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# Ethephon- and Jasmonate-Elicited Pathogenesis-Related Ribonucleases in Cultured Ginseng Cells

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**Abstract**—Cultured ginseng cells (*Panax ginseng* C.A. Mey strain R-1) produce two proteins exhibiting RNase activity (Pg1 and Pg2), which, on the basis of their amino acid sequences, have been earlier referred to intracellular pathogenesis-related proteins. An immunoenzyme technique for estimation of these proteins was developed. A close correlation was found between the content of these proteins and the RNase activity of the cultured cells. Ethephon and jasmonic acid activated the RNase activity, ethephon being more efficient. Salicylic acid did not activate Pg1 and Pg2; high concentrations of salicylic acid suppressed the RNase activity of the culture. The protein kinase inhibitor, H-7, reduced the content and activity of RNases both in the presence and absence of ethephon. The results obtained permit a suggestion that ethylene and jasmonic acid signaling pathways, which include protein phosphorylation, are involved in the induction of PR-10 proteins.

*Key words: Panax ginseng - ribonucleases - PR-10 proteins - ethylene - methyl jasmonate - H-7 inhibitor of kinases*

## INTRODUCTION

Studies of the RNase activity of ginseng cell cultures were initiated by one of the authors (L.I.F.) early in the 1990s. These studies showed that all cultures, independently of their origin and the explant source, produced great amounts of RNases. Our further investigations were performed with strain R-1 (BCKK-BP 38), one of the most efficient producers of RNases; it grows at a high rate and is best studied [1].

Strain R-1 produces two RNases, Pg1 and Pg2, with mol wts of 16470 and 16400 D, respectively. In RNA, they split 3'-phosphodiester bonds formed by all nucleotides, except for cytidine, and produce nucleoside- and oligonucleoside-3-phosphates [2]. Both enzymes display similar catalytic activity.

The analysis of amino acid sequences of Pg1 and Pg2 showed their high homology to the intracellular PR parsley proteins (PR-10) [2, 3]. Therefore, it was supposed that PR-10 proteins were RNases. This presumption has been widely discussed [4–8], however, it is insufficiently supported by the experimental data [4, 6, 7].

PR-10 proteins belong to a vast family of structurally related polypeptides, which were recently combined into a separate group [9]. These proteins were detected in both dicot and monocot plants. They are synthesized in response to the attack of pathogens, under effect of elicitors, and after wounding [10]. Pollen allergens from birch and food allergens from parsley and apple are similar to these proteins [11]. All these polypeptides have similar mol wts of 16–18 kD, they accumulate in the cytosol and are devoid of signal amino acid sequences. The promoter of the *PR-10a* potato gene directed the expression of the reporter gene in various plant parts in response to the attack of pathogenic fungi, that is, infection induced a response typical of PR-proteins. According to Constabel and Brisson [5], this promoter of the *PR10a* gene was also actively expressed in healthy plants as well, but only in stigma tissues. S-glycoproteins responsible for self-incompatibility are RNases [12].

The regulatory and signal pathways of the synthesis of PR-10 proteins are still obscure. Walter *et al.* [13] showed that dark treatment strongly induced the synthesis of a PR-10 protein in kidney beans. Glutathione and high concentrations of salicylic acid were less efficient. ABA and jasmonic acid activated the genes for PR-10 proteins in lily plants. The deletion analysis of the potato *PR-10a* gene revealed the sequences in the regulatory region of the promoter that could bind the two proteins activating transcription. The activity of one of them was regulated by phosphorylation [10].

Currently, three compounds are considered key regulators of plant defense responses: salicylic acid, jasmonic acid, and ethylene [14]. The objective of this

*Abbreviations*: ACC—1-aminocyclopropane-1-carboxylic acid, DMSO—-dimethyl sulfoxide; H-7—1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; PR-proteins—pathogenesis-related proteins.

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work was to study the effects of these regulators on RNase activity of cultured ginseng cells; to elucidate the mechanism of PR-10 protein activation, as exemplified by Pg1 and Pg2 RNases, and to increase the culture productivity, since RNases are currently actively studied as medicine compounds [15].

## MATERIALS AND METHODS

Ginseng (*Panax ginseng* C.A. Mey, strain R-1, the collection number BCKK-BP 38) cells were cultured as calli on an agar-solidified nutrient medium or in the suspension [1]. Calli were grown on a $W_{4CPA}$ medium [1] containing 0.4 mg/l of 4-chlorophenoxyacetic acid as a growth regulator. Reagents for nutrient media, methyl ether of jasmonic acid, spermidine, and ethephon were purchased from Sigma (United States), H-7 inhibitor (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine) and salicylic acid were obtained from ISN Pharmaceutica (United States).

Ethephon and spermidine were dissolved in distilled water immediately before usage. Methyl ether of jasmonic acid was dissolved in DMSO; H-7 was first dissolved in a small volume of DMSO (one tenth of the total volume of the solution) and then in distilled water. Salicylic acid was added to distilled water and titrated with 5% KOH to pH 5.6. Equivalent amounts of solvents were used in the control treatments. Solutions were filtered through the membrane filter (0.22 μm) and introduced aseptically into flasks with the R-1 suspension.

Cell cultures were maintained in the 100- or 250-ml Erlenmeyer flasks containing 40 or 60 ml of the nutrient medium, respectively, in darkness, at a temperature of 25°C and a relative humidity of 50–70%. The subculturing periods were 30 days for calli and 14 days for suspensions. The inoculum weights were 90–100 mg for calli and 250–300 mg for suspensions.

Growth characteristics were measured in 5–10 replications. Standard errors were estimated using the program package Statistica for Windows 98.

*RNase activity* was assayed spectrophotometrically as described by Abel [16]. High-molecular-weight yeast RNA from ISN was used as a substrate. The amount of the enzyme increasing by a unit the optical density ($D_{260}$) of the supernatant of the reaction mixture (which contained low-molecular-weight products of RNA hydrolysis) for 15 min at 37°C was taken for a unit of enzymatic activity.

*RNase extraction* was performed as described in [2]. The ELISA technique was applied to estimate the content of Pg1 and Pg2 proteins in callus extracts [17].

*Rabbit immunization.* Two rabbits were immunized three times subcutaneously in numerous sites on their backs and intravenously with 0.5 ml of RNase emulsion (0.2 mg/ml) obtained as described in [2] together with 0.5 ml of Freund's complete adjuvant at the intervals of ten days. After 30 days, rabbits were re-immunized three times intravenously with 0.2 ml of protein solution of increasing concentrations (0.2, 0.5, and 1.0 mg/ml, respectively). Blood was collected 15 days after the last immunization; the serum was separated by centrifugation and stored at –4°C.

*Testing the antisera for the presence of anti-RNase antibodies.* Antisera were titrated in polysterene microtiter plates (Dynatech, Switzerland) with preliminary adsorbed protein (0.1 ml, 2 μg/ml of 0.01 M carbonate buffer, 12 h, 4°C). Antisera were serially diluted twofold with 0.01 M phosphate buffer, pH 7.2, containing 0.15% (v/v) Tween-20. Antiserum titers gradually increased, reaching 1/3200 and 1/6400. Pre-immune serum was used in the control treatments. The immunoenzyme analysis was performed as described in [18]. The antibody titer was determined as the last dilution when antiserum absorption is by 0.2 optical units higher than the control value.

*Determination of RNase content in the cell culture by the ELISA technique.* RNases (2 μg/ml of carbonate buffer) were bound to the wells of polysterene microtiter plates at 4°C for 12 h. Unbound antigen was removed. The 0.05-ml aliquots of the RNase solution (10 μg/ml) or cell extracts were added into the wells with serial fourfold dilutions. Then 0.05 ml of anti-RNase serum was added at a dilution of 1 : 400 in phosphate buffer containing Tween-20. Pre-immune serum and antiserum without the inhibitor were used at a dilution of 1 : 800 as the negative and positive control, respectively. The content of RNase in extracts was estimated at the point of 50% inhibition using the calibration curve constructed with initial antigen. The results were processed as described by Kolosova and Blintsov [17]. Each value is a mean from three independent experiments.

## RESULTS AND DISCUSSION

We studied the production of RNases by the R-1 culture over several years. The average value was 2735 unit/g fr wt; the coefficient of variability was equal to 13% (Table 1). However, sometimes the enzyme activity changed substantially during some periods of growth (by three to four times for 2–3 months). These changes were not season-dependent (data not shown).

In order to establish which proteins are responsible for the RNase activity of the culture, we assayed immunologically the amounts of Pg1 and Pg2 proteins [17]. We were the first who produced antibodies against ginseng RNases. Most proteins of this group are strong allergens and exhibit high toxicity. This hampers their investigations as antigens.

The correlation analysis showed close correlation between RNase activity and the content of Pg1 and Pg2 proteins (Table 2). Therefore, we can conclude that RNase activity in cells is mainly determined by the presence of Pg1 and Pg2 proteins.

**Table 1.** RNase activity in ginseng calli, strain R-1, during long-term culturing

| Year | Activity, unit/g fr wt |
|---|---|
| 1996 | 2023 ± 406 |
| 1997 | 3975 ± 410 |
| 1998 | 3107 ± 377 |
| 1999 | 2309 ± 1054 |
| 2000 | 2264 ± 278 |
| Average activity | 2735 ± 358 |
| Variability coefficient | 13% |

**Table 2.** The correlation between RNase activity and the content of Pg1 and Pg2 proteins in ginseng calli

| No. exp. | Variation limits of RNase activity, unit/g fr wt | Variation limits of ribonuclease Pg1 + Pg2 content, μg/g fr wt | *r* |
|---|---|---|---|
| 1 | 1070–3200 | 18–47 | 0.95 |
| 2 | 1730–3470 | 30–58 | 0.71 |
| 3 | 2210–3180 | 30–76 | 0.87 |
| 4 | 1300–3000 | 19–23 | 0.62 |
| 5 | 2380–3540 | 37–98 | 0.95 |

Note: Experiments were performed every three month with five assays in each experiment.

When cells were grown in the presence of ethephon, which is known to be spontaneously destroyed in nutrient media with the release of ethylene [19], we did not observe any significant increase in the culture fresh weight, but recorded a distinct enhancement of RNase activity (at ethephon concentrations of 1 and 10 μM) (Table 3). Ethephon increased the content of RNases and culture productivity as calculated per flask. The highest production of Pg1 and Pg2 proteins was 0.611 mg/flask, i.e., 10.2 mg/l. This value is relatively high and exceeds, for example, RNase production by a specially constructed yeast expressional system, which produced only 0.5–10 mg RNase/l [20].

RNase activity in the R-1 culture increased after 24 h and continued to go up later (Fig. 1, curve *1*). Cycloheximide, the inhibitor of protein synthesis, prevented this rise in the RNase activity (Fig. 1, curve *3*), indicating that it was due to the *de novo* synthesis of Pg proteins. Cobalt chloride, the inhibitor of ethylene synthesis [21], also interfered with the increase in RNase activity (Fig. 1, curve *2*).

Some additional arguments for the involvement of ethylene in the control of RNase content in ginseng cells were obtained in experiments with spermidine, which, like other polyamines, suppresses the synthesis of ethylene in plant tissues and protoplasts by preventing S-adonosylmethionine to ACC conversion leading to ethylene formation [22]. Spermidine (0.01 mM) inhibited RNase activity in the ginseng suspension by three times (Fig. 2). This indicates that endogenous ethylene may be involved in the RNase induction.

Protein phosphorylation is known as a step in the ethylene-dependent development of plant defense responses [23]. The H-7 inhibitor of protein kinases, which is widely used for the elucidation of the protein kinase role in the ethylene signal transduction [24], reduced the content of Pg1 + Pg2 proteins and RNase activity in both the presence and absence of ethephon (Table 4).

Our repeated experiments demonstrated that salicylic acid did not induce Pg1 and Pg2 proteins in ginseng calli. The addition of salicylic acid to the ginseng cell culture did not increase its RNase activity, and the high salicylic acid concentration (1 mM) suppressed this activity more than twofold (Table 5). Since this salicylic acid concentration substantially retarded cell growth, we did not use higher concentrations to avoid unspecific responses related to the general culture suppression. Walter *et al.* [13] reported that common bean leaf treatment with 5 mM salicylic acid activated the expression of the *PR-10* gene. However, this concentration is too high as compared to the typical concentration of endogenous salicylic acid in infected plants (below

**Table 3.** RNase activity, the content of Pg1 and Pg2 proteins, and culture productivity in the R-1 suspension culture in the presence of ethephon

| Ethephon, μM | Cell fr wt, g/flask | RNase activity | | Pg1 + Pg2 protein content | | Productivity, μg/flask |
|---|---|---|---|---|---|---|
| | | unit/g fr wt | % | μg/g fr wt | % | |
| 0 | 10.4 ± 0.6 | 1560 ± 270 | 100 | 23 | 100 | 230 |
| 1 | 13.0 ± 0.8 | 2880 ± 320* | 185 | 37 | 161 | 481 |
| 10 | 13.3 ± 1.0 | 3200 ± 550* | 205 | 47 | 204 | 611 |
| 100 | 12.7 ± 2.7 | 1710 ± 260 | 110 | 32 | 139 | 416 |
| 1000 | 8.9 ± 0.8 | 1070 ± 210 | 68 | 18 | 78 | 162 |

Note: The experiment was run twice with three recordings in each experiment.

* The difference is significant at $P < 0.05$.

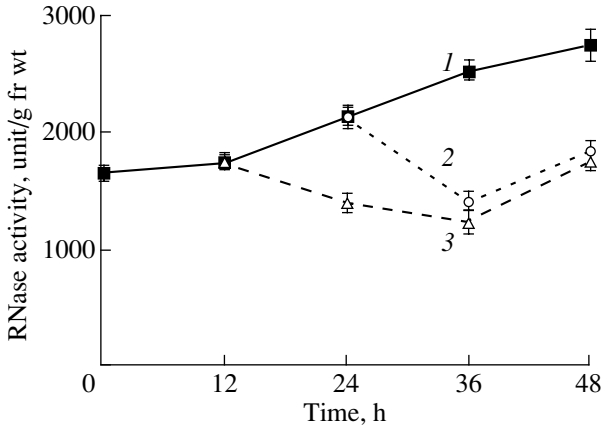


**Fig. 1.** The effects of ethephon, cycloheximide, and cobalt chloride on the RNase activity in the ginseng, strain R-1, suspension culture.

Cell suspension was grown in the medium containing ethephon; cycloheximide was added after 12 h; cobalt chloride was added after 24 h.

(*1*) 10 μM ethephon; (*2*) 10 μM ethephon + 1 mM cobalt chloride; (*3*) 10 μM ethephon + 0. 1 mM cycloheximide.

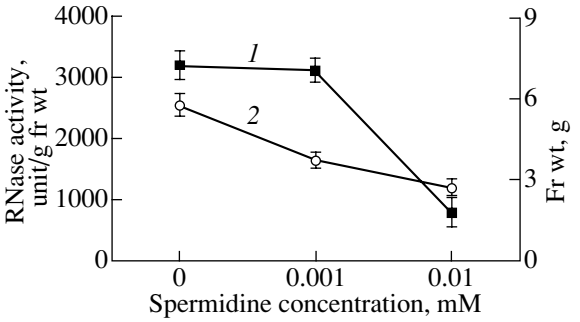


**Fig. 2.** The effect of spermidine on (*1*) RNase activity and (*2*) fresh weight of ginseng cells.

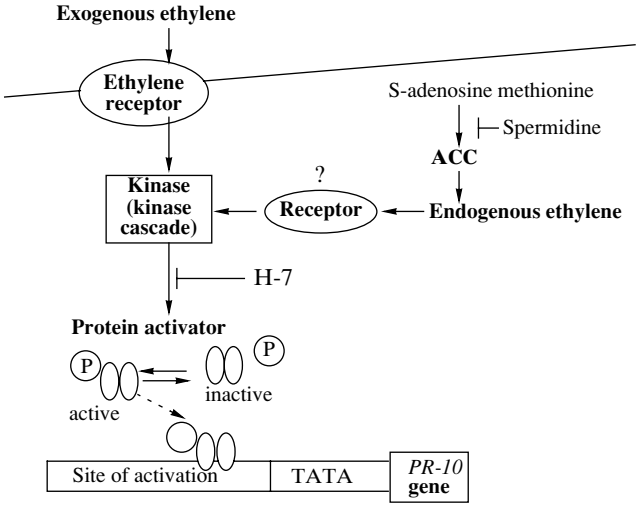


**Fig. 3.** A model of the PR-10 gene activation in ginseng cell culture.

Explanations in the text.

70 μM) [25], and its usage could induce unspecific cell responses.

Methyljasmonate significantly increased the content and activity of ginseng RNases (Table 6). However, its effects were less pronounced than those of ethephon.

One of the first questions arising is why the high RNase level is maintained in cultured ginseng cells. It seems probable that ethylene plays an important role in this process. We could not find any data concerning the factors that stimulate ethylene production in cultured plant cells, excluding those about auxin effects [21, 26]. In our experiments, an increased concentration of 4-chlorophenoxyacetic acid in the cultural medium stimulated RNase activity in the ginseng culture (data not shown).

On the basis of our and literature data, we suggested a model that helps explain the activation of Pg1 and Pg2 protein synthesis (Fig. 3). The protein activator binds to the specific sequence in the promoter of the *PR-10* gene [10]. This protein can function only when it is phosphorylated by the protein kinase, a component of the kinase cascade [27]. Exogenous ethylene activates protein

**Table 4.** RNase activity and the content of Pg1 and Pg2 proteins in the R-1 suspension culture in the presence of ethephon and H-7

| H-7, μM | Ethephon, μM | RNase activity | | Pg1 and Pg2 protein content | |
|---|---|---|---|---|---|
| | | unit/g fr wt | % | μg/g fr wt | % |
| 0 | 0 | 2880 ± 120 | 100 | 58 | 100 |
| | 10 | 3470 ± 111 | 100 | 78 | 100 |
| 5 | 0 | 2448 ± 110 | 85 | 38 | 65 |
| | 10 | 2664 ± 103* | 77 | 44 | 56 |
| 10 | 0 | 1728 ± 170* | 60 | 30 | 52 |
| | 10 | 2160 ± 145* | 62 | 43 | 55 |

Note: The experiment was run twice with three recordings in each series.

* The difference is significant at $P < 0.05$.

**Table 5.** RNase activity and the content of Pg1 and Pg2 proteins in the R-1 suspension culture in the presence of salicylic acid

| Salicylic acid, μM | Cell fr wt, g | RNase activity | | Pg1 and Pg2 protein content | | Productivity, μg/flask |
|---|---|---|---|---|---|---|
| | | unit/g fr wt | % | μg/g fr wt | % | |
| 0 | 5.2 ± 0.5 | 3001 ± 243 | 100 | 23 | 100 | 120 |
| 1 | 3.5 ± 0.4 | 3009 ± 108 | 100 | 22 | 95 | 77 |
| 10 | 3.9 ± 0.3 | 3028 ± 99 | 101 | 22 | 95 | 77 |
| 100 | 3.0 ± 0.4* | 2917 ± 131 | 97 | 19 | 82 | 57 |
| 1000 | 1.3 ± 0.1* | 1255 ± 67 | 42 | 12 | 52 | 16 |

Note: The results of an experiment with five replications.
* The difference is significant at $P < 0.05$.

**Table 6.** RNase activity and the content of Pg1 and Pg2 proteins in the R-1 suspension culture in the presence of methyl jasmonate

| Methyl jasmonate, μM | RNase activity | | Pg1 and Pg2 protein content | |
|---|---|---|---|---|
| | unit/g fr wt | % | μg/g fr wt | % |
| 0 | 2376 ± 114 | 100 | 24 | 100 |
| 1 | 2592 ± 147 | 109 | 37 | 154 |
| 10 | 2880* ± 155* | 121 | 46 | 192 |
| 100 | 3542* ± 204* | 149 | 56 | 233 |

Note: The experiment was run in four replications.
* The difference is significant at $P < 0.05$.

kinase via transmembrane sensor kinase, the first kinase in the kinase cascade [28]. Endogenous ethylene can also induce phosphorylation, but the site of its primary action is unknown. The H-7 inhibitor prevents kinase activity, thus reducing the level of RNases in the cell.

The physiological function of PR-10 proteins is still unclear. Four possibilities were suggested [13]: (1) plant defense responses, (2) mRNA degradation, (3) the reaction of self-incompatibility, and (4) plant senescence. The first function seems preferable. In fact, PR-10 proteins are induced by phytopathogens [29]. RNases exhibit antiviral and antimicrobial activities [30]. According to our data, ethylene and jasmonic acid stimulated PR-10 RNases in ginseng cultures. The involvement of these inducers in plant defense responses during stresses is well known [31]. An enhanced expression of the PR-10 genes in reproductive organs of healthy plants [32] may be explained by the plant attempt to protect these organs against the pathogen attack. However, a genuine physiological role of PR-proteins still remains to be elucidated.

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