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Inhibition of the *JAZ1* gene causes activation of camalexin biosynthesis in *Arabidopsis* callus cultures

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ABSTRACT

Indole alkaloid camalexin has potential medicinal properties such as suppressing the viability of leukemic but not normal cells. Camalexin is not produced in plants and an external factor is required to activate its biosynthesis. In this work, we stimulated camalexin biosynthesis in *Arabidopsis* calli by blocking one of repressors of the jasmonate pathway, the jasmonate ZIM-domain protein 1 (JAZ1) by using amiRNA targeting *JAZ1* gene transcripts. Inhibition of the *JAZ1* gene led to an increase in camalexin content from trace amounts in control culture to 9 μg/g DW in the *jaz1* line without affecting growth. In addition, *JAZ1* silencing enhanced tolerance to cold stress with simultaneous increasing camalexin content up to 30 μg/g DW. Real-time quantitative PCR determination of marker gene expression showed that effects caused by the *JAZ1* silencing might be realized through crosslinking JA, ROS, and abscisic acid signaling pathways. Thus, targeting the distal components of signaling pathways can be suggested as a tool for bioengineering of secondary metabolism, along with standard techniques for targeting biosynthetic genes or genes encoding transcription factors.

1. Introduction

The main specialized metabolites of the plants belonging to the Brassicaceae families are tryptophan derivatives, indolic glucosinolates, and a simple indole alkaloid, camalexin, which plays a key role in the defense of plant against wide spectrum of pathogens (Augustine and Bisht, 2017; Clarke, 2010). Typically, camalexin is found in the plant under pathogen-induced stress as an indicator of the infection (Todesco et al., 2010; Zhang et al., 2014). Camalexin has a powerful antimicrobial activity against bacterial and fungal plant pathogens (Glawischnig et al., 2004) and also against the human protozoan pathogen, *Trypanosoma cruzi* (Mezencev et al., 2009). It has also been shown that camalexin exhibits high selective cytotoxic activity against the human breast cancer cell line SKBr3 (Moody et al., 1997) and induces apoptosis in T-leukemia Jurkat cells (Mezencev et al., 2011). However, camalexin and its derivatives demonstrate minimal cytotoxic effects on the non-tumor cells (Chripkova et al., 2016; Pilatova et al., 2013; Yang et al., 2018). Due to its multitude of biological activities, regulation of the camalexin biosynthesis is an active research area. Therefore, the main subject of most of research is the model plant *Arabidopsis thaliana* from the family Brassicaceae (Augustine and Bisht, 2017). It is especially convenient for such studies to use cell cultures, because under certain conditions other metabolites, except for indole derivatives of tryptophan, are not synthesized in them (Bulgakov et al., 2016).

The upstream enzymes of camalexin biosynthesis, CYP79B2/B3, are strongly regulated by the transcription factors (TFs) MYB34, MYB51, and MYB122 (Frerigmann et al., 2015). If MYB51 is regulated by salicylic acid (SA) and ethylene (ET) signaling, MYB34 and MYB122 are regulated by jasmonic acid (JA) (Frerigmann and Gigolashvili, 2014). The stimulatory effect of JA on secondary metabolism is well-known (De Geyter et al., 2012; Zhou and Memelink, 2016). Jasmonate ZIM-domain proteins (JAZs) play a key role in the regulation the JA signaling pathway (Fernández-Calvo et al., 2011). The main function of JAZ proteins is negative modulation of jasmonate-regulated defense via repression of activity of several TFs, including the basic helix-loop-helix (bHLH) family. JAZs repressed both positive regulation by MYC2 and negative regulation by the JAMs, which are an antagonist to MYC2 (Sasaki-Sekimoto et al., 2013). It was shown that inhibition of JAZ1 could upregulate anthocyanin biosynthesis through distribution of the bHLH–MYB interactions between PAP1-TT8 and GL1-GL3 (Song et al.,

Abbreviations: TFs, Transcription factors; JA –, jasmonic acid; amiRNA, artificial microRNA; RNAi, RNA interference.

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2011; Qi et al., 2011).

13 *JAZ* genes are found in Arabidopsis, and their role in various spatiotemporal structures and functions, as well as their redundancy is not fully understood. One of the proposed phylogenetic classifications is division into groups: group I (*JAZ1/2/5/6*), II (*JAZ10*), III (*JAZ11/12*), IV (*JAZ3/4/9*), and V (*JAZ7/8/13*) and the development of mutant plants in which the whole group is inhibited at once (Guo et al., 2018). Another way to study this system is the multiple inhibition of a large number of *JAZ* genes, for example, mutants *JazQ* (4 genes inhibited), *JazD* (10 genes inhibited), *JazU* (12 genes inhibited) (Liu et al., 2021). The participation of *JAZ* genes in jasmonic acid signaling has been proven, which links them with such parameters as growth and secondary metabolism, or rather, the displacement of plant resources in one direction or the other. Each mutant group carries specificity, moreover, each knockout mutant of *JAZ* gene also has its own characteristics. Simultaneous inactivation of ten *JAZ* genes in *Arabidopsis* plants led to increased indole glucosinolates (IGs) content, which was accompanied by suppression of growth (Guo et al., 2018).

The molecular regulators of the main downstream enzymes of the camalexin biosynthesis, CYP71A12, CYP71A13, and CYP71B15 are also of interest. One of these well- studied regulators is *NAC042*, a member of the NAM, ATAF1/2, and CUC2 (NAC) transcription factor family genes. Expression of *NAC042* is strongly activated by known inducer of camalexin biosynthesis AgNO3. It was shown that NAC042 regulates expression of downstream enzymes (CYP71A12, CYP71A13 and CYP71B15) but not upstream enzymes and it is suggested that expression of *NAC042* might be a downstream event of the reactive oxygen species (ROS) signaling (Saga et al., 2012*)*. WRKY33, member of the large zinc-finger-type WRKY transcription factor family, also plays key roles in the pathogen defense response regulations in plants (Pandey and Somssich, 2009). WRKY33 upregulates camalexin biosynthesis through binding to the promoters of *CYP71B15* and *CYP71A13* (Birkenbihl et al., 2012). Regulation of WRKY33 is carried out through phosphorylation by ROS-induced CPK5/CPK6 and MPK3/MPK6 (Zhou et al., 2020).

Camalexin biosynthesis might not only be regulated by pathogen-induced signaling systems like ROS and JA. Abiotic stresses such as cold and salt treatment significantly increased tryptophan biosynthesis in *Physcomitrella patens*; moreover, tryptophan is the most increased among other amino acids under abscisic acid (ABA) treatment (Arif et al., 2018). This suggests that the ABA signaling pathway and abiotic stress treatment might be involved in camalexin biosynthesis. JA-mediated mechanisms of cold response interact with the ABA-signaling pathway (Cuming, 2019). Besides downregulation of the JA-pathway and cell defense to biotic stress (biosynthesis of specialized metabolites), JAZ1 and JAZ4 repress *CBF-COR* gene expression by interaction with ICE1 to negatively regulate cold tolerance in plants (Hu et al., 2013). In the ABA pathway, the OST1/SnRK2.6-HOS1-ICE1 module controls cold tolerance via the CBF/DREB-dependent cold signaling pathway (Ding et al., 2015). Changes in every echelon of the ABA-signaling pathway are important to understand the plant cell response to any exogenous intervention: receptors (PYR, PYL, RCAR), phosphatases 2 C (PP2C), kinase 2 (SnRK2), and transcription factors (ABI, CBF) (Cuming, 2019). Beside these, other proteins participate in ABA-mediated abiotic stress response: an inducer of CBF expression, ICE1, its inhibitor E3 ubiquitin-protein ligase HOS1 (Liang and Yang, 2015), and ABA-induced the heat stress transcription factors (*HSFs*) family (Baniwal et al., 2004; Udvardi et al., 2007). Besides the ABA-regulon, ROS-signaling is no less an important transductor of the abiotic stresses. Respiratory burst oxidase homolog protein D (RBOHD) plays the key role in this process. Transcription factor ZAT12 plays a central role as negative regulator of the CBF cold response pathway and a positive regulator of the salt tolerance in the cross-talk between ROS- and ABA-dependent abiotic stress responses (Vogel et al., 2004) (Davletova et al., 2005);.

It has been hypothesized that we will be able to activate camalexin biosynthesis through the JA signaling pathway as a cross-talk between ROS and ABA signaling, eliminating the restriction on secondary metabolite biosynthesis. We have chosen miRNA interference for attenuating *JAZ1* since it has recently been described as a new way to regulate secondary metabolism (Bulgakov and Avramenko, 2015) and is considered as indirect method for switching the flow of metabolites between different branches of metabolic pathways (Gou et al., 2011; Yu et al., 2015). In general, manipulations with miRNA and amiRNA are important approaches in the bioengineering of plant adaptions to abiotic and biotic stresses (Paul et al., 2015; Gupta et al., 2017). The main goal of the present study was to evaluate the influence of *JAZ1* gene silencing on specialized metabolism of tryptophan derivatives. We planned not only to fix the new status of the tryptophan metabolism, but also tried to elucidate, as far as possible, the molecular machinery of the JAZ1 cross-link between the JA, ROS, and ABA signaling systems of plant cells.

2. Materials and methods

2.1. Creation of amiRNA to target AtJAZ1

For targeting of the *A. thaliana AtJAZ1* gene (AT1G19180, TAIR) the mature amiRNA sequence (5′-UCGGCUGACGUGAGUUGCCUU-3′) was developed *in silico* using the P-SAMS program (Fahlgren et al., 2015). Expression of this amiRNA based on the unique nucleotide region should not interfere with any other mRNA of the *Arabidopsis*. The creation of amiRNAs was carried out by the method of site-specific mutagenesis (Schwab et al., 2006). Primers pairs containing the mature amiRNA sequence and the cloning sites were designed using WMD3 program (http://wmd3.weigelworld.org) and designated correspondingly to Schwab's protocol and are listed in supplementary table 1. Obtained PCR product and the pSAT6 cassette vector containing the double promoter of the 35S rRNA gene of the cauliflower mosaic virus and the terminator of the 35S rRNA gene were digested with *BamH*I and *Hind*III enzymes (NEB, England) at 5′-GGATCC-3′ and 5′-AAGCTT-3′, respectively and ligated according to protocol of manufacturing (NEB, England). The resulting construction - pSAT6-amiRNA was sequenced to check integrity. Next, the 35S-amiRNA-terminator was transferred from the pSAT6 vector using the PI-PspI (NEB, England) enzyme into the pPZP-RCS2 expression binary vector containing the kanamycin resistance gene (*nptII*) (Tzfira et al., 2005). The resulting binary vector was transferred into *Agrobacterium tumefaciens* strain EHA105 cells (Hood et al., 1993) by electroporation (GenePulser, BioRad, USA). The obtained recombinant strain of *Agrobacterium* was used for genetic transformation of *Arabidopsis* suspension cell cultures.

2.2. Plant cell material

The control, untransformed callus culture of *A. thaliana* Col-0, designated in this work as WT, was obtained previously (Bulgakov et al., 2016). WT culture was transformed using *A. tumefaciens* EHA105 strain harboring a pPZP vector with amiRNA construction as described (Bulgakov et al., 2010). Transgenic culture, designated as *jaz1*, was obtained after three months of selection on kanamycin. Callus cultures were cultivated in tubes contain 15 ml of solid MS media (Murashige and Skoog, 1962), supplemented with 0.5 mg/L of 6-benzylaminopurine and 0.5 mg/L of 2,4-dichlorophenoxyacetic acid (supplementary table 2), in dark, at 24 °C, with 28-day subculture intervals. To investigate the action of MeJ on camalexin biosynthesis, in cultural medium was added 5 and 50 μ MeJ (Sigma, USA) and cultures were grown under standard conditions for one month.

The effect of cold stress on growth of the callus lines was investigated using following conditions: 24 °C for one week, then 16 °C for three weeks in an environmental chamber (Smolensk, SKTB STC, Russia). The salt stress conditions were created by supplementing the growth media with NaCl (Panreac, USA) in the concentrations 60 mM. The inoculum biomass for stress treatment was 200 mg (20 g/L) (each callus was

weighted using an electronic balance). For analysis of growth of the cultures under stress conditions was used growth index (GI), calculated using the formula:

$$GI=(W_b-W_a)/W_a,$$

where GI, growth index; W_b, weight (g/L) of fresh biomass on the end point per 1 liter of media; W_a, weight of fresh biomass of the inoculum (20 g/L).

For analysis of productivity of the cultures was used followed formula:

$$Productivity=C*W$$

where C, content (mg/g DW) of metabolite per gram of dry biomass; W, weight (g DW/L) of dry biomass on the end point for 1 liter of media.

2.3. PCR conditions

To prove successfulness of transformation, cell culture DNA was extracted as described previously (Veremeichik et al., 2019) and the presence of T-DNA was verified by PCR with primer pairs specific to the *nptII* gene (nptD/nptR) and amiRNA expression cassette (35 S PROM/35 S TERM), primers listed in Supplementary table 1. To perform PCR, we used ScreenMix (Evrogen, Russia) set according to the manufacturer's instructions. For each reaction we set a negative control sample (without sample DNA) and a positive control sample (with the addition of a plasmid used for plant cell transformation). Conditions of PCR reactions were as follows for 30 cycles: denaturation, 96 °C for 30 s, annealing, 55 °C for 30 s, and elongation,72 °C for 60 s. All products were analyzed by gel electrophoresis in 1% agarose with addition of ethidium bromide.

2.4. RNA extraction and cDNA synthesis

Total RNA isolation from calli tissue was performed by the LiCl precipitation method, as shown previously (Shkryl et al., 2016). Resulting total RNA was incubated with 1 U of RNase-free DNase I (Jena Bioscience, Germany) according to the manufacturer's manual. Then DNase enzyme was removed with BlueSorb (Sileks M, Russia) and RNA was precipitated by 96% ethanol. The pellet was cleaned with cold 70% ethanol, and resuspended in 30 μl of RNase-free water. RNA integrity check was carried out by gel electrophoresis in 1% agarose. Purity and concentration checkswere performed by a BioSpec Nano spectrophotometer (Shimadzu, Japan). Samples with 260/280 ratio greater than 1.8 and 260/280 ratio greater than 1.5 were used for subsequent cDNA synthesis and qPCR.

To obtain cDNA from RNA samples, we used an MMLV RT kit (Evrogen, Russia) according to the manufacturer's protocol. A mixture of 2.5 μg total RNA sample dissolved in 5 μl Nuclease-Free water and 0.5 ng of oligo-dT primer was heated up to 72 °C for 5 min, then cooled on ice. Then a solution of 1x MMLV buffer, DTT, dNTP and 100 U of MMLV reverse transcriptase was added to sample. The reaction was performed in a C1000 Thermal Cycler (BioRad, USA) during 1 h at 36 °C, followed by 10 min at 72 °C. Resulting cDNA was diluted five times. Absence of a DNA contamination was checked by PCR with primer pair (ActF+ActR), that spans an intron (443 bp) of the *Actin* gene. PCR conditions were as previously written above. Primers pair to the *Actin* gene are listed in Supplementary table 1.

2.5. qPCR

To conduct quantitative real-time PCR (qPCR), we utilized CFX96 (BioRad, USA) and 2.5x SYBR Green PCR Master Mix (Evrogen, Russia) with accordance to the manufacturer's protocol, with a total volume of 12.5 μl, containing 250 nM of each primer, 1 μl of the diluted cDNA sample and 2 mM $MgCl_2$. For each gene expression analysis, we used three biological replicates and three technical replicates for each biological replicate. All primers used for qPCR were designed to match the annealing temperature of 60 °C and are listed in Supplemental table 1. PCR conditions were as follows: denaturation 98 °C for 30′', annealing 60 °C 30′', elongation 72 °C for 30′'; 40 cycles. BioRad CFX Manager software (Version 3.1; Bio-Rad Laboratories Inc., Hercules, CA) was utilized to analyze qPCR expression data and to conduct melting curve analysis. Melting curve analysis and electrophoresis in a 1% agarose with ethidium bromide were carried out to ensure absence of non-specific PCR products.

To determine the most appropriate reference genes for real-time PCR for a given culture and growth conditions we used the approach described by Xie et al. (2012) which utilizes the simultaneous use of several algorithms such as geNorm, Normfinder, BestKeeper, and the comparative Delta-Ct method (https://www.heartcure.com.au/reffinder/). For validations we used previously described qPCR reference genes for *Arabidopsis* (Czechowski et al., 2005). According to our results (Supplemental Fig. 1), because of the lowest geometric mean the comprehensive rating of RefFinder assumes the use of two reference genes – *AtSAND* (AT2G28390) and *AtPP2A* (AT1G13320). Thus, both genes were used to normalize qPCR results. Heat-maps were built with http://www.heatmapper.ca/tools.

2.6. Sample preparation for LC

All chemicals used for HPLC were of analytical grade. The analytical standards camalexin (Sigma-Aldrich, St. Louis, MO, USA) and glucobrassicin (PhytoLab GmbH & Co.KG, Vestenbergsgreuth, Germany) were purchased. Samples of calli tissue were dried under vacuum conditions at room temperature without light and powdered with a mortar and pestle. 50 mg of powdered samples were extracted with 1 ml of 70% (v/v) aqueous methanol. This mixture was sonicated for 20 min, incubated overnight at room temperature, and centrifuged for 10 min at 15,000 rpm. The residue was re-extracted in the same manner. The extracts were combined and purified with a 0.45 μm nylon membrane (Millipore, Bedford, MA, USA). Aliquots of 2–5 μl were injected into an HPLC apparatus for subsequent analysis.

2.7. LC-UV-MS analysis

LC-UV-MS investigations were carried out using a 1260 Infinity analytical HPLC system (Agilent Technologies, Santa Clara, California, USA) coupled with an ion trap mass spectrometer Bruker HCT ultra PTM Discovery System (Bruker Daltonik GmbH, Bremen, Germany). Separation of the crude extracts was performed on a Zorbax C18 column (150 mm, 2.1-mm i.d., 3.5-mm part size, Agilent Technologies, USA) with the flow rate of 0.2 ml min^{-1} at 40 °C. The mobile phases consisted of aqueous acetic acid (0.1% (v/v); A) and acetonitrile (B). The following elution gradient was used: 0–5 min 0% B; 40 min 50% B; 50 min 100% B; and then B for 60 min. UV spectra were recorded in the λ range from 200 nm to 400 nm using a diode array detector. The chromatograms were acquired at a wavelength of 320 nm for camalexin definition. The quantification of camalexin was performed as we described previously (Bulgakov et al., 2016). The ESI-MS analyzes for the determination of intact glucosinolates in crude extracts of calli tissue were performed in negative ion detection with simultaneously supervising a positive signal with *m/z* 201 to confirm availability of camalexin in our samples. The full scan range of *m/z* was 100–800, the drying gas (N_2) flow rate was 8.0 l min^{-1}, the nebulizer gas (N_2) pressure was 175 kPa, the drying gas temperature was 325 °C. If necessary, the individual ions of interest with the composition $[M-H]^-$ (or $[M+H]^+$ for camalexin) were selected for subsequent MS^2 fragmentation. The glucosinolate identification was based on those published recently (Bulgakov et al., 2016). The single ion monitoring (SIM) mode was used for registration of the abundance of the deprotonated ions of intact glucosinolates for its quantification as presented earlier by Cataldi et al. (2007) and Lelario et al. (2012). All samples were analyzed alternately with standard

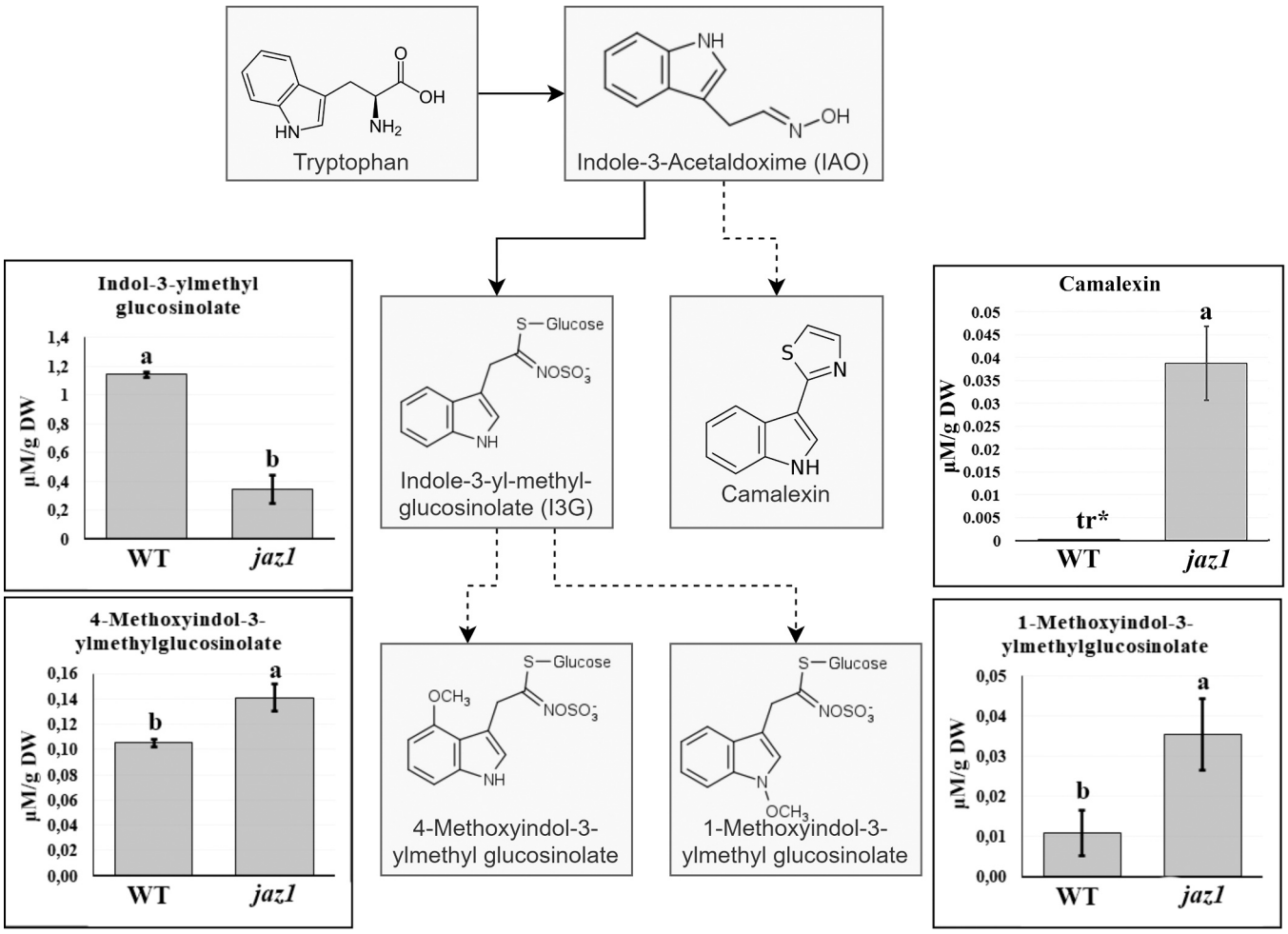

Fig. 1. Impact of the *JAZ1* inhibition on the JA-regulated biosynthesis of tryptophan derivatives. Pathway and concentration (µM/mg DW, dry weight) of the indolic glucosinolates and camalexin in WT and *jaz1* cultures under control conditions. Solid lines represent one reaction, the dotted line represents several reactions.

mixtures of camalexin and glucobrassicin. The quantification study of indole glucosinolates was performed by the external standard calibration method using glucobrassicin as the analytical standard. Camalexin and glucosinolate concentrations are expressed as µmol g^{-1} dry weight (DW).

2.8. Statistics

All values are expressed as the mean ± SE using Statistica 10.0 (StatSoft Inc., USA). A difference of $P < 0.05$ was considered significant. Two independent categories were compared using the Student's *t*-test, while comparisons among multiple groups were achieved by ANOVA followed by a multiple comparison protocol. Fisher's protected least significant difference (PLSD) *post-hoc* test was employed for the intergroup comparison.

3. Results

3.1. Obtaining of the genetic constructs and transgenic cell cultures

A cassette vector containing amiRNA to the *AtJAZ1* gene was generated in silico in the P-SAM program, resulting in a mature RNA sequence (5′-UCGGCUGACGUGAGUUGCCUU-3′); this sequence was then obtained in vitro accordingly to first Materials and Methods chapter (Supplemental Fig. 2). The genetic construct 35 S Promotor-amiRNA-35S Terminator was transferred into binary vector pPZP (Supplemental Fig. 2). The binary vector was then transferred into *A. tumefasciense* strain EHA105 by electroporation.

An *Arabidopsis* control cell culture (designated as WT) was obtained previously from leaves of an *Arabidopsis* Col-0 plant (Bulgakov et al., 2012). In the present study, the WT cell line was transformed via *Agrobacterium*-mediated transformation using a pPZP binary vector with amiRNA to *AtJAZ1*. After selection on kanamycin-supplemented medium (50 mg/L) during three months, a kanamycin-resistant callus line was obtained and designated as *jaz1*. Both cell cultures had a similar morphology, with a yellowish tint and a friable consistency (Supplemental Fig. 3).

To confirm a successful gene transfer and *nptII* gene expression, DNA and cDNA samples were prepared from normal and transformed cell lines. To verify that the cDNA and DNA was native and cDNA was free of genomic DNA RT-PCR amplification with primers corresponding to the *AtActin* gene was performed (Supplemental Fig. 4A). Gene-specific RT-PCR analysis of the DNA samples with primer pairs to an expression cassette showed that gene transfer was successful (Supplemental Fig. 4B). To verify the *nptII* expression, PCR with primer pairs to the *nptII* gene was performed (Supplemental Fig. 4C).

3.2. Shifting in glucosinolates biosynthesis and increasing of camalexin concentration

Because the main target for JAZ is the JA-biosynthetic pathway (Pauwels and Goossens, 2011), we first checked composition of the specialized metabolites in the control and transgenic cell lines using HPLC-MS-UV methods. The transformed culture had different content of

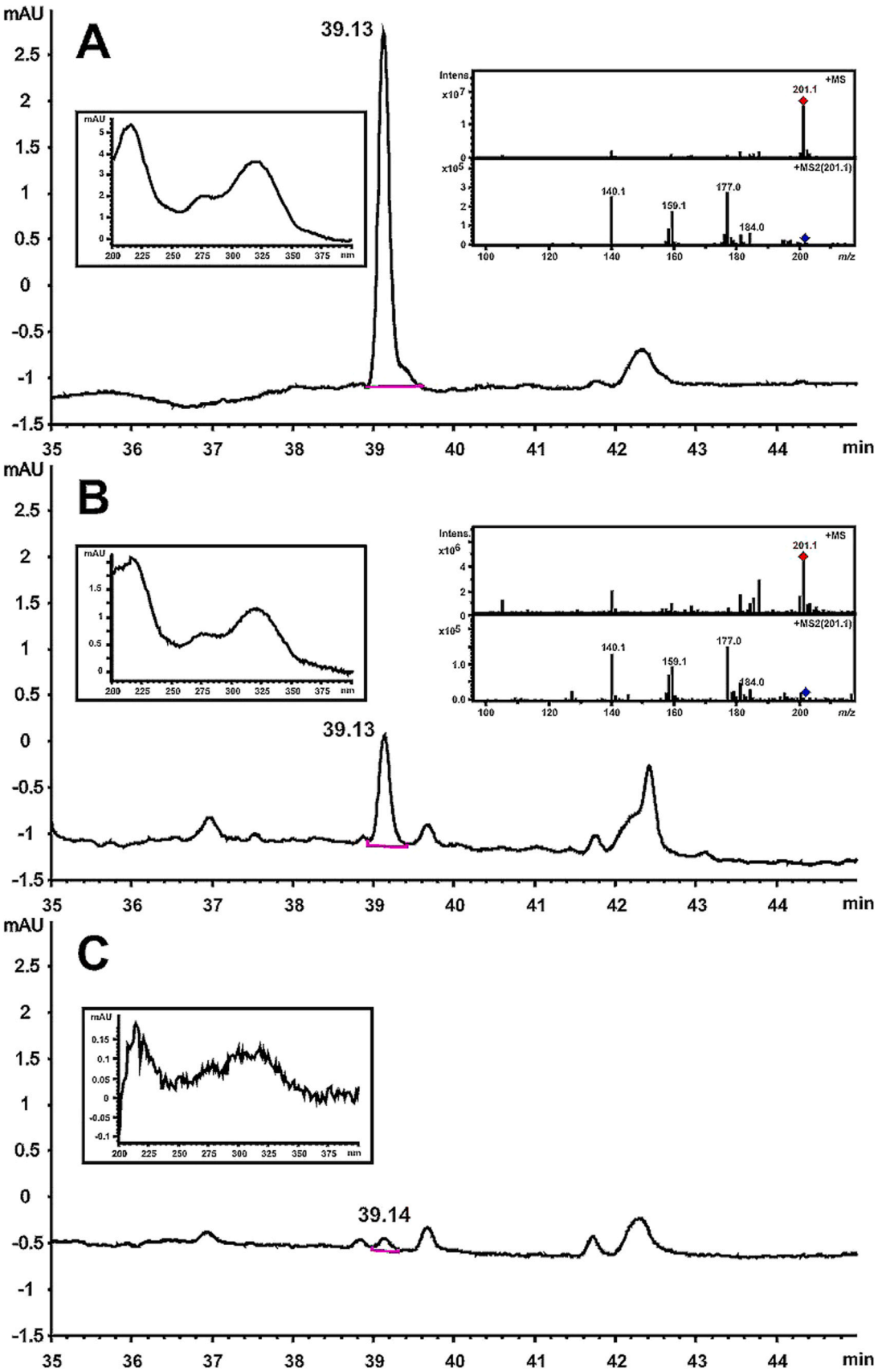

Fig. 2. HPLC-UV-MS determination of camalexin in crude extracts of calli tissue *jaz1*, consists of chromatographic profile of standard solution of camalexin with concentration 0.01 μmol ml-1 (A), in crude extracts of *jaz1* culture (B) and WT culture (C). Chromatograms were recorded at 320 nm, all spectral data were acquired from the top of the peaks of interest, UV spectra (left side) were obtained using diode array detector, full-scan spectra (right side, up) and product ion spectra of [M+H]+ precursor ions at *m/z* 201 (right side, down) were performed by LC-ESI-MS in positive ion mode.

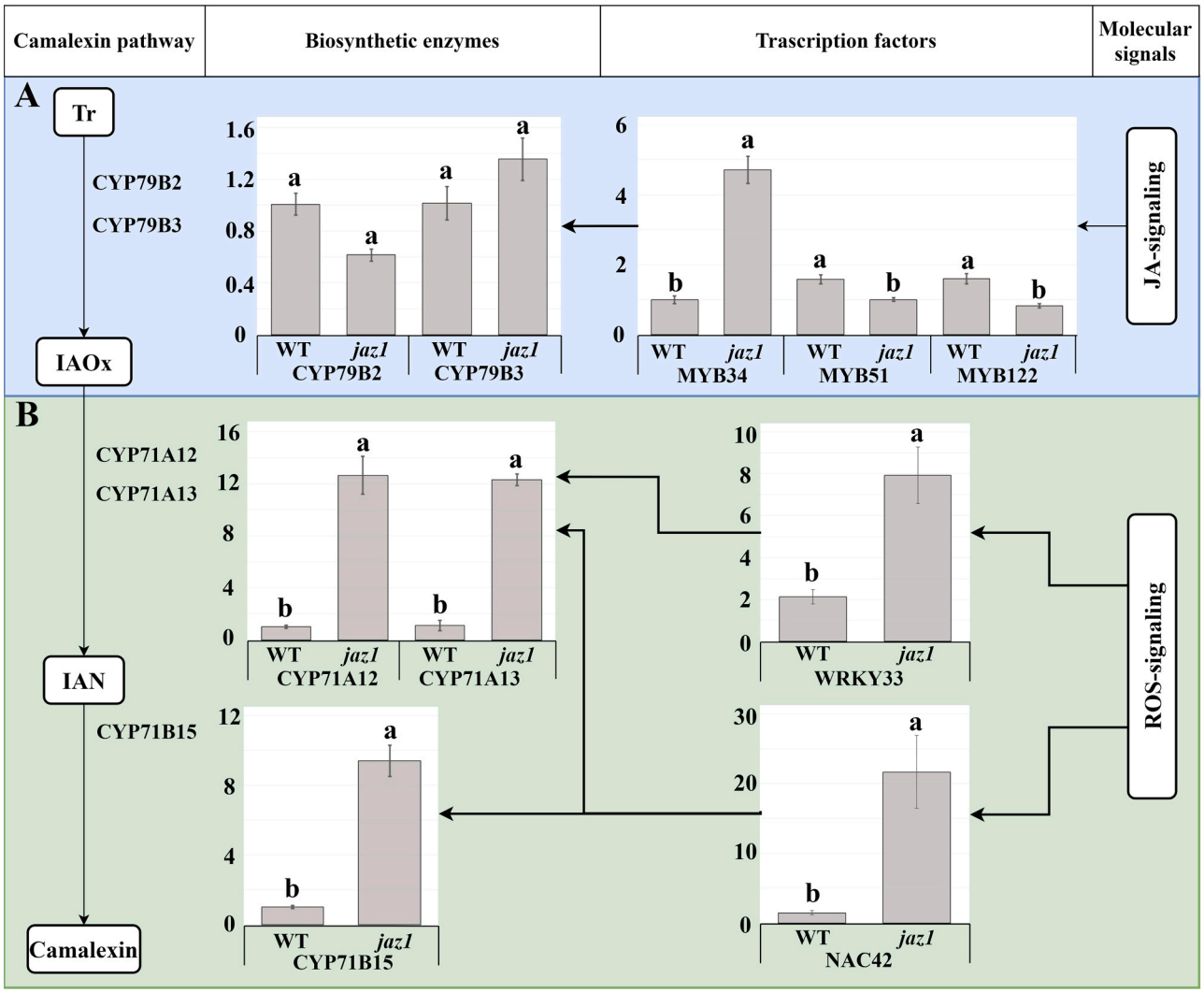

Fig. 3. Camalexin biosynthetic pathway and expression data analysis of key enzymes genes and transcription factors. The Y-axis of all bar-graphs are normalized fold-change in relative expression for WT and *jaz1* cultures. A – Upstream pathway, Tr – tryptophan, IAOx - Indole-3-acetaldoxime, MYB34/51/122 are main regulative TFs of the first steps enzymes, CYP79B2 and CYP79B3; B – downstream pathway, IAN – indol-3-acetonitrile; NAC42 and WRKY33 are TFs that control the last two biosynthetic steps catalyzed by following enzymes CYP71A12, CYP71A13 and CYP71B15. Data are presented as the mean ± SEM. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$, Fisher's LSD). (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

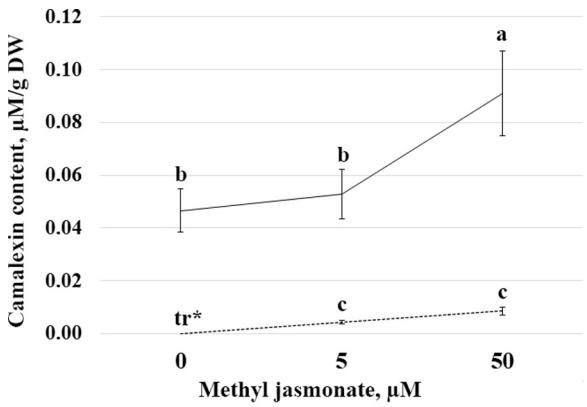

Fig. 4. Effect of methyl jasmonate (5 and 50 µM) on the camalexin content (µM/g DW) in WT and *jaz1*. Data are presented as the mean ± SEM. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$, Fisher's LSD).

the main secondary metabolites of *Arabidopsis*, glucosinolates and camalexin, compared to the control culture (Fig. 1). The concentration of indol-3-ylmethyl glucosinolate, which is a precursor of more active methoxy forms of glucosinolates, was decreased 3.3 times in the transgenic culture compared to the control. The concentration of the methoxy forms, 1-methoxyindol-3-ylmethylglucosinolate and 4-methoxyindol-3-ylmethylglucosinolate, on the contrary, increased in the transgenic culture 2.8 and 1.2 times as compared to the control culture, respectively. The main phytoalexin of *Arabidopsis*, camalexin, was absent in the control culture; however, in the transgenic culture, its concentration was 0.045 µM/mg DW (Fig. 1). The identification of camalexin in crude extracts of *jaz1* calli was performed using the comparison of chromatographic and mass-spectrometric data, such as retention time, UV profile, and MS2 fragmentation patterns of [M+H]+ precursor ions at *m/z* 201 of a standard solution of camalexin (Fig. 2A) and our samples (Fig. 2B and C).

The main transcription factors regulating the camalexin pathway were different in terms of expression between the control and transgenic cultures. The expression of the *MYB34* gene was 4.7 times higher in the transgenic culture; however, *MYB51* and *MYB122* gene expression in the

control culture were significantly higher (Fig. 3A). These MYBs regulate the expression of the upstream enzymes CYP79B2/B3 (Frerigmann et al., 2015). Expression levels of these enzymes were not significantly changed in the *jaz1* cells (Fig. 3A). In contrast, expression of the downstream enzymes CYP71A12, CYP71A13, and CYP71B15 was dramatically increased in the *jaz1* cells compared to WT (12-, 12-, and 8-fold, respectively) (Fig. 3B). Expression of *WRKY33*, which regulates the expression of the CYP71A12 and CYP71A13, was increased 8-fold; expression of the *NAC42*, which regulates the expression of CYP71A12, CYP71A13 and CYP71B15, was increased more than 20-fold in *jaz1* cells compared to WT (Fig. 3B).

Next, we analyzed the action of MeJA on camalexin biosynthesis in the control and *jaz1* cell lines to confirm that JAZ1-blocking of the secondary metabolism was removed. Adding of 5 and 50 μM of MeJA to the medium led to increasing camalexin concentration, but only just above the detection level for WT. Moreover, there are no significant differences between the effects of 5 and 50 μM MeJA on camalexin concentration in the control WT line. Adding of 5 μM of MeJA slightly increased camalexin concentration in *jaz1* line. At the same time, 50 μM of MeJA increased camalexin concentration in *jaz1* almost 2-fold (Fig. 4).

3.3. The effect of cold and salinity on camalexin production

Next, we analyzed the effect of abiotic stress treatments such as cold and salt on camalexin accumulation and production. Increasing of tryptophan biosynthesis under cold, salt, and ABA treatment (Arif et al., 2018) gives reason to assume that the ABA signaling pathway and abiotic stress treatment might be involved in camalexin biosynthesis. Recently it was shown that JA positively regulates the CBF transcriptional pathway and total cold tolerance (Hu et al., 2017). The relationship between the JA signaling pathway and salt tolerance is also notable, and is down-regulated by JAZ3 (Geng et al., 2013; Valenzuela et al., 2016). Therefore, we hypothesize that silencing of the one of JA repressors, JAZ1, might work to change the tolerance of transgenic cells to cold or salt.

We analyzed growth parameters of the control and *jaz1* cell lines under cold and salinity stress treatments.

A temperature of 16 °C was used to cause cold stress throughout the entire passage, because lower temperatures completely suppress the growth of *Arabidopsis* control calli. Growth parameters of cultures under control conditions did not change (Fig. 5A). Under cold stress conditions (16 °C for 14 days), the growth index of the transgenic culture significantly differed from the control, where the growth of the control culture was inhibited by 46%, while the transgenic culture was inhibited by only 22% (Fig. 5A). Under salt stress (60 mM NaCl for 30 days), significant difference between the control and *jaz1* cell cultures was not found. Growth of the both cultures was inhibited by approximately 36% (Fig. 5A).

We analyzed effect of cold and salinity on the camalexin biosynthesis in the *JAZ1*-inhibited line compared to the WT line (Fig. 4B). In the WT culture, the level of camalexin under cold and salt treatment was significantly higher compared to control conditions; the effect of salinity on camalexin content resulted in a 2-fold higher increase compared to the effect of cold stress. However, camalexin levels in the WT control under stress treatment was not higher than in the *jaz1* line without any treatment (Fig. 5B). We have shown that cold stress increased the camalexin content in the transgenic culture by 1.4-fold, from 0.05 μM up to 0.15 μM. The salt treatment had the same effect (Fig. 5B).

We analyzed camalexin productivity in the WT and *jaz1* cell lines. In the control WT line, camalexin was detected under cold and salt treatments. Taking into account cold-induced growth inhibition, camalexin production was 10 μg/L, which is approximately 35% of camalexin productivity of *jaz1* culture (almost 30 μg/L) without any treatment (Fig. 5C). Growth inhibition of WT by salt was similar to cold treatment. Camalexin content in the WT cultures under salt stress was increased

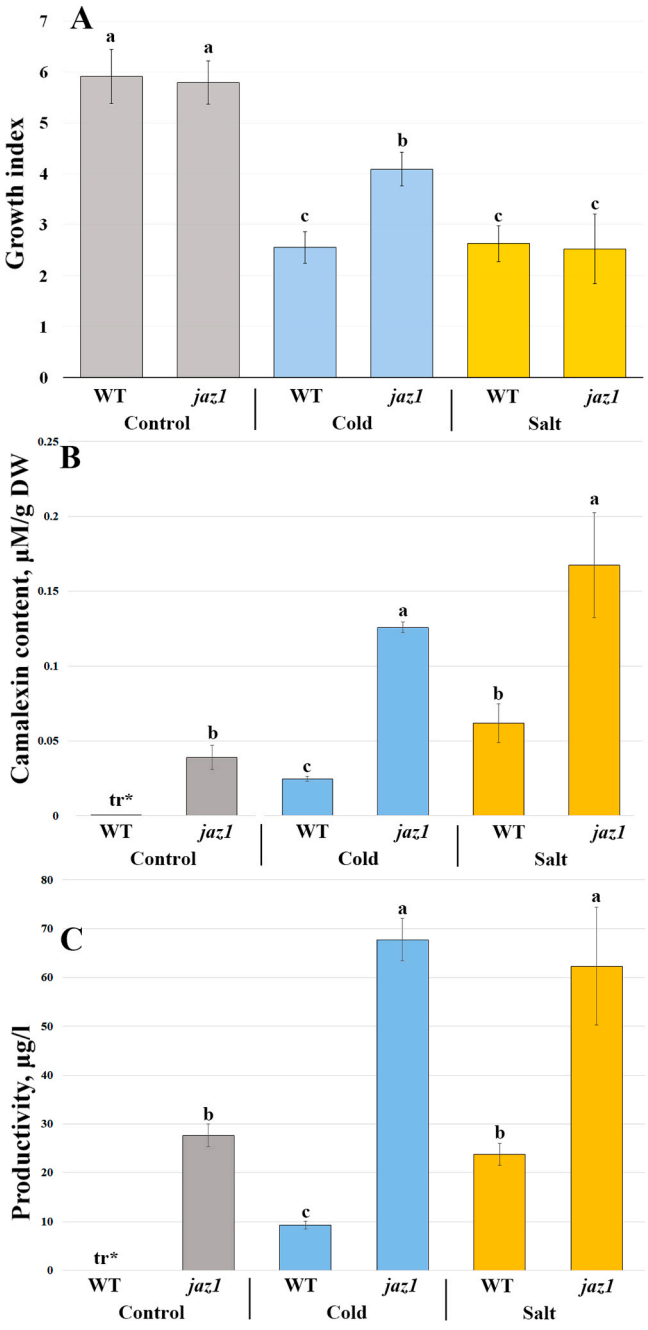

Fig. 5. Growth and camalexin concentration of WT and *jaz1* culture under stress conditions. Growth index (A) and camalexin concentration (B) and were measured for WT (non-transgenic) and *jaz1* (*AtJAZ1*-inhibited) cultures, growing under control conditions (grey bars), 24 °C for 3 weeks; cold (blue bars), 24 °C for 1 week and 16 °C for 2 weeks; salt (orange bars), 60 mM of NaCl for 3 weeks. Camalexin productivity (C) was calculated as ratio of the camalexin concentration to growth. Data are presented as the mean ± SEM. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$, Fisher's LSD). *tr – only trace levels were detected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

and camalexin production under salt treatment was two time more then in normal conditions (Fig. 5, C).

Cold stress enhanced camalexin content in *jaz1* culture, and considering tolerance of the transgenic cell culture to cold stress and slight inhibition of growth, production of camalexin was more than two-fold higher (70 μg/L) (Fig. 5C). Inhibition of *JAZ1* did not change

tolerance to salt treatment, however, and salt did not significantly increase the camalexin content compared to cold treatment. Camalexin production in the *jaz1* mutant under salt stress increased up to concentrations observed during cold stress (70 μg/L) (Fig. 5C).

3.4. Gene expression analysis

We have shown a simultaneous activation of camalexin biosynthesis and cold tolerance, induced by inhibition of *JAZ1*. Moreover, inhibition of the *JAZ1* doubled cold-induced camalexin biosynthesis. To clarify as much as possible the molecular mechanism, we studied the expression of genes associated with jasmonic acid pathway, abscisic acid pathway,

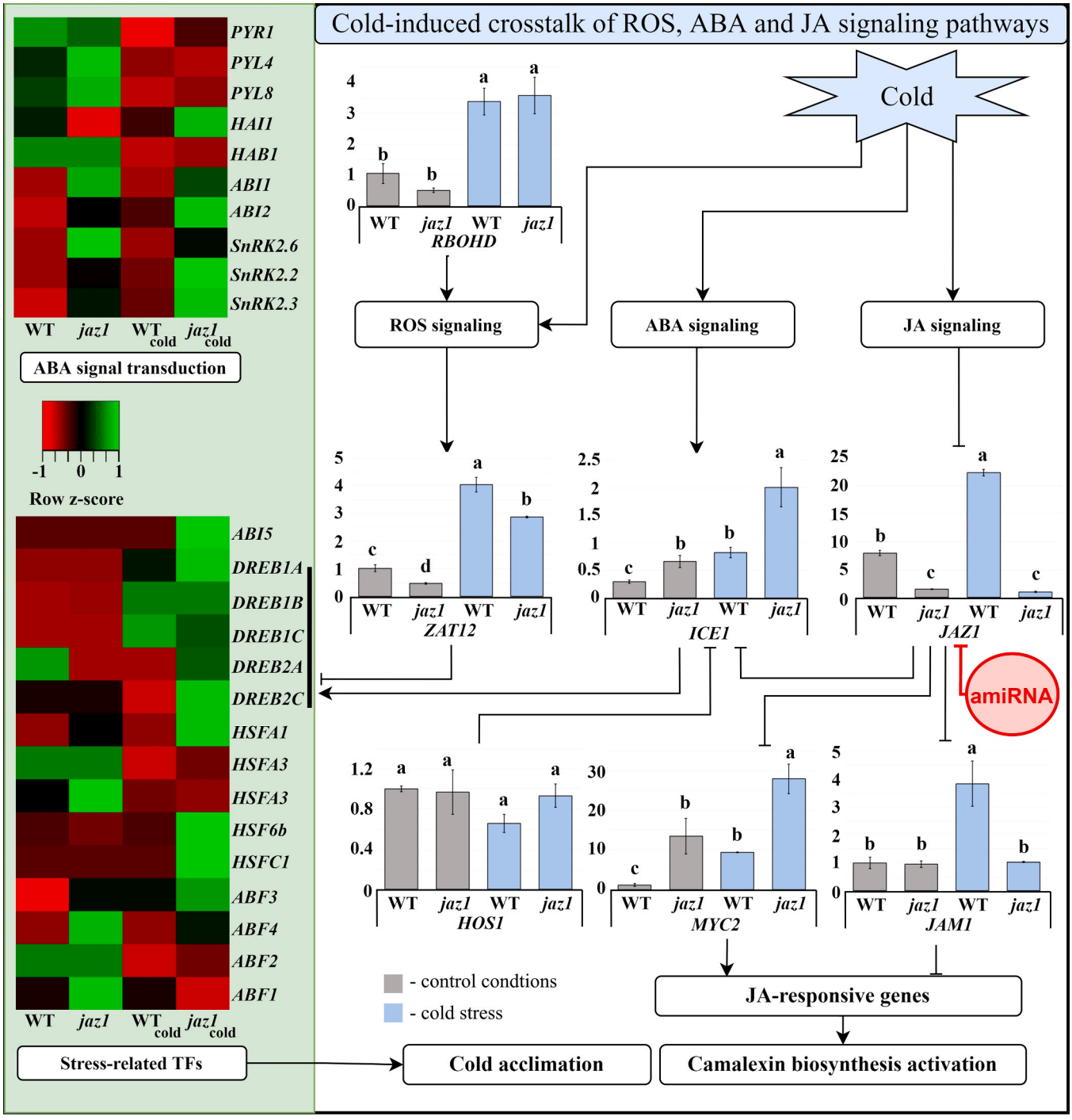

Fig. 6. Genes expression changes of cold-related genes in WT and *jaz1* cultures under control and cold (16 °C for 14 days) conditions. Left panel: heatmaps generated from real-time PCR data of WT and *jaz1* cultures under control and cold (16 °C for 14 days) conditions. ABA signal transduction – genes related to ABA reception and signal transduction. Stress-related TFs – genes that regulated during cold stress. Right panel: Expression data analysis of genes related to cold stress tolerance, ROS-signaling (*RBOHD* and *ZAT12*), ABA-signaling (*ICE1* and *HOS1*) and JA-pathway (*JAZ1*, *MYC2* and *JAM1*). amiRNA stands for RNAi inhibition of *JAZ1* gene. The Y-axis of all histogram represent normalized fold-change relative expression; the blue color indicates the culture was grown under cold stress conditions. Data are presented as the mean ± SEM. Different letters above the bars indicate statistically significant differences of means ($P < 0.05$, Fisher's LSD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

ROS-signaling pathway, and those related to the cold stress signaling in the WT and *jaz1* cell cultures under control and cold treatment (Fig. 6).

Without any treatments, *JAZ1* gene expression was significantly inhibited in the transgenic culture compared to the control (Fig. 5, right panel). Low temperature led to an increase in *JAZ1* expression in the control culture, while in the transgenic culture, the *JAZ1* expression level did not change. Under cold treatment, expression of the *JAZ1*-target transcription factors *MYC2* and *JAM1* significantly decreased and increased in the WT strain, respectively. In the *jaz1* line, expression levels of *MYC2*, an activator of the JA-target genes, was increased compared to WT in both untreated and cold-treated conditions. Expression of the *JAM1*, an antagonist of MYC2, was equal to WT levels and did not change under cold treatment in *jaz1* line (Fig. 6, right panel).

Expression analysis of the ROS-signaling markers, stress-inducible RBOHD and ZAT12, showed probability of interaction between JAZ1 and ROS-signaling. We showed that *RBOHD* expression in *jaz1* cells steadily increased more than 3-fold compared to WT. Cold exposure of sufficient duration (two weeks) did not did not influence RBOHD expression in either WT or transgenic cell lines (Fig. 6, right panel). Expression levels of the CBF negative regulator, *ZAT12*, increased in the both control and transgenic cultures under long cold treatment, but its expression level was significantly higher in WT compared to the *jaz1* line (Fig. 6, right panel).

To assess the role of *JAZ1* inhibition in the function of the OST1/SnRK2.6-HOS1-ICE1 module, and assess the resulting effect of this module on the ICE1-CBF/DREB regulon, we studied the expression of corresponding genes as well as other genes from the core ABA transduction pathway. As expected, *ICE1* expression was higher in *jaz1* lines compared to WT (Fig. 6, right panel). Cold treatment also activated expression of *ICE1* and the simultaneous action of cold and *JAZ1* inhibition caused the maximal additive effect. Expression of the ICE1 repressor, the *HOS1* gene, was equal in both cultures and was not changed under cold treatment. *OST1/SnRK2.6* and related *SnRK2.2* and *SnRK2.3* were upregulated. Genes encoding ABA-associated transcription factors *ABF1*, *ABF3*, and *ABF4* (but not *ABF2* and *ABI5*) were upregulated (Fig. 6, left panel). Surprisingly, none of the *CBF/DREB* genes were activated in *jaz1* lines, neither those responsible for thermotolerance (*DREB2A* and *DREB2C*) nor those responsible for cold resistance (*CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A*). At the same time, cold treatment, in agreement with previous results (Hu et al., 2013), activated the cold resistance genes *CBF1/DREB1B*, *CBF2/DREB1C,* and *CBF3/DREB1A* (Fig. 6, left panel).

Because the ABA pathway functions not only via activation of CBF/DREB genes, we further studied expression of genes encoding PYR/PYL/RCAR ABA receptors (PYLs) and protein phosphatases 2 C (PP2Cs) such as HAI1, HAB1, ABI1, and ABI2 (Fig. 6, left panel). PYL receptor gene expression remained virtually unchanged in *jaz1* lines. Expression of PP2Cs genes was variable: *ABI1*and *ABI2* were activated in *jaz1* lines, while *HAI1* was downregulated (Fig. 6, left panel). Expression of *HAB1* was unchanged. According to the data on the pairing of ABA receptors with PP2Cs (Tischer et al., 2017), it is impossible to distinguish between ABI1/ABI2 and HAI1 at the level of their molecular receptor-coreceptor interactions. However, there is a functional difference, because HAI1 is involved in ABA-independent drought-associated signaling (Bhaskara et al., 2012).

Gene expression of the HSF group was different in both cultures. The genes HSFA1, HSFC1, and HSFA6b were expressed in the transgenic culture similarly to the control culture; under cold stress conditions there was no significant change in the WT, while in the transgenic culture the expression level increased significantly. The expression of the HSFA2 and HSFA3 genes decreased under cold conditions for both cultures, but its level differed under control conditions; in the control culture, the expression level of the HSFA3 gene was higher than in the transgenic line, while HSFA2 it was higher in the transgenic line (Fig. 6, left panel).

4. Discussion

In the present study we describe activation of the JA pathway through repression of the one regulatory element, JAZ1. Our results show that inhibition of the *JAZ1* gene led to an increase in camalexin concentration to 0.045 μM/g DW (30 μg/L) without any additional treatments. Cold and salt treatments led to an increase in camalexin in the *jaz1* line to approximately 0.15 μM/g DW (70 μg/L). Inhibition of *JAZ1* showed a more intensive effect on the camalexin biosynthesis in *Arabidopsis* cell culture compared to overexpression of the *Agrobacterium rolB* gene, a known activator of different plant metabolic pathways (Bulgakov et al., 2011); however, in the case of the *Arabidopsis* cell cultures, there are no effects of *rolB* overexpression on the camalexin biosynthesis (Bulgakov et al., 2016). In order to compare the obtained results with effect of pathogen infection, we identify several examples of pathogen induction of the camalexin concentration in cell cultures. It was shown that treatment with fungi *Cochliobolus carbonum* increased camalexin concentration from zero to 120 μg/L (6 μg per 50 ml) in suspension cell cultures of *Arabidopsis* (Zook, 1998). Elicitation with *Orobanche ramose* led to increasing of camalexin concentration from zero to 150 ng/ml (or 150 μg/L) (El-Maarouf-Bouteau et al., 2008). This suggests that inhibition of *JAZ1* increased camalexin biosynthesis near 10 times less, than pathogen attack.

It is interesting to compare these results with known data of camalexin induction in plants. Treatment of *Arabidopsis* plants with nonspecific pathogen *Erwinia carotovora* led to increasing camalexin concentration from zero to 10 nM/g FW, corresponding to approximately 1 μM/g DW; whereas 10 mM of $AgNO_3$ led to an increase in camalexin concentration to 90 nM/g DW (Brader et al., 2001). Microbe-associated lipopolysaccharides increased camalexin concentration up to 3.7 μg/g FW (Beets et al., 2012). Overexpression of one of the key enzymes of camalexin biosynthesis in transgenic plants, CYP71B15, had no effect on camalexin concentration, but addition of $AgNO_3$ stimulated an increase in camalexin to 200 pM/g FW (Schuhegger et al., 2006). In addition, camalexin biosynthesis was increased up to 0.45 μg/g FW by overexpression of *PdPR5–1* in *Arabidopsis* plants when accompanying treatment with *Alternaria brassicicola* (El-kereamy et al., 2011). All of these approaches for activation of camalexin production generated only minor increases, because the main function of camalexin in plant cells is cytotoxicity against a pathogen; a side effect is a reduction in cell proliferation activity of whole plant or plant cell cultures when the camalexin concentration is high (Shih et al., 2018). We can suggest that inhibition of the *JAZ1* gene was not able to totally mimic the full JA spectrum effect, to the extent that plants usually respond to the presence of JA. However, our results showed that genetic modification of the regulatory apparatus can increase camalexin concentration without any additional exogenous treatment like pathogen elicitation. The JA signaling pathway plays an important role in a plant's response to environmental changes and various stresses by reprogramming secondary metabolism and allocating resources to effectively respond to stimuli for further adaptation and survival. Activation of the JA pathway resulted in inducing many biosynthesis genes of various pathways such as TIA, nicotine, artemisinin, and others (De Geyter et al., 2012).

The *JAZ1* gene studied in our research is part of group I (*JAZ1/2/5/6*), and also studied in multiple mutants *JazD*, *JazQ*, *JazU*. It was previously shown that knockout of ten *JAZ* genes in *jazD* mutant plants led to activation of IG biosynthesis (Guo et al., 2018), and several phenotypic changes such as reduced rosette size, hypersensitivity to JA-induced leaf senescence. Another mutant, *jazQ*, enhances the biosynthesis of anthocyanins and glucosinolates, increases tolerance to pathogens (Major et al., 2017, 2020; Guo et al., 2018). Multiple mutations *jaz1/2/3/4/5/6/7/9/10/11/12* (*jazU*) enhance JA-regulated phenotypes, restore the immunity to Botrytis cinerea in the coi1 mutant. It is likely that such differences are based on different activation of downstream transcription factors, for example, it is assumed that

jaz1/2/5/6 mutations can release more IIId type bHLH factors than MYC. However, the role of each *JAZ* gene and their interactions, redundancy is still to be determined. (Liu et al., 2021). In our study, we demonstrated that inhibition of the individual *JAZ1* gene expression altered camalexin accumulation and reaction to cold stress of an *Arabidopsis* cell culture without impairing cell growth. Moreover, the camalexin accumulation in the control MeJA-treated cell culture was four-fold less than in *jaz1* cultures without any treatment. At the same time, application of 50 μM MeJ led to a twofold increase of camalexin accumulation in the *jaz1* line but not in the control WT line. We have shown that inhibition of the *JAZ1* gene activates secondary metabolism, without affecting growth.

Induction of camalexin biosynthesis in the *JAZ1*-repressed cell line was accompanied with interesting changes in the expression of the TFs that regulate camalexin biosynthesis. Among upstream TFs (MYB34, MYB51, and MYB122), only expression of *MYB34* was significantly increased; the others remained unchanged. This finding correlated with previous data for these MYBs: MYB34 and MYB122 are JA-regulated TFs, while MYB51 is SA- and ET-regulated and MYB34 can repress MYB122 TFs (Frerigmann and Gigolashvili, 2014). We found an increase in the expression level of TFs WRKY33 and NAC042, which regulate expression of downstream enzymes CYP71A12/A13 and CYP71B15. Activation of WRKY33 expression in *JAZ1*-inhibited cells correspond to previous finding where JAZ1 can negatively regulate it (Birkenbihl et al., 2012). The highest increase in expression was shown for NAC42, which increased approximately 20-fold times in *jaz1* cells compared to WT cells. If WRKY33 only regulates CYP71A12/A13, the sphere of NAC042 influence extends also to CYP71B15. Interestingly, both WRKY33 and NAC042 are independent events of ROS signaling (Saga et al., 2012). This suggests that JAZ1 can negatively regulate some elements of ROS signaling pathway.

Additional stress treatments, such as long exposure under salinity or low temperature conditions, increased camalexin concentration in both control and *JAZ1*-inhibited cell cultures. The second side of the *JAZ1*-inhibition effects is enhancing cold tolerance. Cold stress inhibited growth of the WT cell line by two-fold and the *jaz1* cell line only by one third. Simultaneous enhancing of camalexin concentration, cold tolerance and cold-induced camalexin increasing is accompanied with mirror-changes in the expression of such key JA-signaling transcription factors as *MYC2* and *JAM1* (Song et al., 2013). An activator MYC2 and a repressor JAM1 were downregulated and upregulated, respectively, by cold treatment in the WT but not mutant cell lines. *JAZ1* inhibition led to an increase of cold-induced expression of *ICE1* and a decrease in expression of the CBF negative regulator, *ZAT12*. This finding corresponds to previously published data, where it was shown that JAZ1 and JAZ4 repress the transcriptional function of ICE1 (Hu et al., 2013). In this way, our study supports the suggestion that jasmonate functions as a main upstream signal of the ICE-CBF/DREB1 pathway, positively regulating the cold hardiness of *Arabidopsis* cells (Hu et al., 2013). When the transgenic culture was grown under cold conditions, the expression level of DREB1A increased significantly compared to the control culture, while DREB1C level of expression was higher in control culture. However, DREB1C contributes to the activation COR genes as well as other DREBs, and it was shown that DREB1C is a negative regulator of DREB1A and DREB1B expression (Novillo et al., 2004). Lower levels of DREB1C could contribute to the cold stress tolerance. These differences in the CBF/DREB pathway inform us about the altered response to stress in the transgenic culture, and indicate a strong interaction between the jasmonic acid pathway and cold tolerance. From this we can conclude that the transgenic culture is acclimated to cold stress.

The importance of this work lies in the fact that we were able to reproduce the exogenous use of methyl jasmonate by inhibiting a single gene. At the same time, there was no significant deterioration in the growth of callus cultures. Recently it was shown that the most genotypes of *Arabidopsis* have a baseline, trace amount of constitutively produced camalexin; however, some genotypes have constitutively hyper-accumulated camalexin. Abiotic stress and pathogen attack induced camalexin accumulation only in the strains expressing low levels of camalexin and did not change or even reduce camalexin accumulation in the plants with the constitutive camalexin hyper-accumulation. However, plants with the camalexin hyper-accumulation are significantly more resistant to bacterial infection (Zhang et al., 2014); therefore, metabolic engineering of tryptophan pathway is one of the main tools to obtain crops with high pathogen tolerance.

CRediT authorship contribution statement

D. Makhazen: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing, Funding acquisition. **G. Veremeichik:** Methodology, Writing – original draft, Writing – review & editing, Investigation, Validation, Supervision. **Y. Shkryl:** Methodology, Writing – review & editing, Investigation, Validation, Supervision. **G. Tchernoded:** Investigation. **V. P. Grigorchuk:** Methodology, Investigation. **V. Bulgakov:** Writing – review & editing, Conceptualization, Funding acquisition, Supervision.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Dmitrii Makhazen reports financial support was provided by Russian Foundation for Basic Research. Dmitrii Makhazen reports a relationship with Federal Scientific Center of the East Asia Terrestrial Biodiversity of the Far Eastern Branch of the Russian Academy of Sciences that includes: employment.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jbiotec.2021.10.012.

References

Arif, M.A., Alseekh, S., Harb, J., Fernie, A., Frank, W., 2018. Abscisic acid, cold and salt stimulate conserved metabolic regulation in the moss Physcomitrella patens. Plant Biol. J. 20, 1014–1022. https://doi.org/10.1111/plb.12871.

Augustine, R., Bisht, N.C., 2017. Regulation of glucosinolate metabolism: from model plant arabidopsis thaliana to brassica crops. In: Mérillon, J.-M., Ramawat, K.G. (Eds.), Glucosinolates, Reference Series in Phytochemistry. Springer International Publishing, Cham, pp. 163–199. https://doi.org/10.1007/978-3-319-25462-3_3.

Baniwal, S.K., Bharti, K., Chan, K.Y., Fauth, M., Ganguli, A., Kotak, S., Mishra, S.K., Nover, L., Port, M., Scharf, K.-D., Tripp, J., Weber, C., Zielinski, D., von Koskull-DÖring, P., 2004. Heat stress response in plants: a complex game with chaperones and more than twenty heat stress transcription factors. J. Biosci. 29, 471–487. https://doi.org/10.1007/BF02712120.

Beets, C.A., Huang, J.-C., Madala, N.E., Dubery, I., 2012. Activation of camalexin biosynthesis in Arabidopsis thaliana in response to perception of bacterial lipopolysaccharides: a gene-to-metabolite study. Planta 236, 261–272. https://doi.org/10.1007/s00425-012-1606-1.

Birkenbihl, R.P., Diezel, C., Somssich, I.E., 2012. Arabidopsis WRKY33 is a key transcriptional regulator of hormonal and metabolic responses toward botrytis cinerea infection. Plant Physiol. 159, 266–285. https://doi.org/10.1104/pp.111.192641.

Brader, G., Tas, É., Palva, E.T., 2001. Jasmonate-dependent induction of indole glucosinolates in arabidopsis by culture filtrates of the nonspecific pathogen erwinia carotovora. Plant Physiol. 126, 849–860. https://doi.org/10.1104/pp.126.2.849.

Bulgakov, V.P., Avramenko, T.V., 2015. New opportunities for the regulation of secondary metabolism in plants: focus on microRNAs. Biotechnol. Lett. 37, 1719–1727. https://doi.org/10.1007/s10529-015-1863-8.

Bulgakov, V.P., Gorpenchenko, T.Y., Veremeichik, G.N., Shkryl, Y.N., Tchernoded, G.K., Bulgakov, D.V., Aminin, D.L., Zhuravlev, Y.N., 2012. The rolB gene suppresses reactive oxygen species in transformed plant cells through the sustained activation of antioxidant defense. Plant Physiol. 158, 1371–1381. https://doi.org/10.1104/pp.111.191494.

Bulgakov, V.P., Shkryl, Y.N., Veremeichik, G.N., 2010. Engineering high yields of secondary metabolites in rubia cell cultures through transformation with rol genes. In: Fett-Neto, A.G. (Ed.), Plant Secondary Metabolism Engineering. Humana Press, Totowa, NJ, pp. 229–242. https://doi.org/10.1007/978-1-60761-723-5_16.

Bulgakov, V.P., Shkryl, Y.N., Veremeichik, G.N., Gorpenchenko, T.Y., Inyushkina, Y.V., 2011. Application of agrobacterium rol genes in plant biotechnology: a natural phenomenon of secondary metabolism regulation. Genet. Transform. https://doi.org/10.5772/24200.

Bulgakov, V.P., Veremeichik, G.N., Grigorchuk, V.P., Rybin, V.G., Shkryl, Y.N., 2016. The rolB gene activates secondary metabolism in Arabidopsis calli via selective activation of genes encoding MYB and bHLH transcription factors. Plant Physiol. Biochem. 102, 70–79. https://doi.org/10.1016/j.plaphy.2016.02.015.

Cataldi, T.R.I., Rubino, A., Lelario, F., Bufo, S.A., 2007. Naturally occurring glucosinolates in plant extracts of rocket salad (Eruca sativa L.) identified by liquid chromatography coupled with negative ion electrospray ionization and quadrupole ion-trap mass spectrometry. Rapid Commun. Mass Spectrom. 21, 2374–2388. https://doi.org/10.1002/rcm.3101.

Chripkova, M., Zigo, F., Mojzis, J., 2016. Antiproliferative effect of indole phytoalexins. Molecules 21, 1626. https://doi.org/10.3390/molecules21121626.

Clarke, D.B., 2010. Glucosinolates, structures and analysis in food. Anal. Methods 2, 310. https://doi.org/10.1039/b9ay00280d.

Cuming, A.C., 2019. Advances in botanical research. In: Evolution of ABA Signaling Pathways. Elsevier, pp. 281–313. https://doi.org/10.1016/bs.abr.2019.06.003.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., Scheible, W.-R., 2005. Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol. 139, 5–17. https://doi.org/10.1104/pp.105.063743.

Davletova, S., Schlauch, K., Coutu, J., Mittler, R., 2005. The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in arabidopsis. Plant Physiol. 139, 847–856. https://doi.org/10.1104/pp.105.068254.

De Geyter, N., Gholami, A., Goormachtig, S., Goossens, A., 2012. Transcriptional machineries in jasmonate-elicited plant secondary metabolism. Trends Plant Sci. 17, 349–359. https://doi.org/10.1016/j.tplants.2012.03.001.

Ding, Y., Li, H., Zhang, X., Xie, Q., Gong, Z., Yang, S., 2015. OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in arabidopsis. Dev. Cell 32, 278–289. https://doi.org/10.1016/j.devcel.2014.12.023.

El-kereamy, A., El-sharkawy, I., Ramamoorthy, R., Taheri, A., Errampalli, D., Kumar, P., Jayasankar, S., 2011. Prunus domestica pathogenesis-related protein-5 activates the defense response pathway and enhances the resistance to fungal infection. PLoS One 6, e17973. https://doi.org/10.1371/journal.pone.0017973.

El-Maarouf-Bouteau, H., Moreau, E., Errakhi, R., Sallé, G., 2008. A diffusible signal from germinating Orobanche ramosa elicits early defense responses in suspension-cultured Arabidopsis thaliana. Plant Signal. Behav. 3, 189–193. https://doi.org/10.4161/psb.3.3.5545.

Fahlgren, N., Hill, S.T., Carrington, J.C., Carbonell, A., 2015. P-SAMS: a web site for plant artificial microRNA and synthetic trans -acting small interfering RNA design. Bioinformatics 32, 157–158. https://doi.org/10.1093/bioinformatics/btv534.

Fernández-Calvo, P., Chini, A., Fernández-Barbero, G., Chico, J.-M., Gimenez-Ibanez, S., Geerinck, J., Eeckhout, D., Schweizer, F., Godoy, M., Franco-Zorrilla, J.M., Pauwels, L., Witters, E., Puga, M.I., Paz-Ares, J., Goossens, A., Reymond, P., De Jaeger, G., Solano, R., 2011. The arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell 23, 701–715. https://doi.org/10.1105/tpc.110.080788.

Frerigmann, H., Gigolashvili, T., 2014. MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in Arabidopsis thaliana. Mol. Plant 7, 814–828. https://doi.org/10.1093/mp/ssu004.

Frerigmann, H., Glawischnig, E., Gigolashvili, T., 2015. The role of MYB34, MYB51 and MYB122 in the regulation of camalexin biosynthesis in Arabidopsis thaliana. Front. Plant Sci. 6, 654. https://doi.org/10.3389/fpls.2015.00654.

Geng, Y., Wu, R., Wee, C.W., Xie, F., Wei, X., Chan, P.M.Y., Tham, C., Duan, L., Dinneny, J.R., 2013. A spatio-temporal understanding of growth regulation during the salt stress response in Arabidopsis. Plant Cell 25, 2132–2154. https://doi.org/10.1105/tpc.113.112896.

Glawischnig, E., Hansen, B.G., Olsen, C.E., Halkier, B.A., 2004. Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 101, 8245–8250. https://doi.org/10.1073/pnas.0305876101.

Gou, J.-Y., Felippes, F.F., Liu, C.-J., Weigel, D., Wang, J.-W., 2011. Negative regulation of anthocyanin biosynthesis in arabidopsis by a miR156-targeted SPL transcription factor. Plant Cell 23, 1512–1522. https://doi.org/10.1105/tpc.111.084525.

Guo, Q., Yoshida, Y., Major, I.T., Wang, K., Sugimoto, K., Kapali, G., Havko, N.E., Benning, C., Howe, G.A., 2018. JAZ repressors of metabolic defense promote growth and reproductive fitness in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 115, E10768 https://doi.org/10.1073/pnas.1811828115.

Gupta, O.P., Karkute, S.G., Banerjee, S., Meena, N.L., Dahuja, A., 2017. Contemporary understanding of miRNA-based regulation of secondary metabolites biosynthesis in plants. Front. Plant Sci. 8, 374. https://doi.org/10.3389/fpls.2017.00374.

Hood, E.E., Gelvin, S.B., Melchers, L.S., Hoekema, A., 1993. New agrobacterium helper plasmids for gene transfer to plants. Transgenic Res. 2, 208–218. https://doi.org/10.1007/BF01977351.

Hu, Y., Jiang, L., Wang, F., Yu, D., 2013. Jasmonate regulates the inducer of cbf expression–c-repeat binding factor/dre binding factor1 cascade and freezing tolerance in arabidopsis. Plant Cell 25, 2907–2924. https://doi.org/10.1105/tpc.113.112631.

Hu, Y., Jiang, Y., Han, X., Wang, H., Pan, J., Yu, D., 2017. Jasmonate regulates leaf senescence and tolerance to cold stress: crosstalk with other phytohormones. J. Exp. Bot. 68, 1361–1369. https://doi.org/10.1093/jxb/erx004.

Lelario, F., Bianco, G., Bufo, S.A., Cataldi, T.R.I., 2012. Establishing the occurrence of major and minor glucosinolates in Brassicaceae by LC–ESI-hybrid linear ion-trap and Fourier-transform ion cyclotron resonance mass spectrometry. Phytochemistry 73, 74–83. https://doi.org/10.1016/j.phytochem.2011.09.010.

Liang, C.-H., Yang, C.-C., 2015. Identification of ICE1 as a negative regulator of ABA-dependent pathways in seeds and seedlings of Arabidopsis. Plant Mol. Biol. 88, 459–470. https://doi.org/10.1007/s11103-015-0335-9.

Liu, B., Seong, K., Pang, S., Song, J., Gao, H., Wang, C., Zhai, J., Zhang, Y., Gao, S., Li, X., Qi, T., Song, S., 2021. Functional specificity, diversity, and redundancy of Arabidopsis JAZ family repressors in jasmonate and COI1–regulated growth, development, and defense. New Phytol. 231, 1525–1545. https://doi.org/10.1111/nph.17477.

Major, I.T., Guo, Q., Zhai, J., Kapali, G., Kramer, D.M., Howe, G.A., 2020. A phytochrome B-independent pathway restricts growth at high levels of jasmonate defense. Plant Physiol. 183, 733–749. https://doi.org/10.1104/pp.19.01335.

Major, I.T., Yoshida, Y., Campos, M.L., Kapali, G., Xin, X.-F., Sugimoto, K., de Oliveira Ferreira, D., He, S.Y., Howe, G.A., 2017. Regulation of growth-defense balance by the Jasmonate zim-domain (JAZ)-MYC transcriptional module. New Phytol. 215, 1533–1547. https://doi.org/10.1111/nph.14638.

Mezencev, R., Galizzi, M., Kutschy, P., Docampo, R., 2009. Trypanosoma cruzi: antiproliferative effect of indole phytoalexins on intracellular amastigotes in vitro. Exp. Parasitol. 122, 66–69. https://doi.org/10.1016/j.exppara.2009.01.013.

Mezencev, R., Updegrove, T., Kutschy, P., Repovská, M., McDonald, J.F., 2011. Camalexin induces apoptosis in T-leukemia Jurkat cells by increased concentration of reactive oxygen species and activation of caspase-8 and caspase-9. J. Nat. Med. 65, 488–499. https://doi.org/10.1007/s11418-011-0526-x.

Moody, C.J., Roffey, J.R., Stephens, M.A., Stratford, I.J., 1997. Synthesis and cytotoxic activity of indolyl thiazoles. Anti-Cancer Drugs 8, 489–499. https://doi.org/10.1097/00001813-199706000-00012.

Murashige, T., Skoog, F., 1962. A Revised Medium For Rapid Growth And Bio Assays With Tobacco Tissue Cultures. Physiol. Plant 15, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x.

Novillo, F., Alonso, J.M., Ecker, J.R., Salinas, J., 2004. CBF2/DREB1C is a negative regulator of CBF1/DREB1B and CBF3/DREB1A expression and plays a central role in stress tolerance in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 101, 3985–3990. https://doi.org/10.1073/pnas.0303029101.

Pandey, S.P., Somssich, I.E., 2009. The role of WRKY transcription factors in plant immunity. Plant Physiol. 150, 1648–1655. https://doi.org/10.1104/pp.109.138990.

Paul, S., Datta, S.K., Datta, K., 2015. miRNA regulation of nutrient homeostasis in plants. Front. Plant Sci. 6, 588. https://doi.org/10.3389/fpls.2015.00232.

Pauwels, L., Goossens, A., 2011. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell 23, 3089–3100. https://doi.org/10.1105/tpc.111.089300.

Pilatova, M., Ivanova, L., Kutschy, P., Varinska, L., Saxunova, L., Repovska, M., Sarissky, M., Seliga, R., Mirossay, L., Mojzis, J., 2013. In vitro toxicity of camalexin derivatives in human cancer and non-cancer cells. Toxicol. Vitr. 27, 939–944. https://doi.org/10.1016/j.tiv.2013.01.006.

Qi, T., Song, S., Ren, Q., Wu, D., Huang, H., Chen, Y., Fan, M., Peng, W., Ren, C., Xie, D., 2011. The Jasmonate-ZIM-domain proteins interact with the WD-repeat/bHLH/MYB complexes to regulate jasmonate-mediated anthocyanin accumulation and trichome initiation in Arabidopsis thaliana. Plant Cell 23, 1795–1814. https://doi.org/10.1105/tpc.111.083261.

Saga, H., Ogawa, T., Kai, K., Suzuki, H., Ogata, Y., Sakurai, N., Shibata, D., Ohta, D., 2012a. Identification and characterization of ANAC042, a transcription factor family gene involved in the regulation of camalexin biosynthesis in arabidopsis. MPMI 25, 684–696. https://doi.org/10.1094/MPMI-09-11-0244.

Sasaki-Sekimoto, Y., Jikumaru, Y., Obayashi, T., Saito, H., Masuda, S., Kamiya, Y., Ohta, H., Shirasu, K., 2013. Basic helix-loop-helix transcription factors Jasmonate-associated MYC2-LIKE1 (JAM1), JAM2, and JAM3 are negative regulators of jasmonate responses in arabidopsis. Plant Physiol. 163, 291–304. https://doi.org/10.1104/pp.113.220129.

Schuhegger, R., Nafisi, M., Mansourova, M., Petersen, B.L., Olsen, C.E., Svatoš, A., Halkier, B.A., Glawischnig, E., 2006. CYP71B15 (PAD3) catalyzes the final step in camalexin biosynthesis. Plant Physiol. 141, 1248–1254. https://doi.org/10.1104/pp.106.082024.

Schwab, R., Ossowski, S., Riester, M., Warthmann, N., Weigel, D., 2006. Highly specific gene silencing by artificial MicroRNAs in Arabidopsis. Plant Cell 18, 1121–1133. https://doi.org/10.1105/tpc.105.039834.

Shih, P.-Y., Chou, S.-J., Müller, C., Halkier, B.A., Deeken, R., Lai, E.-M., 2018. Differential roles of glucosinolates and camalexin at different stages of Agrobacterium-mediated transformation: GS in Agrobacterium-mediated transformation. Mol. Plant Pathol. 19, 1956–1970. https://doi.org/10.1111/mpp.12672.

Shkryl, Y.N., Veremeichik, G.N., Makhazen, D.S., Silantieva, S.A., Mishchenko, N.P., Vasileva, E.A., Fedoreyev, S.A., Bulgakov, V.P., 2016. Increase of anthraquinone content in Rubia cordifolia cells transformed by native and constitutively active forms of the AtCPK1 gene. Plant Cell Rep. 35, 1907–1916. https://doi.org/10.1007/s00299-016-2005-z.

Song, S., Qi, T., Fan, M., Zhang, X., Gao, H., Huang, H., Wu, D., Guo, H., Xie, D., 2013. The bHLH subgroup IIId factors negatively regulate jasmonate-mediated plant defense and development. PLoS Genet. 9, e1003653 https://doi.org/10.1371/journal.pgen.1003653.

Song, S., Qi, T., Huang, H., Ren, Q., Wu, D., Chang, C., Peng, W., Liu, Y., Peng, J., Xie, D., 2011. The Jasmonate-ZIM domain Proteins Interact with the R2R3-MYB transcription factors MYB21 and MYB24 to affect Jasmonate-regulated stamen development in arabidopsis. Plant Cell 23, 1000–1013. https://doi.org/10.1105/tpc.111.083089.

Tischer, S.V., Wunschel, C., Papacek, M., Kleigrewe, K., Hofmann, T., Christmann, A., Grill, E., 2017. Combinatorial interaction network of abscisic acid receptors and coreceptors from Arabidopsis thaliana. Proc. Natl. Acad. Sci. U.S.A. 114, 10280–10285. https://doi.org/10.1073/pnas.1706593114.

Todesco, M., Balasubramanian, S., Hu, T.T., Traw, M.B., Horton, M., Epple, P., Kuhns, C., Sureshkumar, S., Schwartz, C., Lanz, C., Laitinen, R.A.E., Huang, Y., Chory, J., Lipka, V., Borevitz, J.O., Dangl, J.L., Bergelson, J., Nordborg, M., Weigel, D., 2010. Natural allelic variation underlying a major fitness trade-off in Arabidopsis thaliana. Nature 465, 632–636. https://doi.org/10.1038/nature09083.

Tzfira, T., Tian, G.-W., Lacroix, B., Vyas, S., Li, J., Leitner-Dagan, Y., Krichevsky, A., Taylor, T., Vainstein, A., Citovsky, V., 2005. pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol. Biol. 57, 503–516. https://doi.org/10.1007/s11103-005-0340-5.

Udvardi, M.K., Kakar, K., Wandrey, M., Montanari, O., Murray, J., Andriankaja, A., Zhang, J.-Y., Benedito, V., Hofer, J.M.I., Chueng, F., Town, C.D., 2007. Legume transcription factors: global regulators of plant development and response to the environment. Plant Physiol. 144, 538–549. https://doi.org/10.1104/pp.107.098061.

Valenzuela, C.E., Acevedo-Acevedo, O., Miranda, G.S., Vergara-Barros, P., Holuigue, L., Figueroa, C.R., Figueroa, P.M., 2016. Salt stress response triggers activation of the jasmonate signaling pathway leading to inhibition of cell elongation in Arabidopsis primary root. J. Exp. Bot. 67, 4209–4220. https://doi.org/10.1093/jxb/erw202.

Veremeichik, G.N., Bulgakov, V.P., Shkryl, Y.N., Silantieva, S.A., Makhazen, D.S., Tchernoded, G.K., Mischenko, N.P., Fedoreyev, S.A., Vasileva, E.A., 2019. Activation of anthraquinone biosynthesis in long-cultured callus culture of Rubia cordifolia transformed with the rolA plant oncogene. J. Biotechnol. 306, 38–46. https://doi.org/10.1016/j.jbiotec.2019.09.007.

Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G., Thomashow, M.F., 2004. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of Arabidopsis: Arabidopsis low temperature transcriptome. Plant J. 41, 195–211. https://doi.org/10.1111/j.1365-313X.2004.02288.x.

Xie, F., Xiao, P., Chen, D., Xu, L., Zhang, B., 2012. miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. Plant Mol. Biol. 80, 75–84. https://doi.org/10.1007/s11103-012-9885-2.

Yang, Y., Wang, G., Wu, W., Yao, S., Han, X., He, D., He, J., Zheng, G., Zhao, Y., Cai, Z., Yu, R., 2018. Camalexin induces apoptosis via the ROS-ER stress-mitochondrial apoptosis pathway in AML cells. Oxid. Med. Cell. Longev. 2018, 1–14. https://doi.org/10.1155/2018/7426950.

Yu, Z.-X., Wang, L.-J., Zhao, B., Shan, C.-M., Zhang, Y.-H., Chen, D.-F., Chen, X.-Y., 2015. Progressive regulation of sesquiterpene biosynthesis in Arabidopsis and Patchouli (Pogostemon cablin) by the miR156-targeted SPL transcription factors. Mol. Plant 8, 98–110. https://doi.org/10.1016/j.molp.2014.11.002.

Zhang, N., Lariviere, A., Tonsor, S.J., Traw, M.B., 2014. Constitutive camalexin production and environmental stress response variation in Arabidopsis populations from the Iberian Peninsula. Plant Sci. 225, 77–85. https://doi.org/10.1016/j.plantsci.2014.05.020.

Zhou, J., Wang, X., He, Y., Sang, T., Wang, P., Dai, S., Zhang, S., Meng, X., 2020. Differential phosphorylation of the transcription factor WRKY33 by the protein kinases CPK5/CPK6 and MPK3/MPK6 cooperatively regulates camalexin biosynthesis in arabidopsis. Plant Cell 32, 2621–2638. https://doi.org/10.1105/tpc.19.00971.

Zhou, M., Memelink, J., 2016. Jasmonate-responsive transcription factors regulating plant secondary metabolism. Biotechnol. Adv. 34, 441–449. https://doi.org/10.1016/j.biotechadv.2016.02.004.

Zook, M., 1998. Biosynthesis of camalexin from tryptophan pathway intermediates in cell-suspension cultures of arabidopsis. Plant Physiol. 118, 1389–1393. https://doi.org/10.1104/pp.118.4.1389.