOSPHATE 2), ubiquitin-protein ligase (1)
hvu-MIR444a	MIR444	UUGCUGCCUCAAGCUUGCUGC	21	MIKC-type MADS-box transcription factor WM32A (3)

miRNA	miRNA family	Sequence	Length	Putative target(s) and reference(s)
hvu-MIR444b		UGCAGUUGCUGUCUCAAGCUU	21	
hvu-MIR1120	MIR1120	ACAUUCUUAUAUUAUGGGACGGAG	24	TAK14 (2)
hvu-MIR1130		UCUGUAACUUAAUAUAAGACG	21	unknown
hvu-MIR1436		ACAUUAUGGGACGGAGGGAGU	21	unknown
hvu-MIR5048a	MIR5048	UAUUUGCAGGUUUUAGGUCUAA	22	Resistance gene? Serine/threonine kinase? (4)
hvu-MIR5048b		UAUUUGCAGGUUUUAGGUCUAA	22	
hvu-MIR5049a	MIR5067	UCCUAAAUACUUGUUGUUGGG	21	Tubby protein-like (2)
hvu-MIR5049b		AGUAUUUAGGUACAGAGGGAG	21	non-specific liquid-transfer protein (2)
hvu-MIR5049c		AGACAAUUAUUUUGGGACGGAGG	23	resistance-related receptor-like kinase (4)
hvu-MIR5049d		UACAAUUAUUUAGGAACGGAG	21	
hvu-MIR5049e		AAUUAUUUAGGUACAGAGGGA	21	lipase class 3-like (4)
hvu-MIR5049f		AAUUAAUAUGGAUCGGAGGGA	21	
hvu-MIR5050		UUGAGGUCGUUCAACCAGCAA	21	ETS-like protein? (4)
hvu-MIR5051		UUUGGCACCUUGAAACUGGGA	21	unknown
hvu-MIR5052		ACCGGCUGGACGGUAGGCAUA	21	unknown
hvu-MIR5053		CGCAGCUGUAGUCGCCGGCGU	21	Myb-related protein (4)
				Chlorophyll a/b binding protein CP29 precursor (2)
hvu-MIR6176		GAAGCUGUAGUGCAGCCGGCGUU	23	unknown
hvu-MIR6177		UACCAUGGACAGAAGGCACUUA	22	unknown
hvu-MIR6178		CACGGAACUGGGCGGUUGCACU	22	unknown
hvu-MIR6179		AACCAGUCGAGGCCAGGGGGUU	22	unknown
hvu-MIR6180		AGGGUGGAAGAAAGAGGGCG	20	unknown
hvu-MIR6181		UGCUCUUCAUGGACUGCGGCGCC	23	unknown
hvu-MIR6182		UGAGUGUGUGAUGGAUGGCUUU	22	unknown
hvu-MIR6183		UGAGCGAGUUGGCUGCAAGUUC	22	unknown
hvu-MIR6184		CGGCGUCGGAUCUGGCCGGCCU	22	unknown
hvu-MIR6185		UCUGGCAGCGACGGGAACAUA	21	unknown
hvu-MIR6186		CGAGGAAGGCGCUGAGAGAGA	21	unknown
hvu-MIR6187		UGAACAGGUUCGGCGACCUCA	21	unknown

Table 2. (Continued)

miRNA	miRNA family	Sequence	Length	Putative target(s) and reference(s)
hvu-MIR6188		GGUGGAUCGAUGAACCCGGCGA	22	unknown
hvu-MIR6189		AGGUGAUGCUGUGGUGAUCU	20	unknown
hvu-MIR6190		CGAGGAAAGGAAGAAGCCAUG	21	unknown
hvu-MIR6191		UAGAUUUGUCUAGAUAUGAA	20	unknown
hvu-MIR6192		UAGGAGAGGGGGGAAGGGAUCU	22	unknown
hvu-MIR6193		CUCUGCCACCGGUCCAUGACGAC	23	unknown
hvu-MIR6194		UAUGGGGAUCUGACAGACGAG	21	unknown
hvu-MIR6195		UGAGUACGUAGUAGGGAUGAG	21	unknown
hvu-MIR6196		AGGACGAGGAGAUGGAGAGGA	21	unknown
hvu-MIR6197	MIR1120	UCUGUUCCUAAAUGUAAGACG	21	unknown
hvu-MIR6198		GCUCUGUCUUGGAUGGUCAUUC	22	unknown
hvu-MIR6199		CCACAGAAUUCUCACAGUGAUGG	23	unknown
hvu-MIR6200		UUUGGCCAACUAGAUCUAUGA	21	unknown
hvu-MIR6201		UGACCCUGAGGCACUCAUACCG	22	unknown
hvu-MIR6202		UGAAGAUUUUAAGCAUUGAA	20	unknown
hvu-MIR6203		AGACGAUUAAGAAGACCUGCAA	22	unknown
hvu-MIR6204		AGGAGAAUAAUUAGAGCUGUGA	22	unknown
hvu-MIR6205		AGGAUGUUUGGAUACGUUUUAGU	23	unknown
hvu-MIR6206		GGCACACGGGCUGCAGGCAUAG	22	unknown
hvu-MIR6207		UGGACGACCUGGGCGCCGACG	21	unknown
hvu-MIR6208		GUGCAUCAAGAUCGGCUCAUCU	22	unknown
hvu-MIR6209		UGAGAUCAGAAAGAUGUGCG	20	unknown
hvu-MIR6210		ACUCCUUGGUUAUCAACUUCGA	22	unknown
hvu-MIR6211		CAGAUCAAGACGCUCCGGCA	20	unknown
hvu-MIR6212		AUACAGUUUACAAUGCACGAU	21	unknown
hvu-MIR6213		ACAGAUUGCUACAGACUGGUC	21	unknown
hvu-MIR6214		CGACGACGACGAGCACGACA	20	unknown

*http://www.mirbase.org. Question mark (?) indicates candidate targets for novel barley miRNAs. References: (1): Dryanova et al., 2008; (2): Hackenberg et al., 2012; (3): Lv et al., 2012; (4): Schreiber et al., 2011.

3.4.2. Translational Inhibition (Translational Repression)

This mechanism likely occurs in cases of imperfect base pairing between miRNAs and target mRNAs; however, there are differences in the activity of miRNAs, and the rate of inhibition appears to be related to the number binding sites of miRNA on mRNA. Several studies (Liu et al., 2005; Sen and Blau, 2005; Huang et al., 2011) noted that miRNA-RISC is located at the cytoplasmic foci (P bodies), where mRNA can be stored or degraded. Binding of the target mRNA to miRNA-RISC at P body sites suggested that mRNA can be moved from translation to degradation. A different explanation was reported later (Guleria et al., 2011), that the miRNAs bind at the 5' UTR, ORF, or 3' UTR of the target mRNA and prevent the movement of ribosomes, leading to translational suppression. An example of this mechanism is found in Arabidopsis, wherein expression of miR399 was up-regulated under low phosphate. Its induction decreased the expression of its target, the UBC24 mRNA (which has a role in protein degradation) directly by binding to its 5'UTR and causing translational repression. Thus the level of UBC24 expression regulated Pi uptake in response to phosphate stress (Chiou et al., 2006).

3.4.3. mRNA Decay

This process may occur when miRNAs include a small sequence of defined length, similar to mRNAs.

The miRNAs speed up the process of polyA tail removal from mRNAs, resulting in instability and mRNA decay. However, mRNA decay has been confirmed only in animals (Guleria et al, 2011).

4. ROLES OF MIRNAS IN ABIOTIC STRESS RESPONSES OF PLANTS

miRNAs have been shown to be involved in the regulation of many plant processes such as development (Reinhart et al., 2002), flowering (Aukerman and Sakai, 2003), auxin signalling (Malloy et al., 2005), responses to abiotic (Sunkar and Zhu, 2004) and biotic stresses (Zhang et al., 2006), or regulation of their own biogenesis (Dugas and Bartel, 2004). The ability of plants to respond *in situ* to abiotic stresses such as salinity, drought, heavy metals, temperature extremes or nutrient deprivation is critical for their survival, as unlike animals, they are immobile. A number of miRNAs have been shown in the last few years to have key roles in regulation of plant responses to environmental factors; these are summarised below. Please refer to Table 1 for miRNAs shared between different species and Table 2 for barley miRNAs.

4.1. Dehydration Stress

Adequate water supply is needed for proper plant growth and development, and over- or under-supply can cause significant damage, as discussed earlier. Many researches have demonstrated crucial roles of miRNAs in responding to water stress. In Arabidopsis, microarray analysis showed that some miRNAs (miR157, miR167, miR168, miR171, miR408, miR393, miR396) had increase in expression levels during drought (Liu et al.,

2008), while only miR169 was down-regulated (Li et al., 2008). The target of miR169 was found to be NFYA5 (a member of NFYA family), a transcription factor with a role in stress response, a decrease in miR169 expression leading to increase in NFYA5 transcript levels (Li et al., 2008). In rice, eight families (miR395, miR474, miR845, miR851, miR854, miR901, miR903, miR1125) showed significant up-regulation and 11 families (miR170, miR172, miR397, miR408, miR529, miR896, miR1030, miR1035, miR1050, miR1088, miR1126) showed down-regulation using microarray analysis, confirmed by quantitative PCR (qPCR) (Zhou et al., 2010b). In *Populous trichocarpa*, all ten miRNA families detected via microarray to respond to drought stress (miR1446, miR1444, miR1447, miR1450, miR1711, miR482, miR530, miR827, miR1445, miR1448) showed a decrease in expression (Lu et al., 2008), while in *Medicago truncatula*, two families (miR398, miR408) were found by qPCR assay to be up-regulated under water stress (Trindale et al., 2009). A recent study of Kantar et al. (2011) using qPCR analysis in wheat under drought treatment found 13 miRNA families responded to drought stress. Of these, eight families (miR1867, miR398, miR474, miR156, miR894, miR1432, miR1881, miR1450) showed an increase, four families (miR528, miR166, miR171, miR396) decreased, and miR896 fluctuated. The targets of miR398, miR474, miR166, miR156, miR171 and miR396 were found to be genes involved in plant development and/or abiotic stress responses, i.e., copper superoxide dismutases, kinesin, HD-ZipIII, Squamosa-promoter binding protein (SBP), GRAS domain and growth regulating factor-like (GRL) transcription factor. Study of barley under drought treatment by qPCR also showed that miR156, miR166, miR171 and miR408 were induced (Kantar et al., 2010), while miR164, miR166, miR167, miR168, miR169, miR172, miR319, miR396 and miR827 were down-regulated (Hackenberg et al., 2012). The contradictory data for miR166 in these studies need further investigation. In maize, microarray data showed 39 miRNAs responded to submergence, and the bioinformatically predicted targets of 38 of these appeared to be involved in signal transduction, cell defence, differentiation and carbohydrate metabolism (Zhang et al., 2008).

4.2. Salt Stress

Soil salinity is one of the most common stress factors worldwide and severely affects plant growth and crop yield, as discussed earlier. In Arabidopsis, expression of a number of miRNAs (miR396, miR397, miR168, miR167, miR165, miR319, miR159, miR394, miR156, miR393, miR171, miR158, miR169) was found up-regulated during salt stress by microarray analysis (Liu et al., 2008), while only miR398 was down-regulated under qPCR assay (Jagadeeswaran et al., 2009). Induction of miR397 was found to down-regulate its targets, laccase-like protein (LAC) and a regulatory subunit of casein kinase (CKB3), by direct cleavage of their mRNA (Zhu et al., 2007). The targets of miR398 are Cu/Zn superoxide dismutases CSD1 and CSD2 (Sunkar et al., 2006), increases in their expression levels being related to down-regulation of miR398. In rice, miR169g and miR169n (from the miR169 family), as detected by qPCR, increased dramatically under salt (Zhao et al., 2009) and were found to regulate NF-YA5 (a CCAAT-box binding transcription factor) by direct cleavage of its mRNA (Li et al., 2008). MiR169 was also induced upon salt treatment in Arabidopsis (Zhao et al., 2009). Another study using qPCR found 98 miRNAs from 27 families in maize root responded to salt stress (Ding et al., 2009). The targets of miR159a/b, miR164a/b/c/d and

miR1661m were identified as transcription factors Myb and NAC and a homeodomain-leucine zipper protein (HD-ZIP) (Ding et al., 2009). Recent study using qPCR also found 8 families (miR159, miR160, miR167, miR174, miR399, miR408, miR1124, miR1133) to be up-regulated during salt stress in wheat (Lu et al., 2011).

4.3. Cold Stress

Low temperature extremes such as chilling and freezing cause severe stress in unadapted plants, as discussed earlier. In Arabidopsis, microarray data showed several cold-responsive miRNAs to be up-regulated (miR165/166, miR169, miR172, miR393, miR396, miR397, miR402, miR408) (Zhou et al., 2008) while miR398a levels decreased (Sunkar and Zhu, 2004). MiR172 was found to regulate the transcription factor AP2 (APETALA) by translational attenuation (Zhou et al., 2008). In *P. trichocarpa*, expression of 15 miRNAs assessed through microarray was found to be up-regulated during cold stress, while miR156g-j, miR475a, b and miR476a were down-regulated (Lu et al., 2008). In rice, also using microarray analysis, 18 miRNA families responded to cold, most of them decreasing in expression, except miRNA171 members which showed varying expression (Lv et al., 2010). Study of Thebaut et al. (2012) on sugarcane by qPCR noted that miR319 was induced but decreased after 48h of cold treatment, and that miR319 regulated its targets, the Myb and TCP transcription factors, by direct cleavage.

4.4. Hypoxia

Hypoxia can affect mitochondrial respiration and change the respiratory mechanism in plants from aerobic to anaerobic. Forty-six miRNAs from 19 families in Arabidopsis showed altered expression during hypoxia (Moldovan et al., 2009). In maize, the microarray showed varied expression patterns; while miR167, miR166, miR171 and miR396 were induced at early stage, miR159, miR395, miR474 and miR528 were down-regulated during early stage but increased after long term exposure (Zhang et al., 2008). The findings need further studies for the understanding of the mechanism of response.

4.5. Oxidative Stress

Excessive accumulation of ROS can occur under diverse stress conditions and can damage cells, as discussed earlier. Oxidative stress can be reduced by superoxide dismutases (SOD), a group of peroxidase and catalase enzymes which can detoxify superoxide radicals into H_2O_2 (Sunkar, 2010). In Arabidopsis, miR398 was found to be oxidative stress responsive and could cleave CSD1 and CSD2 transcripts, resulting in oxidative tolerance (Sunkar et al., 2006).

4.6. Abscisic Acid (ABA) Stimulation

The plant stress hormone ABA plays a key role in certain abiotic response signalling networks. However, ABA-responsive miRNAs have not been studied in detail yet. In Arabidopsis, Sunkar and Zhu (2004) found miR393 expression to be up-regulated during ABA stress, and this miRNA targeted mRNA that encoded the transport inhibitor response 1 (TIR1). TIR1 is involved in proteolysis of Auxin/Indole 3-Acetic Acid (AUX/IAA), which has a role in plant growth and development (Dharmasiri and Estelle, 2002). Accumulation of miR393 cleaves TIR1 mRNA or prevents its translation, resulting in negative effects on auxin signalling and plant development. MiR417 was found by qPCR to be up-regulated at early stage upon ABA treatment but down-regulated later, and led to reduce seed germination and seedling survival (Jung and Kang, 2007). MiR159 was also induced by ABA stress, and its accumulation increased the degradation of MYB101 and MYB33 transcripts that encode ABA-responsive positive regulators, thus diminishing ABA signalling (Reyes and Chua, 2007). However, further understanding of these miRNAs and their targets is essential, as ABA is central to abiotic stress response pathways in plants.

4.7. Nutrient Stress

4.7.1. Sulphate (Over) Stimulation/Exposure

Plants absorb inorganic sulphate and transport it into root and leaf xylem cells where it is assimilated into cysteine, which then takes part in synthesis of proteins and other molecules (Rausch and Wachter, 2005). In Arabidopsis, miR395 was the first sulphate-responsive miRNA identified, and its targets found to be ATP sulphurylases (APS) (involved in sulphate assimilation) (Jones-Rhoades and Bartel, 2004) and AST68 (a transporter that assists with sulphate translocation) (Allen et al., 2004). Over-expression of miR395 reduced APS expression and led to decrease in sulphate metabolism during sulphate deficiency (Jones-Rhoades and Bartel, 2004).

4.7.2. Phosphate Starvation

Inorganic phosphate (Pi) is one of the most important components in the structures of nucleic acids and cell membranes as well as in other biological functions. The targets of Arabidopsis miR399 were identified to be transcripts of the phosphate transporter (PHO2) (Jones-Rhoades and Bartel, 2004) and ubiquitin conjugating enzyme (UBC24; with roles in phosphate homeostasis) (Sunkar and Zhu, 2004). MiR399 was up-regulated upon Pi starvation, its accumulation decreasing the ubiquitin/proteasome pathway that represses the transporter expression and increases Pi loading (Chiou et al., 2006). The transcription factors MYB and phosphate starvation response 1 (PHR1) were involved in miR399 response to Pi deficiency; they share a MYB domain that binds to the GNATATNC cis-element of miR399 and up-regulates its expression (Chiou et al., 2006; Pant et al., 2008).

4.7.3. Copper Deficiency

Copper, a trace element, is essential for key processes including photosynthesis and respiratory electron transport. In Arabidopsis, miR398 showed increased expression under

copper starvation (Yamasaki et al., 2007), leading to negative regulation of CSD1 and CSD2 mRNAs. Burkhead et al. (2009) found that miR397, miR408 and miR857 were also up-regulated during copper starvation and decreased the expression of their targets, the mRNAs of plantacyanin and laccases, making copper available to other vital proteins.

The above-summarised roles of miRNAs in plant development and responses to diverse environmental factors make a strong case for the need to study miRNA-regulated gene expression events in important crop species.

5. MICRORNAS IN BARLEY: AN EMERGENT FIELD

Since the identification of miRNAs in Arabidopsis (Reinhart et al., 2002), a number of plant miRNAs have been identified and deposited in miRBase v19.0 (http://www.mirbase.org), which now contains more than 5,000 entries, belonging mainly to Arabidopsis (291), rice (581), maize (172), sorghum (171) and Brachypodium (142) (Lv et al., 2012).

The study of barley (*Hordeum vulgare*) miRNAs is in early stages compared to other species. The first large scale work on barley miRNAs was by Schreiber et al. (2011), who discovered up to 100 miRNAs through deep sequencing. However, this work only investigated the leaf tissue. Lv et al. (2012) addressed this limitation by analysing miRNAs of barley from different tissues, i.e., roots, stems, leaves and spikes at different stages of development, leading to 126 conserved and 133 novel miRNAs. However, our searches of this database found only 47 of these to be registered (http://www.mirbase.org; last accessed December 2012).

Currently, there are 99 mature miRNAs that match barley miRNA sequence query on miRBase. Of these, 67 are classified as Hvu-miRNA (Table 2), while the other 32 are predicted homologues of species such as rice, wheat or Brachypodium. However, it should be noted that the barley genome sequence has only become available in November 2012 (The International Barley Genome Sequencing Consortium, 2012), and needs to be searched for miRNA-encoding genes. Twenty three of the 67 barley miRNAs belong to 12 families. All are 20-24 nucleotides in length, released from stem-loop structures of about 70-300 nucleotides. Especially in miR168 and miR171 families, each stem-loop appears to have two mature miRNAs, located at the 5’ and 3’ of it, that complement to each other. Based on current literature (Gao et al., 2012; Jones-Rhoades et al., 2006; Lacombe et al., 2008; Schreiber et al., 2011, Sunkar et al., 2005; Yao et al., 2007), barley appears to have 17 conserved miRNA families shared with other species such as rice, wheat, Brachypodium, Arabidopsis, populus, maize and Sorghum (Table 1, Table 2). However, our analysis found only nine of these in miRBase, the other eight being unregistered. The conserved miRNAs are well-supported to have important roles in response to biotic and abiotic stress (Kruszka et al., 2013). Schreiber et al. (2011) also described 44 other putative miRNAs in barley, not known to be expressed in other species and also lacking prior bioinformatic predictions. The candidate targets of these novel miRNAs are transcription factors or vital enzymes that suggest these miRNAs may also have roles in regulation of plant development.

The role of miRNA in regulating responses of barley to the challenging and changing environments is the field of current interests. MiRNA capability of regulating response to drought stress in barley is supported by two studies. Kantar et al. (2010) noted that miR156,

miR159, miR166, miR171 and miR408 were induced under drought stress and inversely correlated to their targets, SQUA promoter binding protein, MYB33, ARF, SCL6 and blue copper protein, respectively, that play role in plant development and architecture. Hackenberg et al. (2012) analysed the expression of miRNAs under the impact of TaDREB3 (a transcription factor) in transgenic barley. Over-expression of TaDREB3 resulted in survival of barley under drought or cold stress. Expression of a number of miRNA in both transgenic and non-transgenic plants was analysed, and miR156 had greatest induction in both plants, indicating it to be a strong candidate for drought tolerance. In addition, a recent study showed that over-expression of miR171 decreased its target, SCL (scarecrow-like) transcription factor, affecting shoot development and flowering timing, and intriguingly, it could activate miR156 regulation (Curaba et al., 2013). The studies supported previous results (Curaba et al., 2012) that barley miRNAs may regulate early development of seed by targeting mRNAs involved in cell differentiation, energy mobilization, signalling pathways and defence responses to biotic and abiotic stresses. Beside barley, the role of miRNA in regulating vernalisation has been also studied in Arabidopsis (Oh et al., 2007) and peach (*Prunus persica*) (Barakat et al., 2012). Together with the strong evidence of roles of siRNA in flowering regulation (Groszmann et al., 2011), these small RNAs seem to have significant roles in hastening vernalisation in plants. The recent work of Kruszka et al. (2013) is the first major report investigating barley miRNA biogenesis as well as regulation of intron-derived miRNAs. Study of pri-miRNA of eight mature miRNAs (miR156g, miR159b, miR166n, miR168a, miR171e, miR397b, miR1120, miR1126) of barley showed that miR397b was generated from an intronless gene while miR156g and miR1126 were produced from introns of non-encoding MIR156g and MIR1126 genes. MiR168a had two different sequences in the same precursor, miR168a-5p and miR168a-3p, that were complementary to each other and also expressed as mature miR168a. Interestingly, the target of miR168 was AGO1 (described by Vaucheret et al., 2006) (see above and Figure 2), showing the role of miR168 in negative regulation of miRNA biogenesis. The miR156g and miR1126 precursors were also present in introns. The MIR156g gene has six exons and five introns, with the first intron containing miR156g-miR156g* duplex, while the MIR1126 gene contains seven exons and six introns, the miR1126-miR1126* duplex being located in the third intron.

The above reports thus contribute highly significant information on miRNA-mediated regulation of barley growth and development.

6. Some Common Putative Targets of Barley MiRNAs Involved in Response to Abiotic Stresses

6.1. Squamosa (SQUA) Promoter-Binding Protein

This transcription factor family facilitates flowering in plants (Xie et al, 2006). The proteins contain a highly conserved DNA-binding domain, a zinc finger motif with two binding sites (Cys-Cys-His-Cys and Cys-Cys-Cys-His). The transcript of this protein is the target of hvu-miR156 (Dryanova et al., 2008). Interestingly, Squamosa promoter binding protein like 7 (SPL7) was detected as a regulator under copper deficiency in Arabidopsis. Unlike other Squamosa members, the DNA-binding domain of SPL7 has a C4 zinc finger

motif that binds to the GTAC motif in the promoter region of miR398 gene and activates its transcription (Yamasaki et al, 2009).

6.2. MYB Transcription Factors

MYB proteins contain a highly conserved DNA-binding 'MYB domain' (Rubio et al., 2001) and have diverse functions in plants including developmental and metabolic processes, cell fate as well as biotic and abiotic stress regulation (Dubos et al., 2010). The transcript encoding a MYB33 transcription factor was identified as the target of hvu-miR159 (Dryanova et al., 2008). Study of 60 MYB genes from wheat (Zhang et al., 2012) showed that they responded to different stresses, 20 of these responded to multiple stresses, and 15 of these were regulated by ABA. Thus investigation of gene regulation by miRNAs under stresses is an important direction for wheat production.

6.3. GRAS Family Transcription Factors

These factors regulate gene transcriptions and signal transductions during plant development (Bolle, 2004). The GRAS genes are reported from many species including Arabidopsis, rice, *Medicago truncatula* and *Lotus japonicas.* The GRAS proteins contain 400-700 amino acids forming motifs in the C-terminal such as the leucine heptad repeat I (LHR I) and repeat II (LHR II), VHIID and SAW, and are classified into eight groups (Hirsch and Oldroyd, 2009). The transcripts encoding GRAS family are targets of hvu-miR171 (Dryanova et al., 2008).

6.4. Homeodomain Leucine Zipper (HD-Zip) Proteins

These proteins have a homeo-domain (HD) and a leucine zipper motif (Zip) (Lee and Chun, 1998), and are divided into four subclasses according to the structures, unique domains and functions. HD-Zip I responds to abiotic stresses such as drought and light, HD-Zip II has roles in phototropism and auxin response, HD-Zip III in morphogenesis, and HD-Zip IV in processes such as epidermal fate, trichome formation and anthocyanin assimilation (Elhiti and Stasolla, 2009). The transcripts encoding this family are targets of hvu-miR166 (Hackenberg et al., 2012); it cleaves its targets directly, resulting in organ formation and abiotic stress response regulation.

6.5. CCAAT-Binding Transcription Factor (NF-Y, CBF or HAP)

This factor is also known as nuclear factor Y (NF-Y), or CBF or HAP. The NF-Y family has roles in abiotic stress responses. NF-YB was reported to have a role in drought tolerance (Nelson et al, 2007), while NFYA5 controls stomatal aperture, its expression being induced by drought and ABA treatment (Li et al., 2008). NF-YA was also found to respond to salt

stress (Zhao et al., 2009). However, the roles of many members of the family remain unclear. An NF-Y protein is made of three subunits (NF-YA, NF-YB and NF-YC), each of which is encoded by around 10 genes (Nelson et al, 2007). An mRNA encoding NF-YA is the target of hvu-miR169 (Dryanova et al., 2008). Induction of miR169 results in cleavage of NF-YA, thus regulating the transcription of a number of downstream genes (Zhao et al., 2009).

6.6. Ubiquitination

The process of ubiquitination of proteins has important roles in response to abiotic stresses such as phosphate starvation (Sunkar and Zhu, 2004) and salt and drought (Zhou et al., 2010a). Ubiquitination systems contain three enzymes, the ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin-protein ligase (E3), and the mRNAs encoding these are targets of hvu-miR399 (Dryanova et al., 2008).

6.7. Laccases

These copper-containing enzymes participate in lignin synthesis, with lignin having functions in water transport restriction. Laccases have diverse functions in plant development (Cai et al., 2006) and responses to biotic stresses such as pathogens and abiotic stresses such as salinity and copper homeostasis (Sunkar, 2010). The transcripts encoding laccase 2, 4 and 17 are targets of hvu-miR397 (Sunkar and Zhu, 2004), with the miRNA directly cleaving its targets, leading to tolerance to salt stress (Zhu et al., 2007).

PERSPECTIVE

Barley is a highly nutrient cereal food for both humans and animals, and recent investigations also show it is a functional food. However, the crop is under threat in many parts of the world due to challenges such as salinity, drought or soil nutrient limitations, with additional demands on yield due to the ever-increasing world population. Therefore, selection and/or improvement of barley lines are necessary to keep it abundant and affordable. Along with selections for, or modifications of, stress-responsive genes, miRNAs with roles in stress responses are ideal candidates for such considerations. Recent studies have contributed significant information, as summarised above, thus firmly establishing miRNAs as excellent candidates for improvement of barley. It is expected that several hundred further miRNAs are yet to be discovered in barley, based on miRNA families in other plant species. Further investigations could focus on analysing the miRNA transcriptomes and degradomes of different tissues and lines, and under different stress conditions, identifications of novel miRNAs, analyses of the target genes and mechanisms of action of select miRNAs, as well as finding any genetic variants in select miRNAs and their target genes and assessing their relationships to stress tolerance.

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Chapter 9

DYNAMICS OF OXIDATIVE PROCESSES AND ACTIVATION OF ANTIOXIDANT SYSTEMS IN BARLEY LEAVES UNDER DROUGHT CONDITIONS OF SOIL

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ABSTRACT

The aim of the research is to study the systematic action of antioxidants in barley under drought conditions of soil, giving special emphasis on the expression of genes encoding antioxidant enzymes and defense related proteins. In green barley leaves cultivated under drought conditions of soil during germination processes the overproduction of reactive oxygen species (ROS) was recorded as cellular damages, which paralleled to the increase in malonedialdehyde (MDA) contents in cellular membrane systems in leaves. In leaves ROS accumulation in cytosol and lipid peroxidation in membrane systems were accompanied by growth inhibition and suppression of rate of photosynthesis. Activation of barley defense systems under severe drought conditions of soil was also shown. This severe condition in barley was evident from the increases in the concentrations of α- and γ-tocopherol and total glutathione, and from the reduction of ascorbic acid and in the activation of main antioxidant enzymes such as superoxide dismutase, ascorbate peroxidase and catalase. Gene expression analysis allowed us to reveal a set of genes encoding defense proteins, antioxidant and subsidiary enzymes overexpressing under water-limiting conditions in soil. The correlation between intensity of drought and osmolytes supply was demonstrated.

Keywords: Barley, reactive oxygen species, low-molecular weight antioxidants, antioxidant enzymes, dehydrins, gene expression, drought conditions of soil

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ABBREVIATIONS

APX	ascorbate peroxidase
AsA	ascorbate
CAT	catalase
DHAR	dehydroascorbate reductase
Dhn	dehydrine
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
MDA	malonedialdehyde
POX	non-specific phenolic peroxidase
ROS	reactive oxygen species
SOD	superoxide dismutase
γ-TMT	γ-tocopherol methyltransferase

INTRODUCTION

Drought is one of the most significant factors limiting crop growth and productivity worldwide. It has been estimated that up to 45% of the world agricultural areas are subjected to drought [1]. It is well known that photosynthesis and cell growth are among the primary processes to be affected by drought [2-4]. However, extensive study on oxidative stress has demonstrated that exposure of plants to adverse environmental conditions, namely to drought, induces the overproduction of ROS, such as superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$) in plant cells [5-7]. ROS are highly reactive to membrane lipids, protein, and DNA [8, 9], so they are believed to be the major factors contributing to stress injuries and causing rapid cellular damage.

Plants have evolved both enzymatic and non-enzymatic mechanisms to scavenge ROS rapidly evolved under drought stress. Enzymes including superoxide dismutase, catalase, peroxidases (ascorbate peroxidase and non-specific phenolic peroxodase) and glutathione reductase, and non-enzymatic antioxidants such as tocopherols, ascorbic acid, glutathione, carotenoids and phenolics [10-15] work in concert to detoxify these ROS.

The relation between drought stress and activation of antioxidant system has been extensively studied in different plant species for past few decades since modulation of activity of antioxidant system in agricultural plants could provide enhanced tolerance to drought [16]. Nevertheless, a general pattern of activation of antioxidant system under drought conditions has not been yet revealed, because plant responses to water deprivation (WD) are dependent upon stress duration and intensity, plant susceptibility and method of drought simulation.

In current study we have tried to reveal a general pattern of activation of antioxidant system in barley plants under drought conditions of soil with different intensity.

I. PLANT TREATMENTS

In present study green barley seedlings (*Hordeum vulgare* L.) were used. Barley seeds were first germinated on wet filter paper for 24 h and then planted in plastic pots filled with humid soil, and soil moisture content at the beginning of experiment was about 53.7 ± 0.8%. Barley was grown under temperature at 25±1°C and 14 : 10 light : dark regime (light intensity 175 μmol quanta·m^{-2}·s^{-1}). Control plants were grown under constant irrigation. Moisture content in pots with control plants was maintained at 51.9 ± 0.3%. For drought simulation experimental plants were not watered after planting so that within 19 days soil moisture in pots with drought treated plants gradually decreased to 22.0 ± 1.7% (Table 1). Leaf samples were collected after 7, 10, 13, 16 and 19 days.

Samples were taken to determine relative water content and water deficit. It was shown that barley seedlings grown under constant irrigation maintained their relative water content (RWC) on the level of 92 − 93% over the course of experiments.

The water deficit (WD) in control plants was 5.2 ± 0.4% on average, which corresponds to the optimal water content of plant tissue [17]. In leaves of 7 − 10-day-old seedlings grown under drought conditions of soil the RWC did not differ from that of control plants. First reduction of the RWC was recorded in 13-day-old drought treated seedlings (by 2%), whereas leaf WD therein was 19.4 ± 0.4% (Figure 1). This process was manifested in the partial wilting of seedling leaves (Figure 2, A).

Leaf RWC decreased depending on drought intensity. In 19-day-old drought treated seedlings the RWC was 20% lower than in control plants. WD in these plants was 68.4 ± 0.5%, and plants wilted completely (Figure 2, B).

Table 1. Soil moisture content (%) in pots with control (Control) and drought-treated (Drought) barley

	Control	Drought
7 days	52.5 ± 0.5	43.4 ± 0.9
10 days	53.0 ± 0.3	38.9 ± 1.3
13 days	51.9 ± 0.3	29.1 ± 0.8
16 days	51.3 ± 0.4	23.7 ± 1.1
19 days	50.7 ± 0.9	22.0 ± 1.7

Table 2. The degrees of drought intensity

Drought intensity	Age of seedlings
Mild drought	7, 10 days
Moderate drought	13 days
Severe drought	16, 19 days

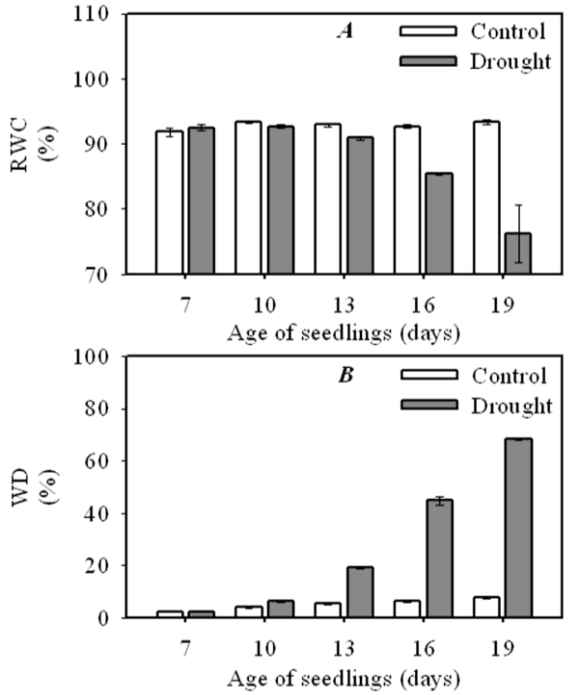
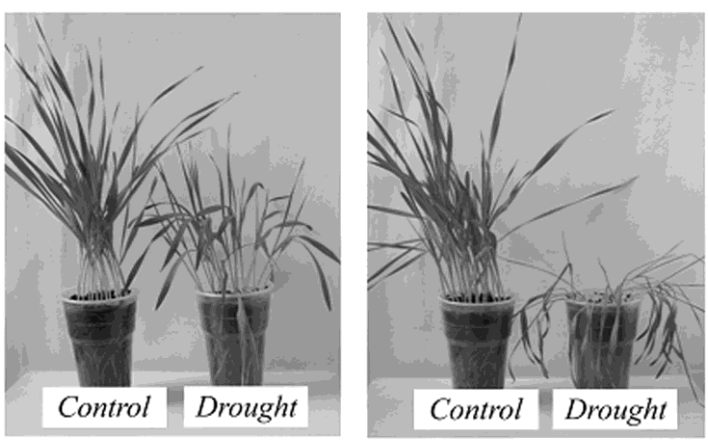


Figure 1. Relative water content (A) and water deficit (B) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).



(A) (B)

Figure 2. The appearance of 13-day-old (A) and 19-day-old (B) barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Based on the results of the extent of water loss by plants we analyzed the differences in the degrees of drought intensity affecting barley seedlings in particular periods of the experiment (Table 2).

II. Drought Effect on Plant Growth and Concentration of Pigments

Soil drought was accompanied by inhibition of growth processes in barley. It was evident from reduction of plant height and weight. The height of barley seedlings grown under moderate and severe drought was 25 – 30% lower than that of control seedlings (Table 3). Drought induced inhibition of growth was not apparent on the first leaf that developed in the absence of water deficit in plant (7 – 10 days). However, development of the second leaf was severely delayed by drought conditions, so that the length was only 30 – 40% that in control plants. In addition, the development of third leaf in plants grown under drought conditions of soil was completely inhibited, though all 19-day-old barley seedlings grown under constant irrigation had third leaf.

The weight of stem of plant was also severely reduced under drought conditions. It was already evident under mild drought. Thus the weight of 10-day-old drought treated plants was 20% lower in comparison with control barley (Table 3), and by 16th day of experiment this difference increased by 70%. The reduction of plant weight of barley seedlings under drought resulted not only from growth inhibition but also from water loss by plants.

Photosynthesis is one of the most drought sensitive processes in plant cells. As was shown in earlier research drought stress affects plant photosynthesis by reducing leaf area, enhancing stomatal closure, causing ultrastructural changes in chloroplasts and destruction of photosynthetic pigments, inhibiting the electron transport and CO_2 assimilation reactions, and altering the rate of photosynthesis in tissue [18-21]. However, nature and extent of the effects of water deprivation on photosynthesis depend upon drought intensity and duration. It is now established that the rate of photosynthesis under mild drought is preferentially controlled by CO_2 assimilation rate in leaves, while under severe stress structural changes of chloroplasts and pigment destruction contribute to reduce the rate of photosynthesis [22-24].

To assess the extent of drought-induced damage of photosynthetic apparatus in current research the concentrations of photosynthetic pigments, chlorophyll ($a + b$) and carotenoids, were determined.

Table 3. Plant height (cm) and weight (g) of barley seedlings grown under constant irrigation (Control) and under drought condition of soil (Drought)

		Control	Drought
Height (cm)	7 days	16.9 ± 0.2	16.6 ± 0.1
	10 days	18.7 ± 0.4	18.2 ± 0.4
	13 days	25.2 ± 0.6	18.7 ± 0.2
	16 days	29.9 ± 0.6	19.5 ± 0.3
	19 days	32.4 ± 1.2	19.7 ± 0.3
Weight (g)	7 days	0.19 ± 0.01	0.19 ±0.01
	10 days	0.27 ± 0.01	0.22 ± 0.01
	13 days	0.33 ± 0.01	0.18 ± 0.01
	16 days	0.34 ± 0.02	0.11 ± 0.01
	19 days	0.36 ± 0.01	0.10 ± 0.01

Analysis of photosynthetic pigments showed that under mild drought there was no statistically significant reduction in the concentration of chlorophyll (*a* + *b*) in seedlings, but under severe drought conditions the concentrations of chlorophyll in barley decreased by 72% those of control of leaves. The amount of chlorophyll (*a* + *b*) further reduced depending on the intensified drought conditions and on 19th day of experiment it was 45% lower than that in control plants (Figure 3, A). It was also found that chlorophyll *a*: chlorophyll *b* ratio tended to increase under severe drought condition (Table 4) indicating that primary destruction of chlorophyll *b* occurred. Carotenoid concentration in barley seedlings also decreased under drought conditions. Thus, in 13-day-old barley grown under drought conditions −of soil carotenoid concentration was 22% lower than that in control plant, while severe drought conditions brought the reduction of carotenoid concentrations by 2-fold (Figure 3, B).

These results were in agreement with those obtained previously using karst plants [25], cotton plants [26], wheat [27], euonymus [28], and sunflower [29] showing drought-induced reduction of photosynthetic pigments as well as the increase in the chlorophyll *a*: chlorophyll *b* ratio. Authors suggest that drought-induced increase in chlorophyll *a*: chlorophyll *b* ratio indicates a decline in the amount of active peripheral light-harvesting complexes of photosystem II (LHC II) [30]. This process is adaptive and meant for reduction of ROS formation coming from over-reduction of electron acceptors in chloroplast.

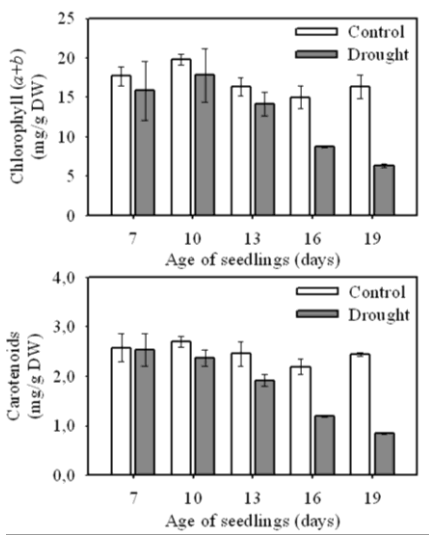


Figure 3. Chlorophyll (*a* + *b*) (A) and carotenoid (B) concentrations in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Table 4. Chlorophyll *a*: chlorophyll *b* ratio (rel. units) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

	Control	Drought
7 days	3.28 ± 0.03	3.32 ± 0.04
10 days	3.51 ± 0.17	3.52 ± 0.21
13 days	3.27 ± 0.03	3.18 ± 0.01
16 days	3.25 ± 0.03	3.56 ± 0.17
19 days	3.26 ± 0.15	3.37 ± 0.12

III. DYNAMICS OF OXIDATIVE PROCESSES IN PLANT UNDER DROUGHT CONDITIONS

One of the primary events in plant response to stress conditions is the change in redox state of plant cells identified to be over production of ROS. The main sources of ROS under drought are: overload of electron transport chain in chloroplasts, intensification of photorespiration in peroxisomes associated with inhibition of CO_2 fixation reactions and activation of respiration in mitochondria [31-33].

The dynamics of oxidative processes in barley seedlings under drought conditions of soil was examined using the test with dichlorofluorescein diacetate [34] and the test with scopoletin [35].

It was found that total ROS concentration of barley seedlings exposed to drought conditions was higher than in control plants (Figure 4, A). The difference in ROS concentration between control and drought-treated barleys increased at the drought growth. On the early stage of drought conditions of soil the concentration of ROS was similar in control and drought treated seedlings. After 10 days of drought conditions of soil the ROS content in plants was 28% higher than that in control barley, and in 16-day-old plants – 60% higher. It should be mentioned that on the 19th day of experiment total ROS concentration in barley was reduced severely, but still remained higher in the drought-treated plants. It could be explained by ageing-induced inhibition of metabolic reactions in the 1st leaf of barley plants. The results of experiments on total ROS changes in barley cells under drought conditions of soil are in good agreement with published data [36], and indicate the increase in intensity of ROS evolution under drought conditions of soil.

On the contrary, hydrogen peroxide (H_2O_2) concentration decreased depending on the plant age both under constant irrigation and under drought (Figure 4, B). However, in drought-treated seedlings the amount of H_2O_2 was still low. As seen on Figure 4, B, the amount of H_2O_2 was 35-55% lower in barley seedlings exposed to mild drought conditions as compared with barley plant grown under control condition. Under moderate drought conditions the amount of H_2O_2 was only 6% that in the amount recorded in control plants, and H_2O_2 was remained at a low concentration under severe drought conditions. H_2O_2 reduction under drought conditions was also shown in [37], which may be the result of detoxification of H_2O_2 by antioxidant system. It was shown [38] that H_2O_2 formed under drought conditions was actively converted into hydroxyl radical in Haber-Weiss reaction. The intensity of Haber-Weiss reaction increases dramatically due to accumulation of so-called "catalytic iron" (free Fe^{2+}). "Catalytic iron" forms hydroxyl radical as a result of protein proteolysis by the ROS, which are overproduced under stress conditions [38].

ROS play a dual role in cells responding to stress. On the one hand, they are endogenous signaling molecules involved in the development of plant resistance to stress factors [39-41]. On the other hand, high reactivity of ROS is responsible for their toxicity to cell interior. ROS toxicity to cell membranes consists in the induction of lipid peroxidation reactions and de-esterification of fatty acids resulting in an increase in membrane permeability to some metabolites and ions [42]. Intensity of lipid peroxidation as well as membrane permeability represents good tests for determining the amounts of plant injury under stress conditions.

In current study, the increase in ROS level in plant under drought conditions of soil was accompanied by induction of lipid peroxidation [43]. Although the differences in the amounts

of ROS between control and drought treated plants was recorded under mild drought, the induction of lipid peroxidation occurred under moderate drought conditions. Thus, in 7-10-day-old seedlings grown under drought conditions malonedialdehyde (MDA) concentrations were similar to those in control plants, after 13 days of experiment it was 16% higher and after 16-19 days it was 2-fold higher as compared with control plants of corresponding ages (Figure 5, A).

Despite of the fairly high levels of ROS and lipid peroxidation products in barley seedlings exposed to drought conditions of soil during 7-16 days, the electrolyte leakage from the tissue remained similar to that in control plants. And on the 19th day of experiment the amount of electrolyte leakage from drought-treated barley exceeded by 7-fold that in control plant (Figure 5, B).

Interestingly, the permeability of cell membranes to free nucleotides was remained unchanged in both plants in control and in drought conditions (Table 5). This was probably due to the lack of significant structural damage to the cell membranes in barley seedlings under drought conditions.

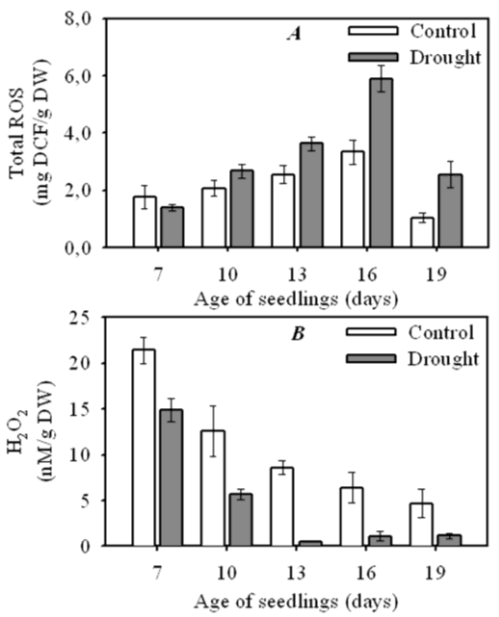


Figure 4. Total ROS (A) and H_2O_2 (B) concentrations in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Table 5. Free nucleotide leakage (rel. units) in barley seedlings grown under constant irrigation (Control) and under drought condition of soil (Drought)

	Control	Drought
7 days	0.0125 ± 0.0010	0.0111 ± 0.0010
10 days	0.0200 ± 0.0030	0.0166 ± 0.0026
13 days	0.0186 ± 0.0025	0.0206 ± 0.0049
16 days	0.0118 ± 0.0027	0.0178 ± 0.0053
19 days	0.0241 ± 0.0039	0.0284 ± 0.0051

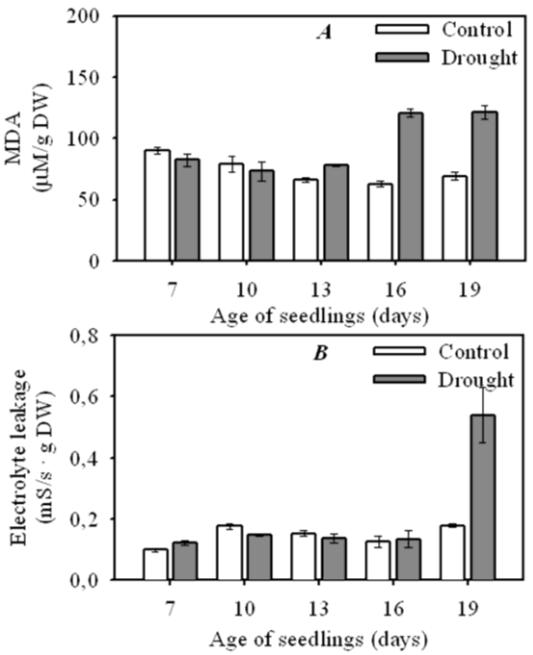


Figure 5. MDA content (A) and electrolyte leakage (B) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

IV. ANTIOXIDANT ENZYMES

Investigation of activation of antioxidant system in plant under drought conditions was started from analysis of superoxide dismutase activity. SOD represents the first line of plant cell protection from oxidative damage. It catalyses reaction of $O_2^{\bullet-}$ disproportionation, resulting in H_2O_2 and O_2 formation, which blocks the oxidation of cellular components at the stage of initiation [14].

Analysis showed that barley seedlings exposed to mild drought conditions of soil already had 75% higher total SOD activity as compared with control plants (Figure 6). Under moderate drought a slight decrease in SOD activity was shown. However, barley seedlings exposed to severe stress had increased activity of SOD.

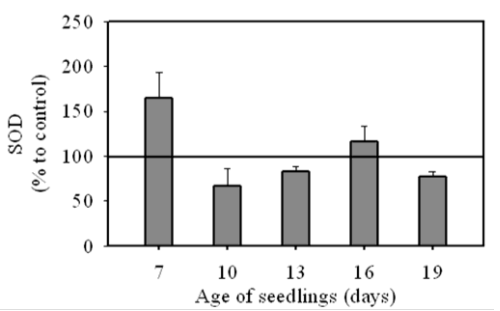


Figure 6. Total SOD activity (% of control) in barley seedlings grown under drought conditions of soil.

By means of native PAGE four isoforms of SOD were detected: Mn-SOD, Fe-SOD, cytosolic Cu/Zn-SOD (*cyt*Cu/Zn-SOD) and chloroplast Cu/Zn-SOD (*chl*Cu/Zn-SOD) [44]. The greater part of SOD activity is covered by *cyt*Cu/Zn-SOD and *chl*Cu/Zn-SOD, so we focus our attention on drought effects on these two isoforms (Table 6). Comparative analysis showed that the increase in total SOD activity under plant exposure to mild drought was provided with activation of both isoforms of the enzyme, but to a greater extent with the activation of cytosolic isoform. Thus, the activity of *cyt*Cu/Zn-SOD in 7-day-old drought-treated plants exceeded control activity by 82%, at the same time the activity of *chl*Cu/Zn-SOD was only 44% higher as compared with plants grown under constant irrigation. Similarly, increased activity of SOD under severe drought was also related to *cyt*Cu/Zn-SOD activation by 1.2-fold, whereas activity of *chl*Cu/Zn-SOD in these plants was similar to that in control.

High activity of *chl*Cu/Zn-SOD and *cyt*Cu/Zn-SOD in 7-day-old drought-treated barley plants suggests that active formation of $O_2^{\bullet-}$ occurs in both compartments under mild drought conditions. Under severe drought conditions cytosol became the main site of $O_2^{\bullet-}$ production and preferential activation of cytosolic isoform of SOD in seedlings exposed to drought conditions for 16 days confirmed this suggestion.

Hydrogen peroxide (H_2O_2) formed in the reaction of $O_2^{\bullet-}$ dismutation further undergoes reduction to H_2O in enzymatic reactions catalyzed by peroxidases. There are two types of peroxidases participating in H_2O_2 destruction: first type uses ascorbate as electron donor, which is ascorbate peroxidase (APX), and the second type is less specific to ascorbate and uses a wide range of phenolic compounds which is non specific phenolic peroxidase (POX) [45, 46]. We investigated the influence of drought on both APX and POX activities in barley plants.

It was shown that total APX activity was similar in barley plants grown both under constant irrigation and under mild and moderate drought condition of soil. However, in 16- and 19-day-old seedlings exposed to drought conditions activities of enzyme were 8% and 25% higher than those in control plants (Figure 7). This result indicates activation of hydrogen peroxide (H_2O_2) formation in seedlings under severe drought conditions.

Table 6. Activity of cytosolic (*cyt*Cu/Zn-SOD) and chloroplast (*chl*Cu/Zn-SOD) isoforms of SOD in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

		Control	Drought
*cyt*Cu/Zn-SOD (rel. units)	7 days	0.77 ± 0.06	1.40 ± 0.29
	10 days	1.41 ± 0.32	0.74 ± 0.12
	13 days	1.11 ± 0.26	1.08 ± 0.22
	16 days	0.98 ± 0.19	1.20 ± 0.24
	19 days	1.09 ± 0.13	1.04 ± 0.10
*chl*Cu/Zn-SOD (rel. units)	7 days	0.66 ± 0.06	0.96 ± 0.25
	10 days	1.60 ± 0.30	0.82 ± 0.14
	13 days	1.32 ± 0.26	0.99 ± 0.28
	16 days	1.07 ± 0.19	1.16 ± 0.27
	19 days	1.67 ± 0.21	1.04 ± 0.19

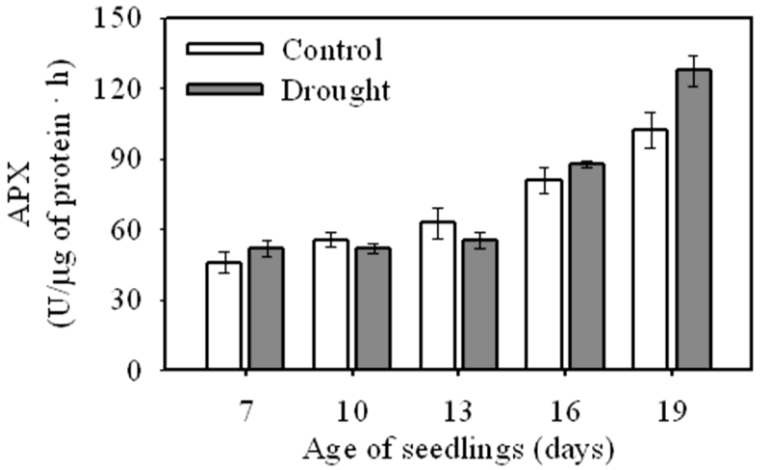


Figure 7. Total APX activities in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

By means of native PAGE we examined the activities of two APX isoforms-cytosolic (*cyt*APX) and chloroplast (*chl*APX) [47]. It was shown that the increase in total APX activity in 16-day-old drought-treated seedlings was due to chloroplast isoform activation. In 19-day-old plant induction of *cyt*APX occurred, so that increase in APX activities were due to both *cyt*APX and *chl*APX activation (Table 7).

The results showed that induction of H_2O_2 detoxification involving APX occurs in severe stress (16 – 19 days). Cytosolic as well as chloroplast APX isoform activation indicates that H_2O_2 was generated in both compartments.

Study of non-specific phenolic peroxidase (POX) in barley grown under constant irrigation and drought conditions of soil showed a statistically significant increase in POX activities in seedlings grown under mild drought (Figure 8). In barley seedlings grown under constant irrigation total POX activity increased depending on the plant age. In 19-day-old seedlings total POX activities were 5-fold higher than those in 7-day-old seedlings. Total POX activities were less significant in drought-treated plants: in 19-day-old barley grown under water deprivation POX activities were only 2.5-fold higher than those in 7-day-old seedlings. In the previous research an increase in the activities of POX under drought conditions were associated with non-specific defense response in plants. The increase in POX activities accompanied with the increase in water-soluble phenolic compound concentrations were shown in white clover (*Trifolium repens* L.) grown under water deficit for 7 days [48]. Authors suggest that increase in POX activities as well as water-soluble phenolics concentrations were associated with activation of cell wall lignification in clover plants and results in reduced cell wall permeability to water.

Using PCR analysis we investigated the amount of expression of *POX8*, a gene encoding cell-wall peroxidase in barley. Interestingly, PCR product of this gene was found only in the 7-day-old drought-treated barley (Figure 9). It is likely that *POX8* expression is responsible for the increase in POX activity in barley under mild drought. These results demonstrated the activation of cell-wall lignification processes in barley seedlings on early stages of drought conditions.

Table 7. Activities of cytosolic (*cyt*APX) and chloroplast (*chl*APX) isoforms of APX in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

		Control	Drought
*cyt*APX (rel. units)	7 days	8.9 ± 0.3	9.8 ± 0.7
	10 days	7.2 ± 0.8	7.2 ± 0.7
	13 days	9.8 ± 1.6	8.8 ± 1.5
	16 days	9.3 ± 3.7	9.8 ± 2.1
	19 days	8.9 ± 1.0	11.3 ± 1.7
*chl*APX (rel. units)	7 days	16.2 ± 0.3	16.8 ± 0.4
	10 days	19.2 ± 0.8	17.9 ± 1.3
	13 days	19.6 ± 2.7	15.3 ± 1.0
	16 days	11.4 ± 1.7	15.3 ± 2.2
	19 days	15.4 ± 1.8	18.2 ± 2.6

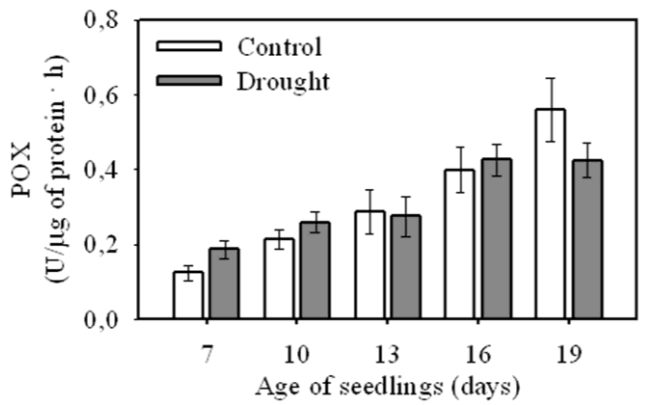


Figure 8. Total POX activities in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

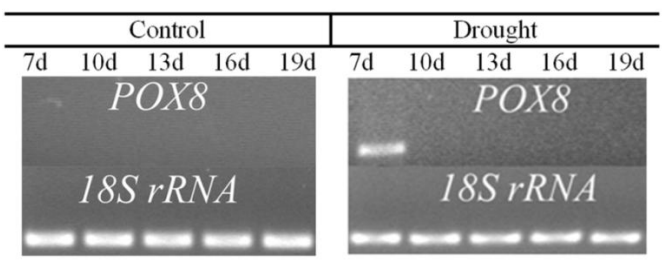


Figure 9. Expression of *POX8* in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

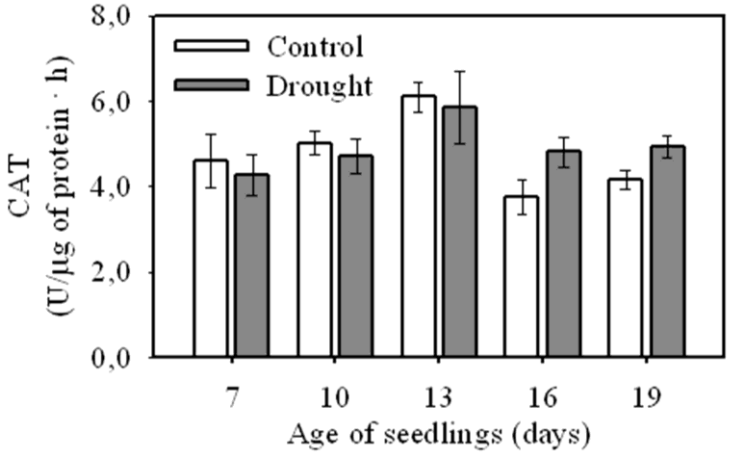


Figure 10. Total CAT activities in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

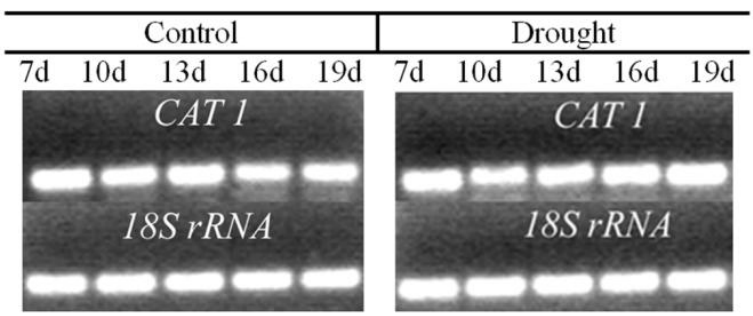


Figure 11. Expression of *CAT1* in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

H_2O_2 generated in peroxisomes is eliminated by catalase (CAT). Noctor et al. [33] showed that peroxisomes are the main sources of ROS under water deficit, so that CAT could play a key role in the protection of plant under oxidative stress caused by drought conditions.

Analysis of total CAT activity showed that under mild drought conditions enzyme activities were equal in control and drought-treated plants (Figure 10). However, under severe drought (16 – 19 days) there were significant increases in CAT activities by 28 and 18%, respectively, compared with those in control CAT activities.

There are three classes of genes responsible for CAT synthesis in plant: *CAT1* is expressed in adult leaves, and is responsible for the synthesis of CAT eliminating H_2O_2 produced in peroxisomes during photorespiration; *CAT2* is expressed in the vascular tissues, and product of *CAT3* expression was found in microsomes of seeds and seedlings.

It was shown that expression of the gene encoding peroxisomal CAT (*CAT1*) is induced under severe drought (Figure 11). The results suggest that activation of H_2O_2 synthesis in photorespiration occurs only under severe drought conditions.

V. Low-Molecular Weight Antioxidants

In current research we also investigated the concentrations of low-molecular weight antioxidants, such as ascorbic acid (AsA), glutathione, tocopherols and water-soluble phenolics, as well as subsidiary enzyme activities in barley seedlings under drought conditions of soil.

Total pool of AsA increased with age in barley seedlings grown under constant irrigation (Figure 12). The maximum of AsA content was registered in 16-day-old control plants. In 19-day-old seedlings total pool of AsA reduced. In barley plants grown under mild and moderate drought conditions of soil the total concentrations of AsA did not differ from those in control plants. However, under severe drought conditions there was a significant reduction in the concentration of AsA in drought-treated plants as compared with control plants. Thus, in 16-day-old seedlings grown under drought conditions the concentrations of AsA were reduced by 62% those in control plants. Further increase in duration of drought conditions did not result in a significant change of AsA concentration: in 19-day-old drought-treated seedlings total AsA did not differ from the concentration recorded in 16-day-old seedlings, and was equal to the concentration in the control plants. Depletion of AsA under drought conditions was demonstrated earlier [37, 49]. Authors supposed that drought-induced reduction of AsA concentration is associated with carbohydrate metabolism impairing [50].

Analysis of reduced AsA concentration showed that reduced AsA was decreased in barley plant under mild and moderate drought conditions and increased under severe drought conditions. As observed in Table 8, concentrations of reduced AsA were 13-16% higher in barley plants exposed to drought conditions of soil for 16-19 days, which were compared with those in control plants. The reduced concentration of AsA in plant cell is a result of several processes. On the one hand, AsA oxidation occurs in a wide range of enzymatic and non-enzymatic reactions of ROS detoxification. On the other hand, reduced AsA pool enriches by the processes both owing to *de novo* synthesis and to recruit of oxidized form. Declining of reduced AsA under mild and moderate drought conditions suggests that AsA consumption in the reactions of ROS detoxification prevails over its *de novo* synthesis and recruiting by the reduction, while increasing of reduced AsA part in total pool under severe drought conditions indicates the activation of recruiting processes.

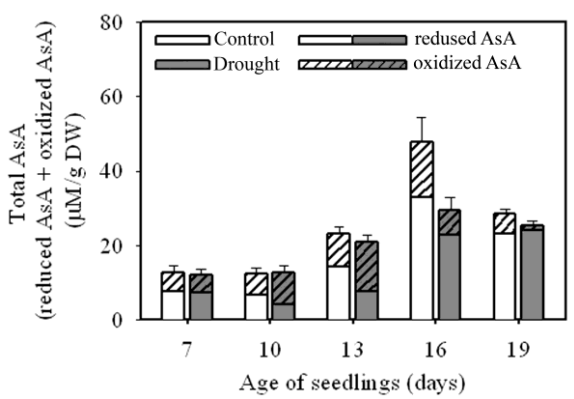


Figure 12. Total AsA (reduced AsA + oxidized AsA) concentrations in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Table 8. Part of reduced AsA (%) in total pool in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

	Control	Drought
7 days	60.4 ± 2.6	60.0 ± 4.3
10 days	55.3 ± 2.1	32.3 ± 3.0
13 days	61.2 ± 3.1	37.1 ± 3.0
16 days	69.3 ± 3.4	78.2 ± 5.1
19 days	81.4 ± 2.8	94.5 ± 2.4

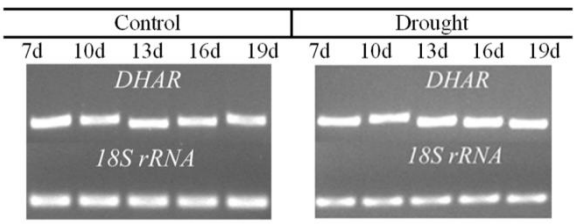


Figure 13. Expression of *DHAR* in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

To confirm this suggestion we investigated the expression level of gene, encoding dehydroascorbate reductase (DHAR), an enzyme participating in oxidized-to-reduced AsA recovery. It was shown that *DHAR* expression was 25 − 40% higher in 10-19-day-old seedlings grown under drought conditions as compared with control plants (see Figure 13). Obtained results indicate that activation of the oxidized-to-reduced AsA regeneration occurred under mild drought conditions.

As mentioned above, H_2O_2 formed by $O_2^{\bullet-}$ dismutation in chloroplasts was further reduced by AsA in enzymatic reaction catalyzed by APX. This leads to the formation of dehydroascorbate. Dehydroascorbate is then reduced by DHAR, which uses glutathione as electron donor. Under oxidative stress glutathione prevents protein sulfhydryl group oxidation by ROS. In recent research signal function of glutathione under stress conditions is also considered [51, 52].

We showed that in 19-day-old barley seedlings total glutathione (GSH + GSSG) concentrations in control plants gradually reduced, which may be due to ageing process (Figure 14). In barley grown under drought conditions of soil total amount of glutathione was reduced during the first few days of observation, and was almost constant in seedlings exposed to drought for 10 – 19 days. Total amounts of glutathione under drought conditions were always higher than the values recorded in control plants. Under mild drought conditions total glutathione contents exceeded only by 10 % those in control plants, and in 19-day-old drought-treated seedlings it was 4-times higher than that in control plants. The increase in total glutathione pool in barley under severe drought conditions is likely to be the results of supply by *de novo* synthesis.

Key characteristics of glutathione pool under natural and stress conditions are redox potential, which are estimated as the ratios of reduced to oxidized glutathione (GSH: GSSG). Comparative analyses showed that GSH: GSSG ratios in seedlings grown under drought conditions were higher compared with those in control plants (Table 9). These results indicate

that under drought stress condition higher GSH concentrations were kept up in barley seedlings.

As in the case of reduced AsA content, GSH concentrations in plant cells depended upon intensity of oxidation in ROS-scavenging reactions, *de novo* formation of it and recruiting activity from oxidized form. The intensity of GSH recruiting processes is characterized by activity of glutathione reductase (GR).

Kinetic study showed that under mild and moderate drought conditions common glutathione reductase (GR) activities did not differ both in control and drought-treated plants. However, GR activity in seedlings exposed to severe drought conditions exceeded 15 − 25% by the activities recorded in control plants (Figure 15). These results suggest that under mild drought conditions the increase in GSH concentrations is the result mainly by *de novo* synthesis, while under severe drought conditions recruiting system from GSSG to GSH also participated.

Tocopherols are oil-soluble antioxidants. The main function of tocopherols is termination of lipid peroxidation in plasma membrane under oxidative stress [15, 53].

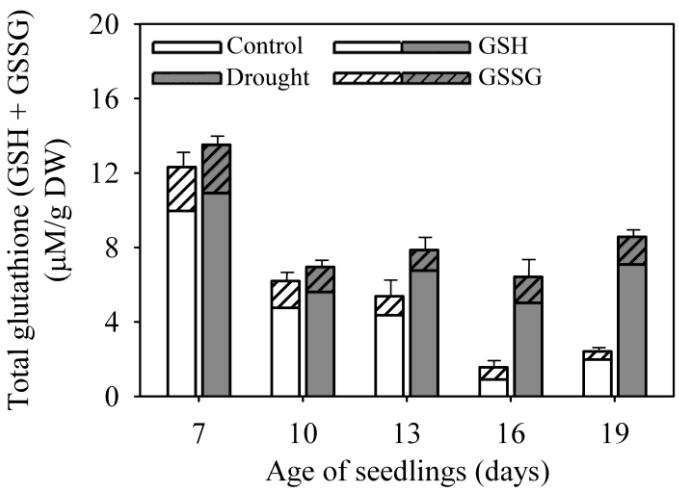


Figure 14. Total glutathione concentrations (GSH + GSSG) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Table 9. GSH: GSSG ratios (%) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

	Control	Drought
7 days	4.2 ± 0.4	4.2 ± 0.2
10 days	3.3 ± 0.2	4.2 ± 0.3
13 days	4.3 ± 0.6	6.2 ± 0.2
16 days	1.4 ± 0.2	3.7 ± 0.3
19 days	4.6 ± 0.3	4.8 ± 0.1

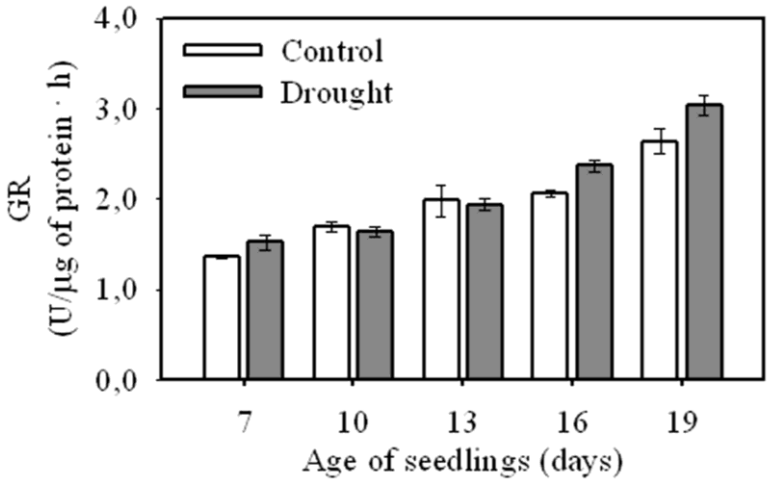


Figure 15. GR specific activity in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

By means of HPLC we detected two major forms of tocopherols – α-tocopherol and γ-tocopherol. The analysis showed that the total pool of tocopherols in barley grown under constant irrigation was increased during the first 13 days and then was reduced (Figure 16). In barley plants grown under drought conditions of soil total tocopherol concentrations increased constantly during the period from 7- to 19-day-old. In seedlings exposed to mild stress total tocopherol concentrations were equal to those in control plants. Tocopherol concentrations were 10 − 30% lower in 13- to 16-day-old seedlings grown under drought conditions than those in control plants, and only on the 19-day-old total tocopherol concentrations exceeded by 26% than those in control plant. The increase in total tocopherol concentrations in drought-treated plants was the results of increase in both α-tocopherol and γ-tocopherol concentration. The amount of γ-tocopherol increased 3-fold in 19-day-old drought-treated seedlings.

In recent studies specific role of γ-tocopherol in plant protection from drought conditions is actively discussed. It is well known that γ-tocopherol is a precursor of α-tocopherol in biosynthetic pathway and mainly accumulates in seeds and leaves of young plants. In adult leaves α-tocopherol homologue prevails, and synthesized γ-tocopherol is immediately converted into α-tocopherol in the transmethylation reaction catalyzed by γ-tocopherol methyltransferase (γ-TMT). Cela et al. [54] reported an increase in γ-tocopherol concentrations in the adult plants of aptenia (*Aptenia cordifolia* (L. f.)) under drought conditions. However, the concentration of α-tocopherol remains unchanged. Studies of Abbasi et al. [55] showed that the mutant lines of tobacco plants with suppressed expression of *γ-TMT* gene had high resistance to osmotic stress induced by the addition of sorbitol in growth medium. Based on these results, authors suggest that the additional function of γ-tocopherol is protection of plant under conditions of water deficit.

We used α-tocopherol to γ-tocopherol ratio (α-tocopherol: γ-tocopherol) to characterize the composition of tocopherol pool in barley under drought conditions (Table 10). It was shown that in control plants α-tocopherol: γ-tocopherol ratio gradually increased, indicating the decline of γ-tocopherol part in the total tocopherol pool. In seedlings exposed to drought for 10 – 19 days α-tocopherol: γ-tocopherol ratio remained constant, and was significantly

lower than that in control plants. Lower value for α-tocopherol: γ-tocopherol ratio in drought treated seedlings indicates preferred accumulation of γ-tocopherol under drought conditions. All of the results mentioned above confirm the results obtained by Cela et al. [54]. On the other hand, a constant ratio between α- and γ-tocopherol homologues indicates that the synthesis of γ-tocopherol and its transformation into α-tocopherol is strictly controlled under drought stress.

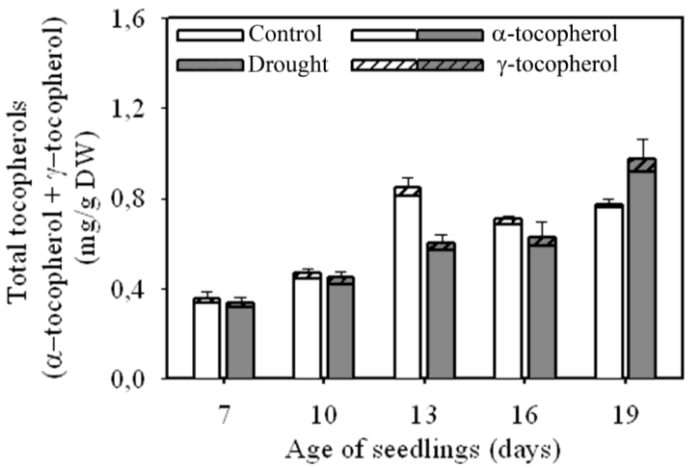


Figure 16. Total tocopherol (α-tocopherol + γ-tocopherol) concentrations in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

Table 10. α-Tocopherol : γ-tocopherol ratio (rel. un.) in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought)

	Control	Drought
7 days	17.6 ± 2.6	19.3 ± 4.2
10 days	18.2 ± 1.7	14.8 ± 0.7
13 days	20.9 ± 0.4	17.5 ± 0.9
16 days	28.5 ± 1.5	15.0 ± 0.5
19 days	43.8 ± 1.2	16.1 ± 4.1

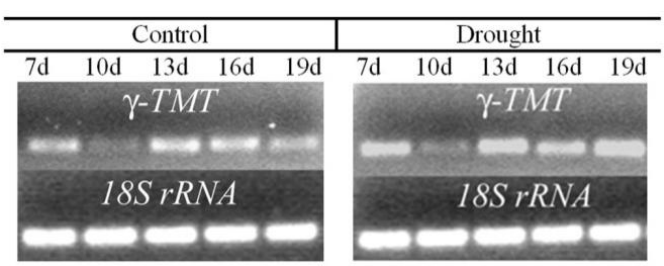


Figure 17. Expression of γ-TMT in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

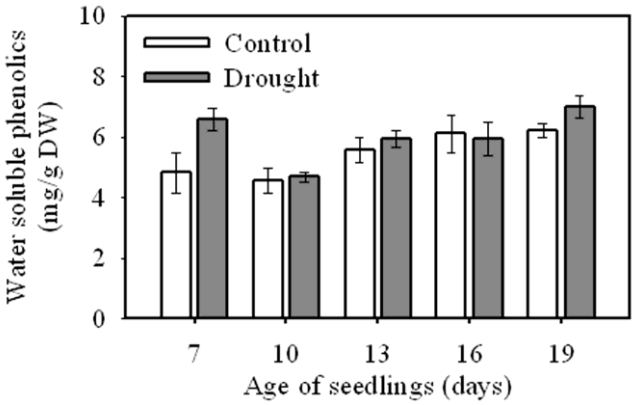


Figure 18. Total water soluble phenolics content in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

We examined the level of *γ-TMT* expression in barley plants under drought conditions and revealed that expression of *γ-TMT* was always lower in barley grown under drought conditions as compared with control seedlings (Figure 17). This indicates that under drought conditions γ-tocopherol to α-tocopherol conversion is inhibited to keep higher content of γ-tocopherol.

Phenolic compounds are involved in processes of ROS detoxification in plant cells. Phenolic compounds can interact directly with ROS and radicals of fatty acids [56, 57]. The ability of phenolics to serve as electron donor in reactions catalyzed by POX is of special interest.

It was shown that the concentration of water-soluble phenolic compounds in seedlings grown under mild drought conditions was 40% higher than that in seedlings grown under constant irrigation (Figure 18). However, in 10-16-day-old drought-treated seedlings the concentrations of phenolic compounds did not differ from the concentrations of control, and only in the 19-day-old plants there was a slight increase in the concentrations of phenolic compounds under drought conditions by 12% those in control plants.

As it was discussed above, under mild drought non-specific defense reaction associated with cell wall hardening occurs in plants. The increase in water soluble phenolic compounds on early stages of drought may also be a part of such response reaction, while under severe drought phenolics pool could enlarge as a result of drought-induced oxidative burst since water-soluble phenol compounds serve as good antioxidative protectants.

VI. OSMOLYTES

Loss of water is non-specific reaction of plant cells to water deprivation. In such conditions, osmolytes, e.g., proline and dehydrins, play an important role in protecting cells from damage caused by dehydration. Osmolytes are involved in maintaining the hydrated state of the cytoplasm, active enzymes conformation and structural integrity of cell membranes [58, 59]. Antioxidative properties of osmolytes are also discussed in literature [60].

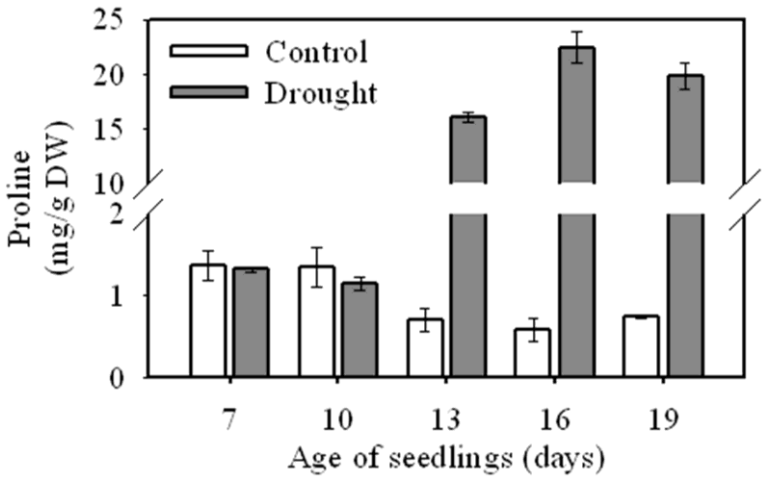


Figure 19. Total proline concentrations in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

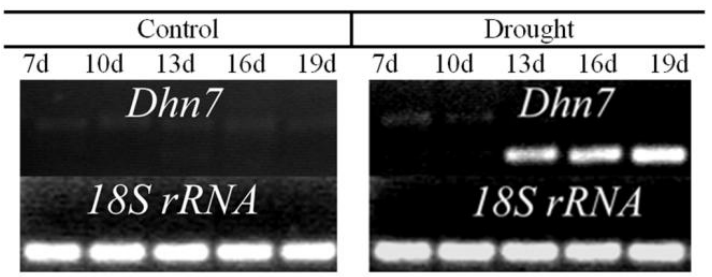


Figure 20. Expression of *Dhn 7* in barley seedlings grown under constant irrigation (Control) and under drought conditions of soil (Drought).

In current research to assess the activity of osmoprotective system in barley plants exposed to drought conditions we used two parameters, which were the concentration of low-molecular weight osmolyte proline and the amount of dehydrin gene expression by the example of *Dhn 7*.

Analysis showed that in barley seedlings exposed to mild drought conditions of soil proline concentration did not differ from that in control plants (Figure 19). The increase in proline concentration was first observed in plants grown under drought conditions for 13 days. However, the maximum concentrations of proline accumulated in 16-day-old drought-treated seedlings, which were 40-fold as high as those in the control seedlings.

PCR product of gene encoding *Dhn7* was not detected in control plants as well as in seedlings exposed to mild drought conditions (Figure 20). Expression of *Dhn7* was first observed in barley seedlings grown under moderate drought conditions. The amount of PCR product for *Dhn7* increased depending on intensity of drought conditions. The 16- and 19-day-old barley seedlings had 49% and 83% higher expression of *Dhn7* as compared with those in 13-day-old seedlings. If compared with previous results on RWC and WD in leaf tissue (see Figure 1) the results indicate that activation of osmoprotective system occurs under decrease of RWC to 90.9 ± 0.3% and WD 19.4 ± 0.4%.

Table 10. General pattern of activation antioxidant system in barley plants under drought condition

	I Mild drought	II Moderate drought	III Severe drought
RWC,	92.5%	90.9%	85.4%
WD	2.5%	19.4%	44.8%
Proline	0	+ 2300%	+ 4000%
Dhn 7	0	expressed	expressed
SOD	+ 75%	0	+ 15%
APX	+ 10%	0	+ 20%
CAT	0	0	+ 30%
POX	+ 40%	0	− 30%
Water-soluble phenolic compounds	+ 40%	0	+ 12%
Total ascorbate	0	0	− 40%
Total glutathione	+ 10%	+ 50%	+ 400%
Total tocopherols	0	− 30%	+ 26%

VII. General Pattern of Activation of Antioxidant System in Plant under Drought Condition

Summarizing the results cited above antioxidant system response on prolonged water deprivation could be divided into three phases depending upon intensity of drought conditions (Table 10).

Phase I (mild drought conditions) is characterized by the absence of significant structural and physiological damage in seedlings caused by drought conditions. Growth and development processes are not inhibited in barley during this phase; there is no water deficit in plant tissues and relative water concentrations correspond to those in the seedlings of control condition; signs of oxidative damage also do not develop in barley under mild stress condition. Nevertheless, under mild drought conditions activation of antioxidant system occurs related to the increase in activity of both cytosolic and chloroplast isoforms of SODs. Accumulation of water-soluble phenolic compounds accompanied by increase in POX activity is also observed during this stage. As was mentioned above this can be attributed to the intensification of cell wall lignification under mild drought.

Phase II (moderate drought) is connected with the reduction of relative water content in plant tissues and with the development of water deficit. During this stage primary oxidative processes are started in plant cells, which is indicated in the increase of total ROS concentrations in barley plants. Interestingly, under moderate drought conditions there is no significant activation of antioxidative system indicating possible adaptation of plant to drought conditions. The activity of major antioxidative ezymes corresponds to control value. Still, under moderate drought the system that is responsible for low-molecular weight antioxidants (AsA and glutathione) *de novo* synthesis and recovery is induced. More of all, the induction of osmoprotective system also occurs during this stage.

Phase III (severe drought conditions) is associated with an increase in drought negative effects on plant. Under such condition plant growth and development are delayed, and there is a strong water deficit in plant tissues. One can observe the destruction of photosynthetic apparatus, which is indicated in the decrease in concentrations of photosynthetic pigments. Biochemical analysis reveals signs of increases in oxidative stresses: malonedialdehyde content increases, in which membranes become permeable to electrolytes. Under severe drought all elements of antioxidant system, with the exception of non-specific phenolic peroxidase, possess maximum activity.

CONCLUSION

The effects of drought conditions of soil on dynamics of oxidative processes and on patterns of activation of antioxidant systems were studied in green barley leaves.

Drought induced inhibition of the growth and development of barley seedlings was registered under severe drought conditions accompanied by reduction of length, weight as well as delay in third leaf formation of the seedlings. Decline in photosynthetic pigments – chlorophyll ($a + b$) and carotenoids and the increase in the ratio of chlorophyll *a*: chlorophyll *b* was also shown indicating the reduction in the intensity of light trapping due to damage of light harvesting complexes of photosystem II.

In barley grown under drought conditions excess generation of ROS accompanied by the intensification of lipid peroxidation was registered. The tendency was revealed for strengthening of ROS formation and lipid peroxidation reaction in seedlings with increasing drought intensity. It was also shown that the content of hydrogen peroxide reduced under drought, which can be explained by the active work of the antioxidant system and increasing intensity of Haber-Weiss reaction. Under severe drought increase in electrolytes leakage was detected indicating an integrity of cell membranes to have barrier properties.

Mild drought was accompanied by activation of SOD and APX cytosolic isoforms suggesting that majority of oxidative processes occurs in cytoplasm during this phase of response. Accumulation of water-soluble phenolic compounds and increase in POX activity were also observed during mild drought indicating the intensification of cell wall lignifications in barley.

Under moderate drought conditions development of water deficit as well as accumulation of ROS started. This stage of response is referred to as adaptive processes since there is no significant activation of antioxidant system in barley plants. Under moderate drought conditions the system that is responsible to low-molecular weight antioxidants, is functioning to support *de novo* synthesis of them and to support recruiting systems of them.

Under severe drought conditions, an increase in drought negative effects on plant was shown. During this stage all elements of antioxidant systems, with the exception of non-specific phenolic peroxidase, possess maximum activities of them.

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Chapter 10

ISOLATION OF HIGH-YIELDING PARAQUAT-RESISTANT LINES OF *HORDEUM VULGARE* CV FIBER SNOW: PHENOTYPE CHARACTERIZATION

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ABSTRACT

We isolated high-yielding paraquat (PQ)-resistant lines of *Hordeum vulgare* cv Fiber Snow. In 2005, the seeds were sterilized by steeping in 1.25% (w/v) sodium hypochlorite solution and chilled overnight in a refrigerator at 4 °C. On the following day, we added 25 mL of Murashige and Skoog (MS) medium (pH 6.3), containing 0 µM, 2 µM, 4 µM, 6 µM, and 8 µM PQ, to each Petri dish. The seeds were incubated for 7 days under an alternating light/dark regime: 8 h of light (160 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 8 °C followed by 16 h of darkness at 7 °C. After germination, the seedlings were transplanted into planters, which were placed in a greenhouse for 7 days at 23 °C and then transferred to a corridor to allow acclimatization to ambient temperatures. Seedlings were subsequently grown under natural field conditions, and transplanted into field ridges spaced at a distance of 1 m. The seedlings were planted in a zigzag pattern, with a distance of 30 cm between each plant. At PQ concentrations of 2 µM, 4 µM, 6 µM, and 8 µM, we obtained 24 high-yielding candidate lines (M0) with larger spike numbers, spike weights, and straw weights. Of these lines, 12 were obtained at 2 µM PQ, 6 were obtained at 4 µM PQ, 2 were obtained at 6 µM PQ, and 4 were obtained at 8 µM. In 2006, we sowed 10 of these candidate lines (M0), to isolate putative homozygous lines. In 2007, we screened 7

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candidate lines for further yield analysis (M1), and in 2008, we established putative homozygous lines (M2). In 2009, the seeds (M3) of the original wild-type sibling and 7 high-yielding lines were tested for growth and yield characteristics, at the Kanagawa Agricultural Technology Center. In 2010, the seeds (M4) of these 7 high-yielding lines were tested by using the spot test, at the Kanagawa Agricultural Technology Center. In 2011, we obtained seeds (M5) at the Kanagawa Agricultural Technology Center. In 2012, we evaluated the PQ-resistance characteristics of the 7 high-yielding lines; 5 lines were confirmed to be PQ-resistant. The 7 high-yielding candidate lines were HvR2-3-13, HvR4-3-2, HvR4-3-5, HvR4-4-11, HvR4-4-26, HvR8-1-3, and HvR8-3-21. Among these, 5 mutant lines —HvR2-3-13, HvR4-3-2, HvR4-4-11, HvR8-1-3, and HvR8-3-21— showed PQ-resistant characteristics. Among them HvR2-3-13, HvR4-3-2 and HvR4-4-11 showed highly resistant characteristics. From 2012, the high-yielding capacity of the 7 candidate lines was assessed, and their growth characteristics at various seed and seedling densities were evaluated. In 2013, HvR4-3-2 and HvR8-1-3 showed yields of 8.3 and 3.9 t/ha, respectively, in a field in Machida, Tokyo.

Keywords: Global warming, High yielding, Nucleoside diphosphate kinase, Paraquat, Photosynthesis, ROS detoxification, Singlet oxygen

ABBREVIATIONS

FAD	Flavin adenine dinucleotide
FMN	Flavin Mononucleotide
miR398	Micro RNA 398
NADH	Nicotinamide Adenine Dinucleotide
NDPK	Nucleoside diphosphate kinase
PQ	Paraquat
PTD	Photo-dynamic therapy
ROS	Reactive Oxygen Species
$^1O_2$	Singlet oxygen
$O_2^{\cdot -}$	Super oxide
$^3O_2$	Triplet oxygen

INTRODUCTION

Global warming has reached a critical state because of an increase in the concentration of atmospheric carbon dioxide, arising from overuse of fossil fuels such as coal, petroleum, and natural gases. In the present study, we investigated the induction of high-yielding crop plants as sources of bio-fuels, including bio-ethanol, bio-oil, and bio-gases. We previously developed a method to induce high-yielding crop lines with resistance to ROS, including singlet oxygen ($^1O_2$), in *Pisum sativum* cv Alaska [1, 2] and *Oryza sativa* cv Koshihikari [3]. The ROS-resistant gain-of-function mutations offer a means of investigating the mechanisms for detoxification of ROS—including $^1O_2$—generated because of excessive sunlight. Furthermore, they represent a valuable tool for analyzing the processes of photosynthesis. These processes are described below, and the implications of the analytical results should be considered strictly from the point of view of energy derived from excessive sunlight.

Excessive sunlight is accepted by chlorophyll contained in the leaves of crop plants; ground-state chlorophyll is excited initially to singlet chlorophyll, and thereafter to triplet chlorophyll. This triplet chlorophyll transfers to triplet oxygen ($^3O_2$) the energy evolved by the following photolytic reaction:

$$2H_2O \rightarrow O_2 + 4H^+ + 4e^-$$

The energy transfer from triplet chlorophyll to $^3O_2$ not only leads to the evolution of $^1O_2$ but also causes the generated $^1O_2$ to move with a flight-pass length of approximately 500 μm, as in photodynamic therapy [4, 5, 6]. In PDT, a porphyrin derivative is injected into cancerous tissue, along with PO_2. Two laser lights focused into the cancer tissue excite the porphyrin derivative. The resulting transfer of energy to $^3O_2$ leads to the evolution of $^1O_2$, which will move with a range of approximately 500 μm, as measured by the phosphoro-fluorescence emission of the reaction:

$$PO_2 + {}^1O_2 \rightarrow PO_4 + \text{phosphoro-fluorescence}$$

This reaction is very similar to that involved in the energy transfer from triplet chlorophyll to $^3O_2$, thereby evolving $^1O_2$. The $^1O_2$ is reasonably estimated to move with a range of approximately 500 μm, and has the ability to remove the evolved $^3O_2$ from the chloroplast, and also to remove the energy accepted by chlorophyll in chloroplast. This process, which should be measured directly, functions during the photosynthetic process.

We previously used *Neurospora crassa*, *Pisum sativum* cv Alaska, and *Arabidopsis thaliana*, to establish that the C-terminal region of nucleoside diphosphate kinase (NDPK) interacts with catalases [2, 7, 8, 9, 10]. Catalases that localize in the cytosol are known to bind $^1O_2$ at the heme-prosthetic group, thereby facilitating the capture of $^1O_2$ evolved from the chloroplasts. We previously showed that nucleoside diphosphate kinase binds reduced nicotinamide adenine dinucleotide (NADH), and provides electrons to the bound $^1O_2$ at the heme-prosthetic group of catalase, thereby releasing superoxide (O_2^-). The O_2^- is converted into H_2O_2 by superoxide dismutase (SOD); thereafter, the resulting H_2O_2 is converted into H_2O and O_2 via the emission of infrared light (thermal emission) by catalases. The $^1O_2$ generated by the transfer of energy from triplet chlorophyll to $^3O_2$ is believed to remain in the chloroplast [6]. However, it remains unclear whether the evolved $^1O_2$ destroys membrane lipids, proteins (e.g., D1 protein), and the chloroplast DNA, leading, ultimately, chloroplast death. Previous studies using photodynamic therapy have established the lifetime of $^1O_2$ to be 0.5–4 μs [4, 5]; therefore, the $^1O_2$ generated by the transfer of energy from triplet chlorophyll to $^3O_2$ will move at a high speed and will not react with ambient molecules. The range is approximately 500 μm, indicating that $^1O_2$ will not react inside the chloroplast.

In the present study, we developed an isolation method for gain-of-function paraquat (PQ)-resistant crop lines. These high-yielding lines accumulate large amounts of carbohydrates and can more efficiently detoxify ROS, including $^1O_2$. Our objective was to obtain large numbers of gain-of-function PQ-resistant lines able to detoxify the hazardous effect of excessive sunlight in the overall processes of photosynthesis and also following reactions producing sucrose. In *Arabidopsis thaliana*, sucrose is able to induce micro RNA 398 (miR398), which represses the mRNAs for 2 Cu/Zn-SODs, thereby reducing the capacity

for detoxification of cellular ROS concentrations. High ROS concentrations suppress the expression of miR398, inducing high activity of 2 Cu/Zn-SODs. Further, miR398 is reported to control the mRNA for chaperonin, which is involved in the integration of Cu into Cu/Zn-SOD, and also to degrade the mRNA for a cytochrome c oxidase subunit [11, 12]. This regulatory function operates during downstream photosynthesis, and includes sucrose production.

MATERIALS AND METHODS

Origin of Seeds

The seeds of *Hordeum vulgare* cv Fiber Snow used in the present study were produced in Australia, and obtained from Yokohama Nursery Co. Ltd (Yokohama) in March 2005.

Mutagenesis of Barley Seeds

The first mutagenesis of barley seeds was performed in April 2005. We sowed 50 seeds per Petri dish, using a total of 20 Petri dishes. The seeds were sterilized by steeping in 1.25% (w/v) sodium hypochlorite solution, and washed 5 times with sterilized deionized water; the seeds in sterilized deionized water were then chilled overnight in a refrigerator at 4 °C. On the following day, we added 25 mL of Murashige and Skoog (MS) medium (pH 6.3), containing 0 μM, 4 μM, 8 μM, 40 μM, or 80 μM PQ, to each Petri dish. The seeds were incubated for 7 days under an alternating light/dark regime: 8 h of light (160 $\mu mol \cdot m^{-2} \cdot s^{-1}$) at 8 °C followed by 16 h of darkness at 7 °C. After germination, the seedlings were transplanted to planters, which were placed in a glass greenhouse for 7 days at 23 °C and then transferred to a corridor to allow acclimatization to ambient temperatures. In May 2005, the plants were grown under natural field conditions; in June, the plants were transplanted into field ridges spaced at a distance of 1 m. The seedlings were planted in a zigzag pattern, with a distance of 30 cm between each plant. The planting was late; further, the chilling treatment of sterilized seeds may have been too short. Consequently, the first plants to show growth became wilted without shoot formation. Figure 1a shows the growth rate of plants in the planters.

The second mutagenesis of barley seeds was performed in December 2005, using the method described above, but with the following concentrations of PQ in the MS medium: 0 μM, 2 μM, 4 μM, 6 μM, or 8 μM. In January 2006, the treated plants were transferred to the field, using the planting method described above.

Growth Characteristics of Putative High-Yielding Mutant Lines

We assessed the candidate mutant lines (M0) for the following growth characteristics: tiller numbers, spike numbers, and spike weights per line. From 2006 to 2007, we established seeds (M1) of the putative homozygous lines. From 2007 to 2008, these seeds (M2) were assessed for spike numbers, spike weights and straw weight per line, at the Kihara Institute

for Biological Research. In 2009, the seeds (M3) obtained in 2008 were transferred to the Kanagawa Agricultural Technology Center. From 2009 to 2010, the seeds obtained in 2009 were grown at the Kanagawa Agricultural Technology Center, to obtain fresh seeds (M4). From 2010 to 2011, the seeds of the mutant lines were assessed for growth characteristics, using 12 dot-planting tests, at the Kanagawa Agricultural Technology Center. Of the 12 plants used in the dot-planting tests, we eliminated those showing the lowest and highest seed weights, and determined the average seed weight of the remaining 10 plants.

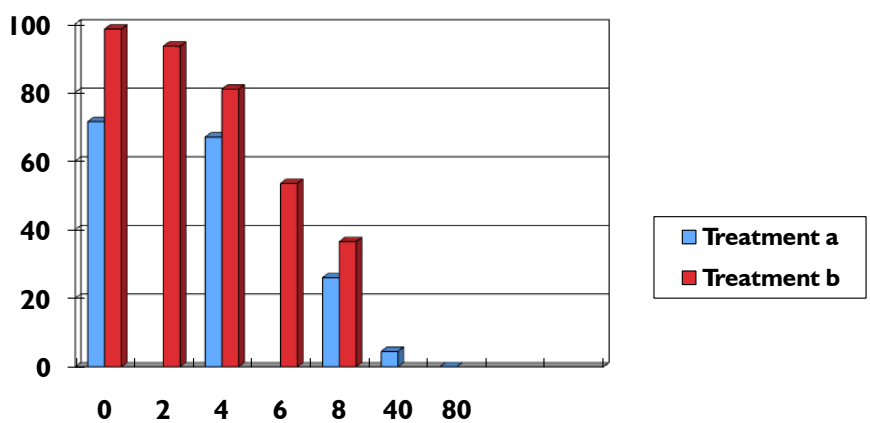
Evaluation of the ROS-Resistant Phenotype and Growth Characteristics

In 2012, the ROS-resistant phenotype was evaluated by using the seeds (M5) from the Kanagawa Agricultural Technology Center. We compared the densities of plants grown in a field in Machida, Tokyo, and also the spike weights of HvR4-3-2 and HvR8-1-3 grown in a field in Machida, Tokyo in 2013.

RESULTS

Growth Characteristics of PQ-Treated Barley Seeds

In the first mutagenesis of barley, the planting season was too late; consequently, we obtained data only on the growth ability of barley (Figure 1 a). Seedlings treated with 0 µM, 4 µM, 8 µM, and 40 µM PQ showed survival rates of 71.5%, 67%, 26%, and 4.5%, respectively. The second mutagenesis of barley seeds was performed in MS medium containing 0 µM, 2 µM, 4 µM, 6 µM, or 8 µM PQ; the surviving plants showed growth abilities of 98.5%, 93.5%, 81%, 53.5%, and 36.5%, respectively (Figure 1 b).



(a) The first mutagenesis of germinating seeds was performed in MS medium containing 0 µM, 4 µM, 8 µM, 40 µM, or 80 µM PQ.

(b) The second mutagenesis of germinating seeds was performed in MS medium containing 0 µM, 2 µM, 4 µM, 6 µM, or 8 µM PQ.

Figure 1. PQ-induced mutagenesis of germinating barley seeds.

The distribution of spike numbers, spike weights, and straw weights and the averages ± S.E. of the original wild-type line (43 plants) are summarized in Figure 2a, b, c and d, respectively. By screening high-yielding lines in MS medium containing 2 µM, 4 µM, 6 µM, and 8 µM PQ, we obtained 12, 6, 2, and 4 mutant candidate lines, respectively; these lines formed larger numbers of spikes than did the original wild-type line. From 2006 to 2007, we screened the first 10 of the 24 mutant candidate lines (Table 1), to obtain putative homozygous lines. In 2009, the seeds of the original wild-type line and 10 mutant candidate lines with putative homozygous states were transferred to Kanagawa Agricultural Technology Center. We subsequently screened 7 of these lines—HvR2-3-13 from 2 µM PQ; HvR4-3-2, HvR4-3-5, HvR4-4-11, and HvR4-4-26 from 4 µM PQ; and HvR8-1-3 and HvR8-3-21 from 8 µM PQ—by using dot-planting test (Figure 3).

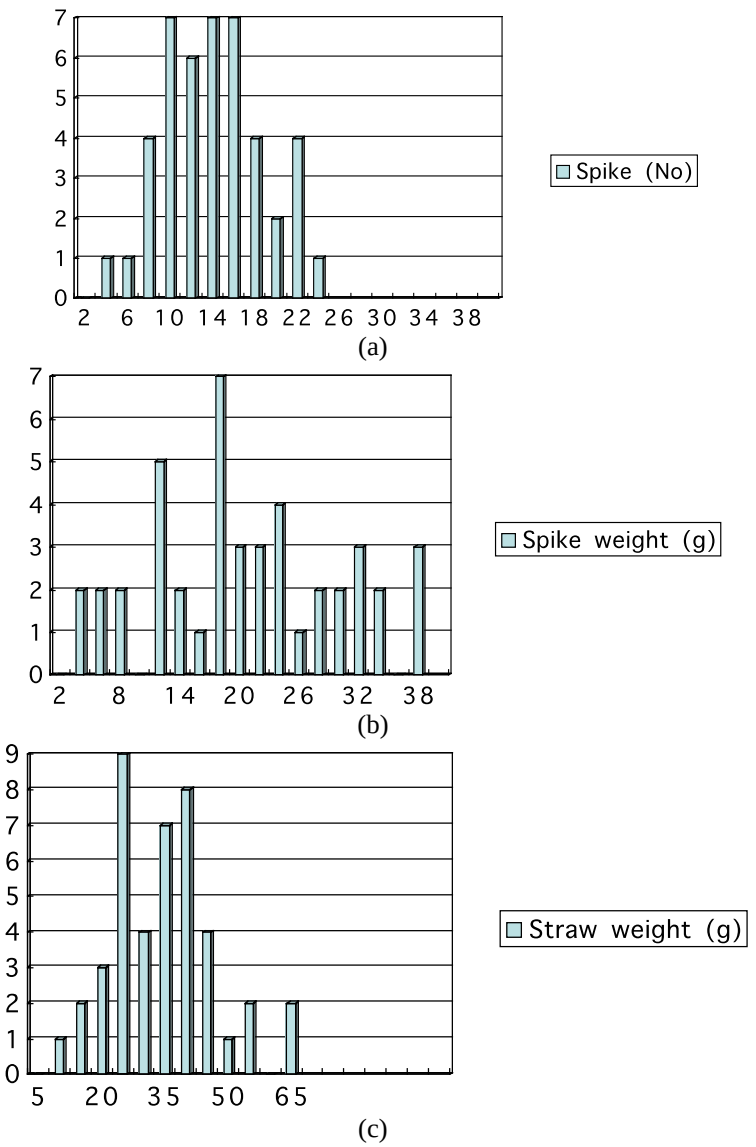
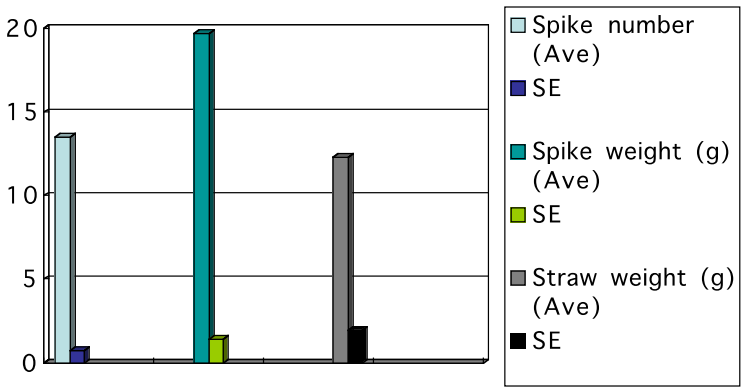


Figure 2. (Continued).



(d)

Figure 2. The distribution of spike numbers, spike weights, and straw weights, and averages ± S.E. in the original wild-type line. (a) Distribution of spike numbers in the original wild-type line. (b) Distribution of spike weights (g) in the original wild-type line. (c) Distribution of straw weights (g) in the original wild-type line. (d) Averages of spike numbers, spike weights (g) and straw weights (g) ± S.E. are shown.

During collation of our results, the far northeast of Japan was hit by a very large earthquake, followed by a tsunami. Our research was severely disrupted, and therefore our data are incomplete, for example, by lacking a control experiment (Figure 3). Despite this, the data show important trends, namely, the very high yield characteristics of the candidate mutant lines. The average seed weights determined in the dot-planting tests showed apparently parallel results, which are presented in the data of original mutant lines (M0) as shown in the latter part of Figure 3. Further, the spike numbers, spike weights, and straw weights of the candidate mutant lines were 2–3-fold than were those of the original wild-type line. Despite the lack of data regarding the seed weight of the original wild-type line, we estimated an approximately 2-fold difference between the seed weights of HvR4-3-2 and HvR8-1-3, and those of HvR4-3-5 and HvR4-4-26.

Table 1. The averages of spike numbers, spike weights (g) and straw weights (g) ± S.E. in the original wild-type (43 lines) are shown, and the spike numbers, spike weights (g) and straw weights (g) in the 10 mutant candidates are presented

Original WT line/ HvR mutant line	Spike No /line (Ave ± S.E.)	Spike weight (g)/line (Ave ±weight	Straw weight (g)/ line (Ave ± S.E.)
Original WT line	13.5 ± 0.7	19.7 ± 1.4	32.5 ± 1.9
HvR2-1-6	27	45	58
HvR2-3-13	36	46	89
HvR4-3-2	28	50	70
HvR4-3-5	32	50	46
HvR4-4-11	29	48	73
HvR4-4-26	39	47	85
HvR6-2-9	27	52	55
HvR8-1-2	31	46	72
HvR8-1-3	42	47	92
HvR8-3-21	31	49	82

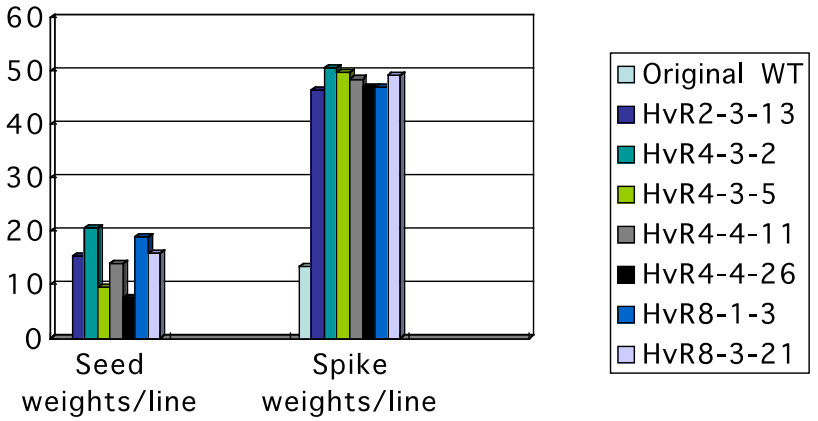
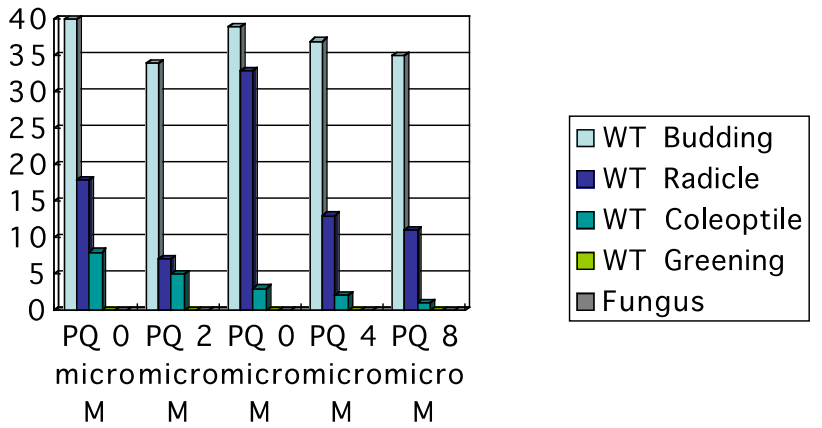


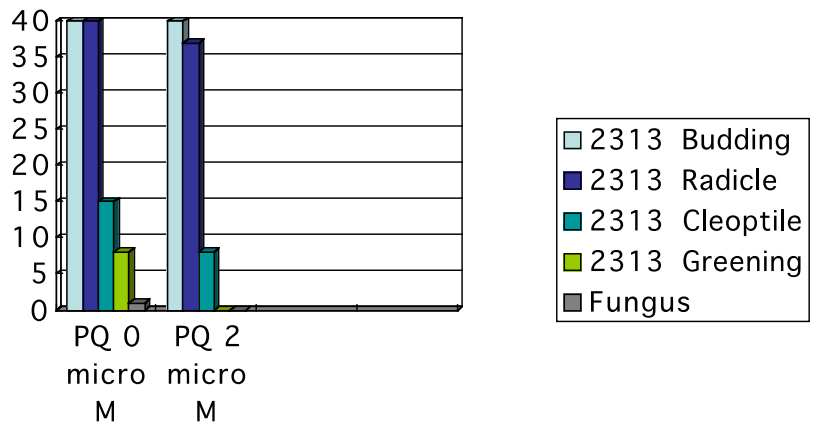
Figure 3. Seed weights of 7 mutant lines subjected to 10 dot-planting tests at the Kanagawa Agricultural Technology Center, and spike weights of the original wild-type line and 7 mutant lines isolated at the Kihara Institute for Biological Research. The average seed weights of 7 mutant lines—HvR2-3-13, HvR4-3-2, HvR4-3-5, HvR4-4-11, HvR4-4-26, HvR8-1-3, and HvR8-3-21 are presented. Correction of seed weights based on seed water content was not performed.

Evaluation of PQ Resistance

We assessed the PQ resistance of seeds of the 7 mutant lines and those of the original wild-type line. In each of 3 Petri dishes (15 cm), we sowed 100 seeds of the original wild-type line (old stock), and 100 seeds of the 7 mutant lines (M5) provided by the Kanagawa Agricultural Technology Center. Next, we added 50 mL of sterilized deionized water, and incubated the seeds for 4 days under light conditions at 7–19 °C. Germinating seeds at approximately the same early stage of budding were transplanted into 8 Petri dishes (9 cm; 10 seeds per dish) and divided into 2 groups. To the first group, we added 25 mL of sterilized deionized water, while to the second group, we added 25 mL of sterilized deionized water containing 2 µM PQ. The seeds were incubated for 7 days under light conditions at 7–19 °C. The germination results are presented in Figure 4 a. In the absence of PQ, the phenotypes of early germinating seeds of the original wild-type line showed continuation of budding, radicle lateral root formation, coleoptile formation, and greening caused by emergence of monocotyledons. The values of radicle lateral root formation were 18 in the absence of PQ, but in the presence of 2 µM PQ the values were reduced to 7, showing decrease by 39% those in the control experiment. We identified the original wild-type line as sensitive to 2 µM PQ. We assessed the sensitivity of the original wild-type line to 4 µM and 8 µM PQ by preparing an additional 3 Petri dishes (15 cm), containing seeds of the original wild-type line and sterilized deionized water. In the absence of PQ, radicle formation was present in 33 out of 39 germinating seeds showing continuation of budding (i.e., 84.6%); in the presence of 4 µM PQ, radicle formation was present in 13 out of 37 germinating seeds (i.e., 35.1%); and in the presence of 8 µM PQ, radicle formation was present in 11 out of 35 germinating seeds (i.e., 31.4%). Therefore, the suppression of radicle formation increased in line with an increase in the PQ concentration, from 0 µM to 8 µM.



(a)



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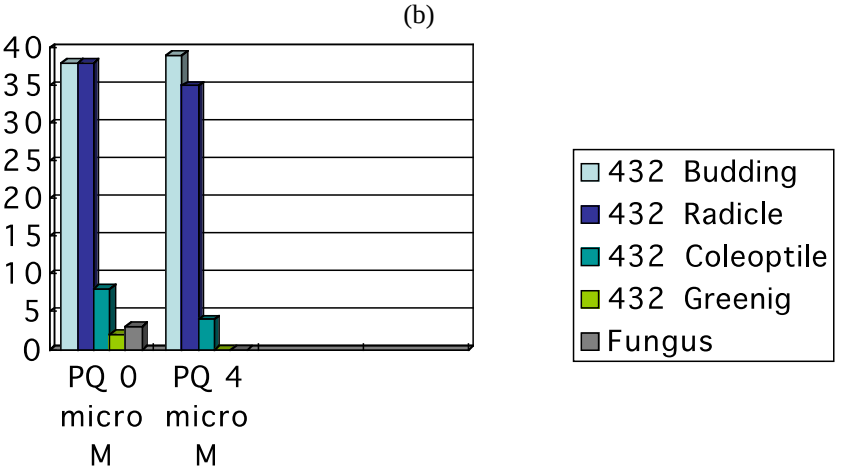
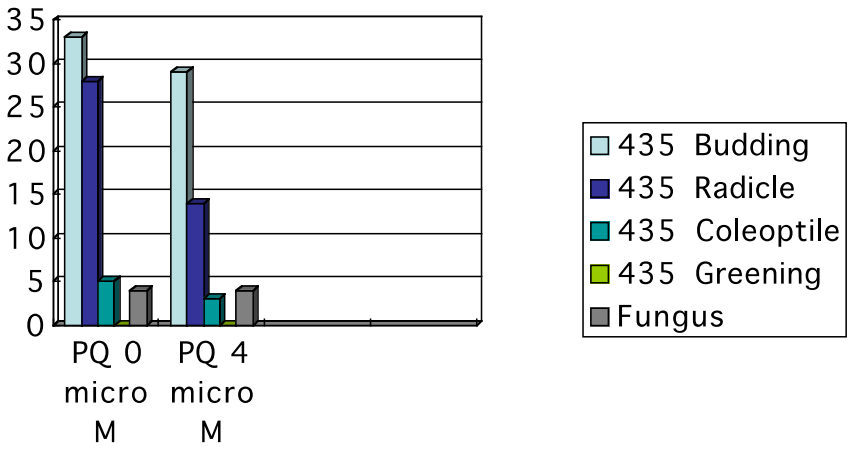


(c)

Figure 4. (Continued).



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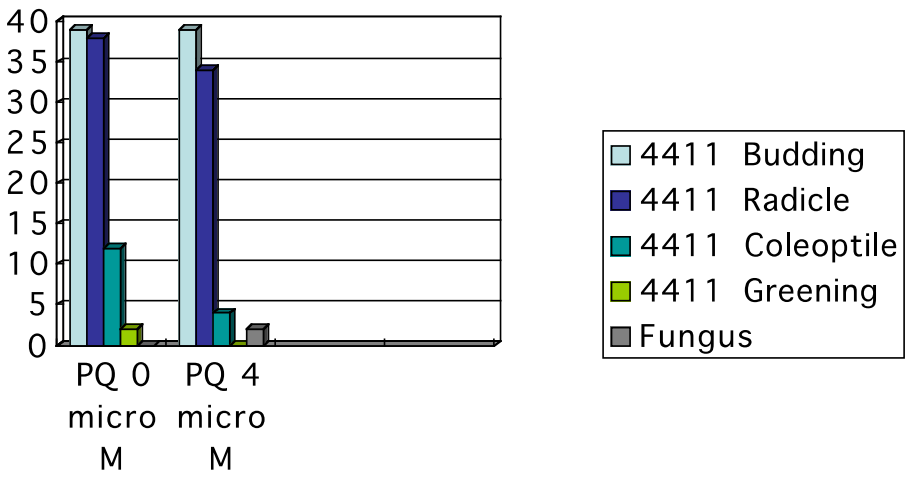
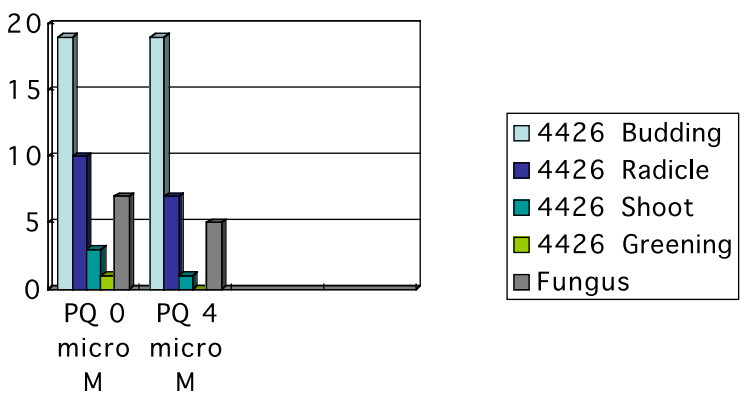


(e)



(f)

Figure 4. (Continued).

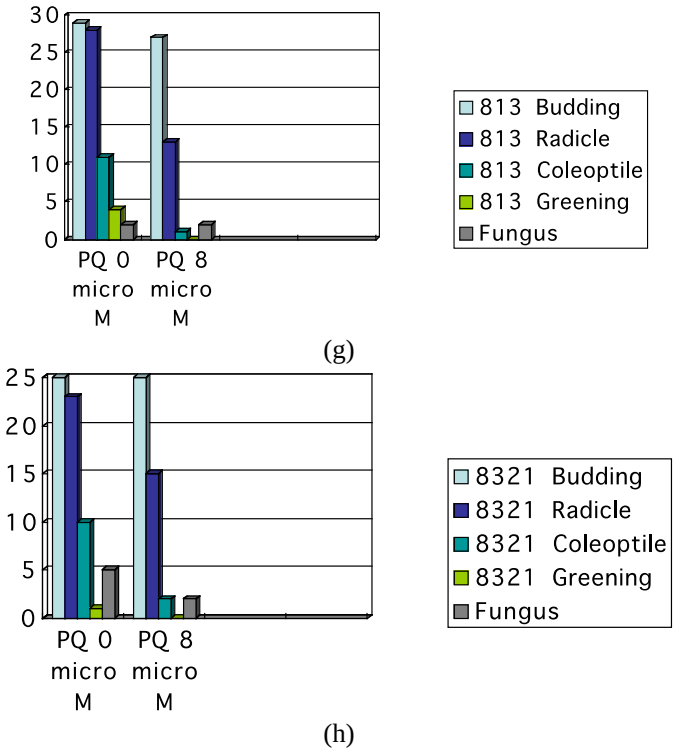


Figure 4. Evaluation of resistant phenotypes, based on the germination processes of seeds isolated from the original wild-type line and from 7 mutant lines. Continuation of budding, radicle lateral root formation, coleoptile formation, green monocotyledon emergence, and fungal infection were tested. (a) The patterns of germination processes in the original wild-type line, in the presence of 0 µM and 2 µM, 0 µM, 4 µM, and 8 µM PQ. (b) The patterns of germination processes in HvR2-3-13 in the presence of 0 µM and 2 µM PQ. (c) The patterns of germination processes in HvR4-3-2 in the presence of 0 µM and 4 µM PQ. (d) The patterns of germination processes in HvR4-3-5 in the presence of 0 µM and 4 µM PQ. (e) The patterns of germination processes in HvR4-4-11 in the presence of 0 µM and 4 µM PQ. (f) The patterns of germination processes in HvR4-4-26 in the presence of 0 µM and 4 µM PQ. (g) The patterns of germination processes in HvR8-1-3 in the presence of 0 µM and 8 µM PQ. (h) The patterns of germination processes in HvR8-3-21 in the presence of 0 µM and 8 µM PQ.

Figure 4 b shows the results for the mutant line HvR2-3-13. In the absence of PQ, radicle formation was present in 40 germinating seeds; in the presence of 2 µM PQ, radicle formation was present in 37 out of 40 germinating seeds (i.e., 93%). Thus, HvR2-3-13 is highly resistant to 2 µM PQ.

Figure 4 c shows the results for the mutant line HvR4-3-2. In the absence of PQ, radicle formation was present in 38 germinating seeds; in the presence of 4 µM PQ, radicle formation was present in 35 out of 38 germinating seeds (i.e., 92%). Thus, HvR4-3-2 is highly resistant to 4 µM PQ.

Figure 4 d shows the results for the mutant line HvR4-3-5. In the absence of PQ, radicle formation was present in 28 germinating seeds; in the presence of 4 µM PQ, radicle formation was present in 14 out of 28 germinating seeds (i.e., 50%). Based on its low sensitivity to 4 µM PQ, we decided not to use HvR4-3-5 in further experiments.

Figure 4 e shows the results for the mutant line HvR4-4-11. In the absence of PQ, radicle formation was present in 38 germinating seeds; in the presence of 4 µM PQ, radicle formation was present in 34 out of 38 germinating seeds (i.e., 89%). Thus, HvR4-4-11 is highly resistant to 4 µM PQ.

Figure 4 f shows the results for the mutant line HvR4-4-26. In the absence of PQ, radicle formation was present in 10 germinating seeds; in the presence of 4 µM PQ, radicle formation was present in 7 out of 10 germinating seeds (i.e., 70%). Further, the germinating seeds showed a high rate of fungal infection. Therefore, we decided not to use HvR4-4-26 in further experiments.

Figure 4 g shows the results for the mutant line HvR8-1-3. In the absence of PQ, radicle formation was present in 28 germinating seeds; in the presence of 8 µM PQ, radicle formation was present in 13 out of 27 seeds (i.e., 48%). Nevertheless, this line showed very good growth in the field, with a high yield in the dot-planting test. Therefore, we decided to use HvR8-1-3 in further experiments.

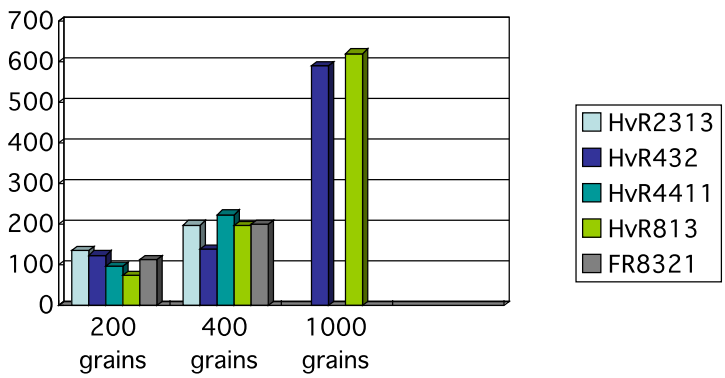


Figure 5. Comparison between density of seeds sown and density of seedlings obtained in a field in Machida, Tokyo. The data of HvR4-4-5 and HvR4-4-26 are not presented.

Figure 4 h shows the results for the mutant line HvR8-3-21. In the absence of PQ, 25 seeds showed continuation of budding, while 23 seeds formed radicles; in the presence of 8 µM PQ, 25 seeds showed continuation of budding, while 15 out of 23 seeds formed radicles (i.e., 65%). Nevertheless, this line showed relatively good growth in the field, with a moderately high yield in the dot-planting test. Therefore, we decided to use HvR8-3-21 in further experiments.

Growth Characteristics of PQ-Resistant Lines, According to Density of Seeds Sown

The growth of plants is known to be dependent on the density of seeds sown. Therefore, we examined the rates of germination and seedling formation in a field in Machida, Tokyo. The field contained ridges (length 7.5 m) from the southern to northern direction; the ridges were spaced at a distance of 60 cm, with a sowing area of 30 cm. On December 2, 2012, we sowed seeds of the 7 PQ-resistant lines. As shown in Figure 5, we sowed 33 g of HvR4-3-2 seeds—corresponding to 1000 grains—in the first ridge, and 6.6 g of HvR2-3-13 seeds—

corresponding to 200 grains—in the second ridge. In the third, fourth, fifth, and sixth ridges, we sowed HvR4-3-2, HvR4-4-5, HvR4-4-11, HvR4-4-26, HvR8-1-3, and HvR8-3-21, respectively, at the same seed density. We performed a second sowing with 13.2 g of seeds in each ridge, following the order described above, except that the first row was original wild-type line, and the final row was sown with 33 g of HvR8-1-3 seeds.

HvR4-3-2
1000 seeds/4.5m^2

HvR2-3-13
200 seeds/4.5m^2

HvR4-3-2
200 seeds/4.5m^2

(a)

HvR4-4-26
200 seeds/4.5m^2

HvR8-1-3
200 seeds/4.5m^2

HvR8-3-21
200 seeds/4.5m^2

(b)

HvR2-3-13
400 seeds/4.5m^2

HvR4-3-2
400 seeds/4.5m^2

HvR4-3-5
400 seeds/4.5m^2

(c)

Figure 6. (Continued).

HvR8-1-3 400 seeds/4.5m^2 | HvR8-3-21 400 seeds/4.5m^2 | HvR8-1-3 1.000 seeds/4.5m^2

(d)

Figure 6. Comparison of seedlings germinated from PQ-resistant mutant lines, under different densities of seeds sown on December 2, 2012. (a) Seedlings of HvR4-3-2, HvR2-3-13 and HvR4-3-2. (b) Seedlings of HvR4-4-26, HvR8-1-3and HvR8-3-21. (c) Seedlings of HvR2-3-13, HvR4-3-2 and HvR4-4-5. (d) Seedlings of HvR8-1-3, HvR8-3-21 and HvR8-1-3.

Table 2 (a) and (b). The data of spike weights (g) of HvR4-3-2 (a) and HvR8-1-3 (b) at the southern part of the ridge are shown. We sowed 1,000 grains each of seeds of HvR4-3-2 and HvR8-1-3.]

HvR4-3-2 S to N	Spike No	Spike weight (g/0.33m)	Spike weight (g/m)	Spike weight (t/ha)	Seed weight (t/ha)
1m-1	125	213			
1m-2	130	202	682	11.4	9.1
1m-3	166	270			
2m-1	121	210			
2m-2	114	205	585	9.8	7.8
2m-3	99	170			
3m-1	99	210			
3m-2	160	278	598	10.0	8.0
3m-3	71	115			
4m-1	233	205			

HvR8-1-3 S to N	Spike No	Spike weight (g/0.33m)	Spike weight (g/m)	Spike weight (t/ha)	Seed weight (t/ha)
1m-1	64	100			
1m-2	51	74	364	6.1	4.9
1m-3	80	120			
2m-1	115	170			
2m-2	70	106	275	4.6	3.7
2m-3	73	96			
3m-1	59	73			
3m-2	39	58	238	4.0	3.2
3m-3	57	90			
4m-1	63	90			

Figure 5 shows a comparison between the density of seeds sown and the density of seedlings obtained. The data of HvR4-4-5 and HvR4-4-26 are not presented. The screened mutant lines HvR4-3-2 and HvR8-1-3 showed germination rates of 60% when sown in the field.

Figure 6 shows the growth patterns of the PQ-resistant mutant lines. The distance between seedlings is of crucial importance in assisting tiller formation and spike formation—tillers are known to represent a stock supply of nutrients for the formation of fruitful spikes with seeds. When we grew 600 seedlings of HvR4-3-2 and HvR8-1-3 in an area of 4.5 m^2 (Figure 6 a and d), the density was too high for sufficient tiller formation to support fruitful spike formation. On the other hand, when we sowed 200 grains of HvR2-3-13, HvR4-3-2, HvR4-4-5, HvR4-4-11, HvR4-4-26, HvR8-1-3, and HvR8-3-21 seeds (Figure 6 a and b), and 400 grains of the same lines in the same order, except that the first row was sown with original wild-type line and the final row was sown with 1000 grains of HvR8-1-3, the densities of seeds were too low to generate high yields (Figure 6 c and d). Therefore, we estimate that a density of 500 seedlings per 4.5 m^2 is adequate for maximum yield.

As shown in Table 2 (a), when we sowed 1000 seeds of HvR4-3-2 in the ridge, the yield of spike weight in 2013 at a distance of 0.33–3.33 m inside the southern part of the ridge, was 11.4, 9.8 and 10.0 t/ha in 1 m each, respectively, corresponding to a seed yield of 9.1, 7.8, 8.0 t/ha, respectively. The average of yield of seed weight was 8.3 t/ha. The growth pattern before harvest is presented in Figure 7 (a). In the corresponding test of HvR8-1-3 as shown in Table 2 (b), the average of yield of seed weight was 3.9 t/ha as shown in Table 2 (b), and the growth pattern before harvest is presented in Figure 7 (b).

DISCUSSION

In the present study, we developed an efficient isolation method for high-yielding PQ-resistant mutant lines. The first isolation step included 24 candidates, from which we finally selected 5 lines of high-yielding PQ-resistant mutant lines. However, our study lacked a control yield test, and we performed a dot-planting yield test by using the average of 10 plants. The 5 PQ-resistant lines—HvR2-3-13, HvR4-3-2, HvR4-4-11, HvR8-1-3, and HvR8-3-21—showed very high yields—up to approximately 2-fold higher—than did those of the remaining 2 lines—HvR4-3-5 and HvR4-4-26. The phenotypes of lines HvR4-3-5 and HvR4-4-26 showed low resistance, as measured by the formation of radicle lateral roots after treatment with 4 μM PQ, and also high rates of fungal infection. Therefore, these 2 mutant lines were appropriate controls for evaluating the performance of the 5 high-yielding mutants. Lines HvR8-1-3 and HvR8-3-21 showed intermediate resistance to PQ; further, HvR8-1-3 showed poor fungal resistance. On the basis of our results, the most suitable high-yielding lines are HvR2-3-13, HvR4-3-2, and HvR4-4-11, which showed high PQ-resistant phenotypes.

HvR2-3-13 **HvR4-3-2** **Original WT**

From left side, HvR2-3-13, HvR4-3-2 and the original wild-type (out side). Seeds of 200, 1,000 and 1,360 (old stock) grains per 4.5 m^2, respectively, were sown. In case of the original wild-type the sowing of seeds was performed in January 2013.

Figure 7 (a). Photograph of HvR4-3-2 before harvest on July 2013.

Nanbuwheat **HvR8-1-3** **HvR8-3-26**

From left side, nanbu wheat, HvR8-1-3 and HvR8-3-21. Seeds of 1,000 grains and 400 grains per 4.5 m^2, respectively, were sown.

Figure 7 (b). Photograph of HvR8-1-3 before harvest on July 2013.

We further evaluated the characteristics of these high-yielding mutant lines by conducting field tests, using different seed-sowing densities. A high seed-sowing density significantly reduces the formation of tillers, which represent a stock supply of nutrients for the formation of fruitful spikes with seeds. Therefore, control of tiller formation is a key factor for control of seed yield. The 5 isolated high-yielding mutants have the capacity for multiple tiller formation, and therefore for supporting high seed yield.

HvR2-3-13, HvR4-3-2, and HvR4-4-11, which showed high PQ-resistant phenotypes and generated high yields, have the capacity to detoxify ROS—including $^1O_2$—during the

processes of photosynthesis; these high PQ-resistant mutants represent valuable tools for further analyzing energy management during the photosynthetic process. However, our study lacked data from a comparable wild-type line, and therefore studies to confirm our results are required.

As shown in Figure 7 (a), HvR4-3-2 showed very good growth in the southern part of the ridge, which received summer monsoon wind. Under such conditions, the yield of seed weight with only basic fertilizer was 8.3 t/ha as presented in Table 2 (a). However, further studies to confirm this finding are required. As shown in Figure 7 (b), HvR8-1-3 showed relatively good growth and the yield of seed weight was 3.9 t/ha as presented in Table 2 (b). Although HvR4-3-2 showed high PQ-resistant phenotype, HvR8-1-3 showed low response. High PQ-resistant phenotype has need for high yielding characteristics. The yield of original wild-type is reported to be 4 t/ha in Japan.

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