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AK and EW designed and carried out the experiments, analyzed the data and wrote the manuscript; GCR and MW were responsible for material collection, RNA isolation, cDNA preparation, and helped with data analysis; PG helped with RT-qPCR reactions and data analysis; JK was responsible for interpretation of the part of the data and preparing the manuscript

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ORIGINAL RESEARCH PAPER

Auxin increases the *InJMT* expression and the level of JAMe – inhibitor of flower induction in *Ipomoea nil*

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Abstract
Interactions among jasmonates and auxin in the photoperiodic flower induction of a short-day plant *Ipomoea nil* were examined. Therefore, we measured changes in jasmonic acid (JA) and jasmonic acid methyl ester (JAMe) levels in the cotyledons of *I. nil* during the inductive night, as well as the effects of indole-3-acetic acid (IAA) on their content. We noticed an interesting result, that IAA applied on the cotyledons of *I. nil* is an effective stimulator of JAMe production in seedlings cultivated under inductive night conditions. IAA treatment also significantly increased the transcriptional activity of *InJMT* (*JASMONIC ACID CARBOXYL METHYLTRANSFERASE*), while did not affect the expression of JA biosynthesis genes (lipoxyganease, allene oxide synthase, 12-oxophytodienoate reductase). These data, as well as the results of our previous research, suggest that exogenous IAA participates in *I. nil* flower induction process by stimulating *InJMT* expression and, as a consequence of that, enhancing the level of JAMe, a flowering inhibitor.

Keywords
flower induction; gene expression; hormone interactions; jasmonates biosynthesis

Introduction

The flowering is the transition from vegetative to generative phase of development and it consists of three main stages: induction, evocation, and flower morphogenesis. Flower induction is controlled both by external factors (e.g., hormones, signal substances) and environmental conditions (e.g., temperature, photoperiod) [1–4].

Methyl jasmonate (JAMe), belonging to the jasmonates (JAs) group, together with ethylene and abscisic acid are considered to be mainly stress hormones participating in the plants adaptation to the changing environmental conditions [5]. Jasmonates also regulate a number of other physiological processes, e.g., time of flowering, flower morphogenesis and their opening, sex differentiation as well as production of viable pollen [6–10]. The identification of mutants incapable of both JA biosynthesis and signaling [*defective in anther dehiscence* (*dad1*), *delayed dehiscence* (*dde1*, *dde2-2*), *fatty acid desaturase* (*fad3-2fad7-2fad8*), *12-oxophytodienoate reductase* (*opr3*), *coronatine-insensitive 1* (*coi1*)] from *Arabidopsis thaliana* and *tasselseed* (*2ts1*, *ts2*) from *Zea mays*, indicates that the presence of these hormones is necessary for a complete reproductive success of the plants [11–15]. The results of research conducted on *Spirodela polyrrhiza* [16], *Wolffia arrhiza* [17], *Nicotiana tabacum* [18], and *Chenopodium rubrum* [19] show that jasmonates inhibit flower induction triggered by the photoperiod. Methyl jasmonate applied both to the cotyledons and roots or shoot apices before

or during the 16-h-long inductive night inhibits flowering in *Ipomoea nil*, whereas JA does not affect that process [20–22]. *Ipomoea nil* is a preferential short-day plant (SDP), which can be induced to flower by a single photo inductive dark period. Short day growth conditions are required for flowering [23]. The results obtained by Kęsy et al. [24] showed that the content of endogenous indole-3-acetic acid (IAA) in the cotyledons of 5-day-old *I. nil* plants during the 16-h-long inductive night is relatively low, but the interruption of the darkness, which inhibits flowering, accelerates the level of this hormone. This suggest that a low content of IAA is necessary for flower induction in *I. nil*. Our earlier works demonstrated that an exogenous IAA, similarly to JAMe, inhibited flowering in *I. nil* [24–26]. The highest auxin effectiveness was observed when IAA was applied to the cotyledons before or during the first half of the inductive night [24]. Moreover, auxin biosynthesis and transport are crucial in the regulation of flower morphogenesis. Auxin biosynthesis mutant *yuc1yuc2yuc4yuc6* in *A. thaliana* produce inflorescence devoid of flower primordia [27]. Additionally, *pin1* auxin transport mutant is defective in initiating flower primordia and forms few abnormal flowers without stamens and fused petals [28,29]. It was also demonstrated that mutation in *ARF3* (*auxin response factor*) gene, cause reduction of the ovary size and disrupts gynoecium patterns [30,31].

Many studies provide a great significance of interactions between IAA and JAs, for instance, stem cell growth, abscission, secondary abscission zone formation, tendril coiling, and wounding [32]. In *A. thaliana*, JAMe inhibits primary root elongation via stimulation of *YUC8* and *YUC9* expression involved in auxin biosynthesis [33]. Gutierrez et al. [34] proposed that auxin, through the positive regulators *ARF6* and *ARF8*, induces the formation of adventitious roots by stimulating the expression of auxin-inducible genes encoding acyl-acid-amido synthetases (*GH3.3*, *GH3.5*, and *GH3.6*), consequently causing JA conjugation and reduction the level of free JA. In *A. thaliana*, JAMe activates the transcriptional activity of *ASA1* (*ANTHRANILATE SYNTHASE α1*) gene, which leads to increased IAA biosynthesis in shoot and root tissues. The defect of *ASA1* affects root and shoot specific synthesis of IAA. Moreover, JAMe decreases the levels of auxin transporting proteins (PIN1 and PIN2). JAMe increases local accumulation of IAA in the root basal meristem and, as a result, promotes lateral root formation [35]. However, the knowledge of the networks controlling IAA-jasmonates relationships in the context of the photoperiodic induction of flowering still remains poorly understood. Due to above-mentioned facts, we investigated the effects of IAA on the level of endogenous JA and JAMe in the cotyledons of *I. nil* during the inductive night, and the changes in the expression of *InLOX2*, *InAOS*, and *InOPR3* – coding for lipoxygenase, allene oxide synthase, and 12-oxophytodienoate reductase, respectively – which are essential enzymes in JAs biosynthesis. We also analyzed the transcriptional activity of *InJMT* (*JASMONIC ACID CARBOXYL METHYLTRANSFERASE*) – the gene responsible for conversion of JA into JAMe.

Material and methods

Plants and cultivation

The plant material (*Ipomoea nil*, synonym *Pharbitis nil*, Chois cv. Violet; Marutane Seed Co., Kyoto, Japan) was prepared according to [26]. Plants were grown in a growth chamber at a temperature of 26 ±1°C under long-day (LD) condition for 5 days (8 h darkness + 16 h light, 130 μmol m^{-2} s^{-1}, cool white fluorescent tubes; Polam, Warsaw, Poland). On the 6th day of cultivation, the seedlings were exposed to a 16-h-long inductive night (SD) to evoke flowering.

In the experiments examining the influence of IAA on JAs level and the expression of genes involved in JAs biosynthesis and metabolism, some plants were treated with IAA at a concentration of 0.5 mM in 0.05 % Tween 20 (v/v) at the beginning of the dark period, whereas control seedlings were treated with a 0.05% Tween 20 (v/v) solution. All manipulations in the darkness were performed under dim green light. The cotyledons (without petioles) were harvested during the inductive night (time of plant material collection is given in Fig. 1–Fig. 3), subsequently were frozen in liquid

nitrogen and stored at −80°C for RNA isolation or JAs determination. All experiments were designed in three independent biological replications.

Determination of endogenous jasmonates

The modified methods of Fan et al. [36] and Gundlach et al. [37] described by Wilmowicz et al. [38] for endogenous JA and JAMe analyses were used. d_2-JAMe (100 ng) and d_5-JA (100 ng) were added to the crude extract as internal standards. SIM GC/MS was performed by monitoring m/z 193, 195, 198, 224, 226, 229. Student's *t*-test was used to calculate the significant differences compared with the controls.

Quantitative real-time PCR (RT-qPCR)

The gene expression analysis was performed by real-time PCR (RT-qPCR) with a LightCycler 2.0 Carousel-Based System (ROCHE Diagnostics GmbH, Germany) and LightCycler TaqMan Master Kit (ROCHE Diagnostics GmbH, Germany). The cDNA templates were prepared according to Glazińska et al. [39], whereas gene-specific primers and UPL probes were designed using Universal ProbeLibrary Assay Design Center (https://lifescience.roche.com/shop/CategoryDisplay?catalogId=10001&tab=&identifier=Universal+Probe+Library) (Tab. 1). The qPCR reactions were made for the following genes: *InAOS* (GenBank acc. No. HM357792.2), *InOPR3* (GenBank acc. No. HM357793.2), *InLOX2* (GenBank acc. No. CJ761975), and *InJMT* (GenBank acc. No. KF573520.1). *InACT4* (GenBank acc. No. HM802138.2) was used as a reference endogenous control for normalization purposes. The qPCR mixtures were made as follows: 1 µL of first strand cDNA, 0.4 µL of the gene specific primers solution (10 µM), 0.2 µL of the gene specific hydrolysis probe, and 4 µL of Master Mix (containing a reaction buffer, dNTP mix, and DNA Polymerase) from LightCycler TaqMan Master (ROCHE Diagnostics GmbH, Germany). The qPCR conditions included an initial denaturation step at 95°C for 10 min followed by 45 cycles at 95°C for 10 s, 58°C for 30 s, 72°C for 1 s, followed by one cycle of cooling at 40°C for 30 s.

Relative quantification was calculated with standard curves from serial dilutions of cDNA. The efficiency tested was >99%. The computer application for the expression analysis was LightCycler Real-Time PCR Systems (ROCHE Diagnostics GmbH, Germany), while for the calculations and graphs – MS Office Excel (Microsoft) and SigmaPlot 2001 ver. 7.0, respectively, were used. Three independent RNA preparations per stage were used for analyses, and data are shown as mean ±*SE* of three samples for each stage.

Results

The level of endogenous JAMe in the cotyledons of *I. nil* seedlings cultivated under inductive conditions was low, stable and amounted to approx. 2 ng g^{-1} fresh weight (Fig. 1). In turn, JA content was subject to slight fluctuations, but accumulated stronger than methyl ester form. At the beginning of the inductive night the level of JA reached 7 times higher value compared with JAMe.

IAA applied to the cotyledons just before the inductive night increased both JA and JAMe level, but observed stimulating effect was stronger in the case of methyl form. JAMe content increased already 2 h after auxin application and at 4, 6, 8, and 10 h reached maximum values – several dozens of times higher than in that not treated with IAA (Fig. 1).

Slightly higher *InLOX2*, *InAOS*, and *InOPR3* expression level in the cotyledons of *I. nil* cultivated under SD conditions was observed at the beginning of the inductive night (Fig. 2). The transcriptional activity of the studied genes decreased at the 2nd hour of cultivation in darkness and was relatively stable till the 16 h. The application of IAA just before the inductive night did not have a significant impact on the expression of genes involved in JA biosynthesis (Fig. 2).

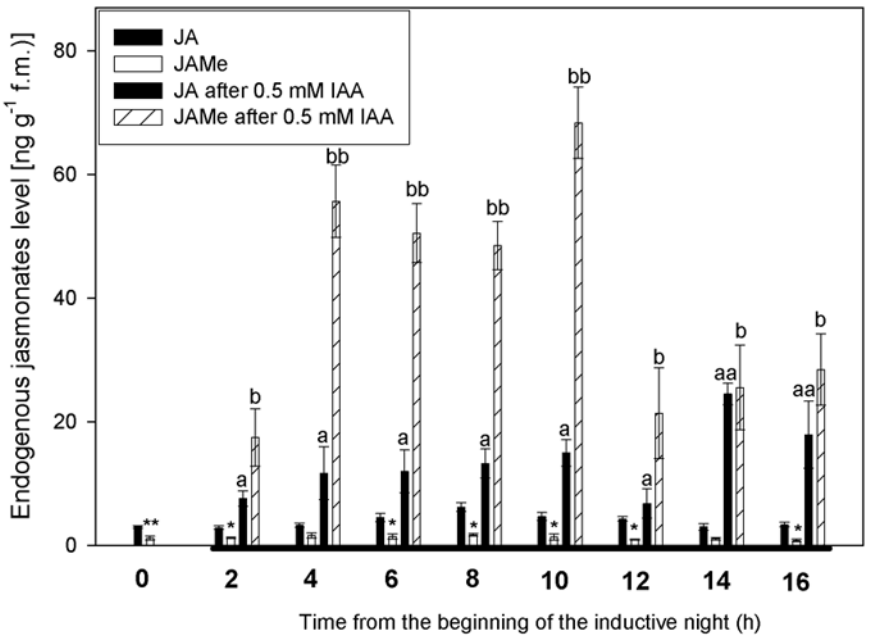

Fig. 1 Changes in JA and JAMe content [ng g^{-1} (f.m.)] in cotyledons of 5-days-old *I. nil* seedlings affected by a 16-h inductive dark period and caused by the dark period plus 0.5 mM IAA. For details on IAA application, see the "Material and methods". The thick horizontal line under the time axis indicates duration of the dark period. Means ±*SE*; $n = 6$. Significant differences in the level of: (***i***) various JAs in the cotyledons are indicated as ** $p < 0.01$ and * $p < 0.05$; (***ii***) JA after IAA application in comparison to JA level in untreated cotyledons are indicated as ^{aa} $p < 0.01$ and ^{a} $p < 0.05$; (***iii***) JAMe after IAA application in comparison to JAMe level in untreated cotyledons are indicated as ^{bb} $p < 0.01$ and ^{b} $p < 0.05$.

The transcriptional activity of *InJMT* during the inductive night was smaller than that of jasmonate biosynthesis genes, whereas the application of exogenous IAA extremely increased the *InJMT* transcript level hundreds of times started from the 2nd hour after treatment (Fig. 2 and Fig. 3).

Discussion

No factor has so far been found which can unequivocally stimulate the flowering of *I. nil* cultivated under non-inductive conditions. However, a number of hormones can inhibit photoperiodic flower induction, e.g., auxins, ethylene, abscisic acid, and methyl jasmonate [20–22,24,40–43]. It was proposed that the exogenous JAMe applied just before the inductive night inhibited the flowering in *Pharbitis nil* probably by stimulating ethylene production [22]. Nevertheless, it turned out that seedlings induced to flowering and treated with JAMe produced comparable amounts of ethylene to control plants, which contradicted the original hypothesis [22]. The deceleration of the endogenous JA/JAMe content in the cotyledons of *Pharbitis nil* seedlings by application of their biosynthesis inhibitor (acetylsalicylic acid) restored the effect of the inductive night. Therefore, a high JA/JAMe level in the cotyledons during the inductive night inhibits flower induction [21,22]. Reaching the jasmonate content appropriate for the control of various processes (including flowering) is made possible by multi-level regulation of their biosynthesis and metabolism, in which *LOX*, *AOS*, and *OPR3*, as well as *JMT*, play a pivotal role. Moreover, the level of jasmonates in particular tissues depends also on their transport [44].

Promoters analyses of genes encoding proteins involved in JA biosynthesis in *A. thaliana* revealed the presence of sequences binding ARF transcriptional factors. The effects of ARF6 and ARF8 on JA biosynthesis can be positive or negative, depending on the process [45]. It was demonstrated that these two members of the ARFs, which

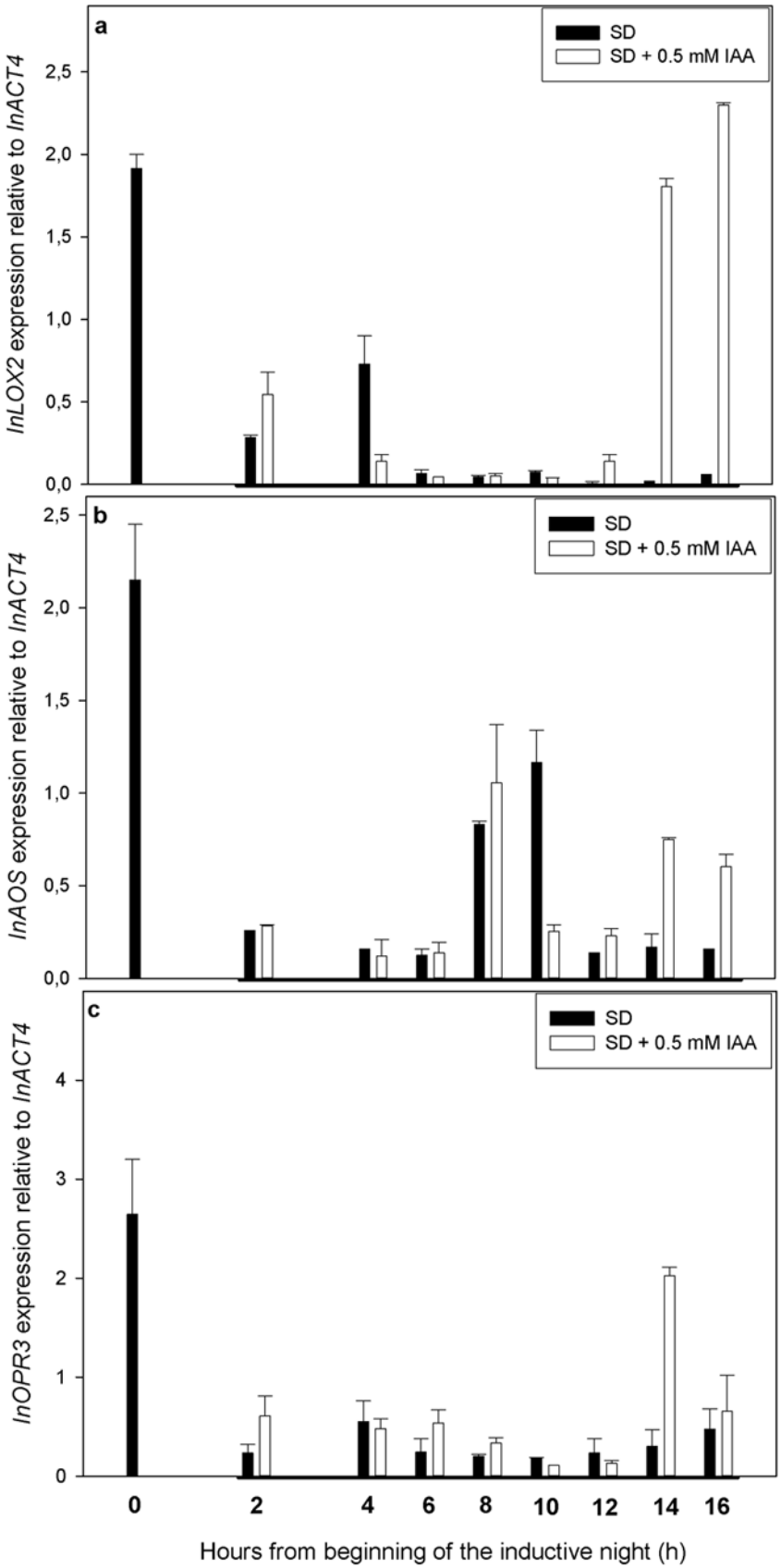

Fig. 2 The expression level (related to *InACT4*) of *InLOX2* (**a**), *InAOS* (**b**), and *InOPR3* (**c**) in the cotyledons of *I. nil* seedlings during inductive night and treated with auxin. Black bars refer to SD conditions, gray bars refer to IAA applied before transfer to SD conditions. Means ±*SE*; $n = 3$. The thick horizontal line under the time axis indicates duration of the dark period.

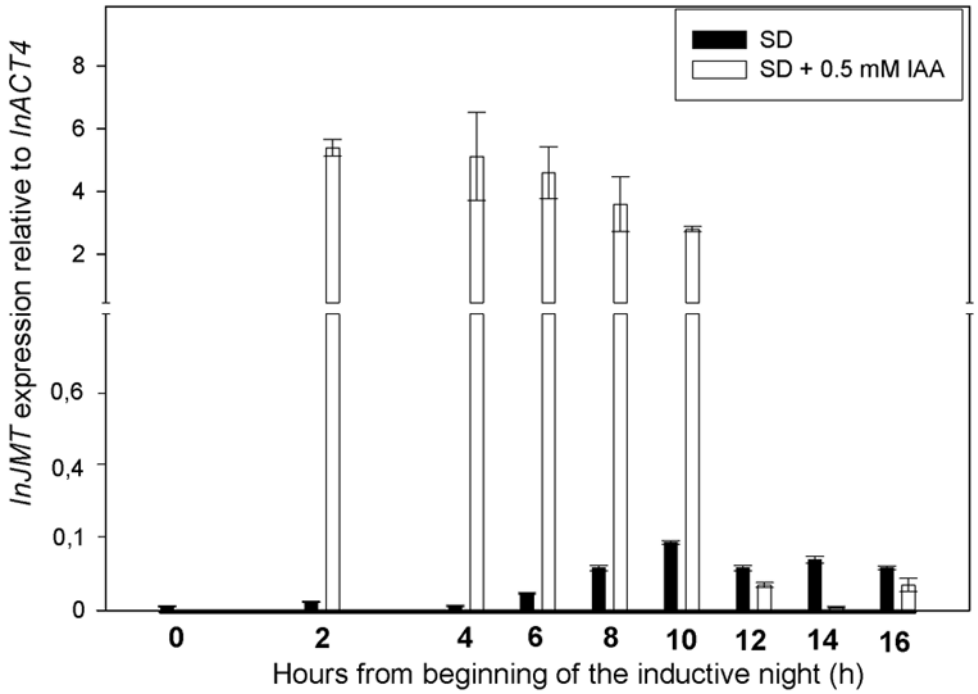

Fig. 3 The transcriptional activity of *InJMT* (related to *InACT4*) in the cotyledons of *I. nil* seedlings during inductive night and treated with auxin. Black bars refer to SD conditions, gray bars refer to IAA applied before transfer to SD conditions. Means ±*SE*; $n = 3$. The thick horizontal line under the time axis indicates duration of the dark period.

Tab. 1 Specific primers and probes used in qRT-PCR reactions.

Name of gene	GeneBank accession No.	Primer sequence 5′–3′	UPL probe No.	Product size (bp)
InLOX2	CJ761975	F: CACGCGGACAAGAAGGAT R: TCAGGATTCCAATCAGGTCA	64	70
InAOS	HM357792	F: CGGAGATGTTTGAGGGTTTG R: CATTCAAGCCGACTTTACCC	134	60
InOPR3	HM357793	F: GTGGGTCGTGCATCTCATC R: GGCTTATCAGTGGAAGATATAGGC	138	68
InJMT	KF573520.1	F: CCAAGAATTCTACTTTGCAGATGA R: AAATGCCGTTTAATGCTTCTTC	67	75
InACT4	HM802138.2	F: GGAAATACAGTGTCTGGATTGGA R: CCACATCTGTTGGAATGTGC	139	65

F – forward primer; R – reverse primer.

mediate auxin-induced gene expression responses, may regulate late stamen development [45]. The *arf6-2 arf8-3* double mutant, as well as male-sterile JA-mutants (*coi1-1*, *opr3*), are defective in anther dehiscence, exhibit shorter filaments and produce unviable pollen grains [13,42,46]. It was revealed that auxin acts through JAs to control proper flower development, because ARF6 and ARF8 are required for the expression of JA biosynthetic pathway genes (*DAD1*, *LOX2*, *AOS*, and *OPR3*) in flower buds and consequently regulate JAs biosynthesis, which in turn activate *MYB21* and *MYB24*. Then MYB induce secondary gene expression responses to JAs stimulating petal and stamen filament elongation and anther dehiscence [45,47,48]. It cannot be excluded that IAA may influence on JA biosynthesis in the cotyledons and this mechanism regulates its flowering of *I. nil*. We showed that applying IAA just before the inductive

night strongly stimulated the expression of *InJMT*, responsible for conversion of JA to JAMe (Fig. 3), which enhanced the level of methyl jasmonate in the cotyledons of *I. nil* seedlings (Fig. 1). On the contrary, this relationship was not observed in the case of *InLOX2*, *InAOS*, and *InOPR3* transcriptional activity. The low expression level of these genes does not exclude their involvement in the regulation of flower induction mediated by IAA. The presence of even very low amount of any transcript can trigger the cascade of events on the cellular level and can lead to a normal physiological response. Another possible way is the transport of JA, synthesized in other tissues and distributed in the cotyledons of *I. nil*. It has been previously suggested by Zhang and Baldwin [49], as well as Heil and Ton [50] that JA is a long distance signaling compound. Moreover, we propose the possible posttranslational modification and activation of enzymes involved in JA biosynthesis, which can be immediately methylated. Gfeller et al. [51] reported that posttranslational modification, potentially modifying the activities of proteins involved in JA production, rather than their levels. A low content of JAMe in the cotyledons seedlings subjected to induction is one of the requirements for the flowering [20,22]. Therefore, the excess of JAMe over JA during flower induction may be a consequence of higher metabolic activity and stability as well as better permeability of the ester form [52]. This provides an explanation that the mechanism of flower inhibition by auxin is dependent on JAMe. Indeed, the interdependent action of both hormones in the regulation of other physiological processes was also observed during root, stamen, and filament growth [32,53,54].

This work confirms that *InJMT* involved in JAs metabolism in *I. nil* demonstrates a high homology to genes identified in other plant species. Exogenous IAA participates in *I. nil* flower induction process by stimulating *InJMT* expression and, as a consequence of that, enhancing the level of JAMe, a flowering inhibitor. Despite the fact that there were previously published data about jasmonates and IAA involvement in flower morphogenesis, our results are the first ones concerning these two classes of phytohormones interactions in flower induction, which paves the way for subsequent flower morphogenesis. Knowledge of the hormonal mechanisms and hormonal interactions controlling the process of flower induction is of great importance for regulation of fertility. Besides a cognitive aspect, it could have a practical one and can be used in future investigations.

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