Phytohormone Profiling of Potato (*Solanum tuberosum* L.) Exposed to French Marigold (*Tagetes patula* L.) Essential Oil

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Abstract

French marigold (*Tagetes patula* L.) is an aromatic plant known for its repellent effects on pests. It is traditionally grown in fields near many vegetable crops, including potato (*Solanum tuberosum* L.). However, the effects of marigold essential oil (EO) on the physiology of neighboring crops have been neglected in research. The aim of this study was to establish, for the first time, a comprehensive phytohormone profile of potato plants exposed to French marigold EO for different time periods (4, 8 and 12h). Endogenous levels of all major phytohormone groups, determined by HPLC-MS analysis, showed altered phytohormone responses of EO-exposed potato plants. The most affected were stress-related phytohormones: abscisic acid-glucose ester, 1-aminocyclopropane-1-carboxylic acid, and jasmonic acid. Increased levels of jasmonic acid, cytokinins, storage form of abscisic acid together with decreased levels of indole-3-acetic acid and ethylene precursor were observed. In most of the analyzed phytohormone groups similar response pattern was observed – an increase in levels after short time exposure (4h), followed by a decrease to control values after prolonged exposure (8h and 12h). Expression levels of genes involved in biosynthesis and catabolism of stress-related phytohormones, obtained by *de novo* bioinformatic processing of data from cDNA microarray analysis, revealed that jasmonic acid biosynthetic pathway was the most affected, with the highest number of altered transcripts and with one of the genes from this pathway (*12-oxophytodienoate reductase 1-like*) exhibiting the highest expression rate.

Keywords: Plant volatiles, plant-to-plant communication, transcriptomics, plant hormonomics, stress-related phytohormones

Introduction

Essential oils (EOs) are complex mixtures of volatile compounds. These chemical signals are emitted into the atmosphere, where other organisms can receive the message they convey (Bouwmeester et al. 2019; Ninković et al. 2021). So far it is fairly unknown how this message affects the physiological responses of neighboring plants. Are phytohormones, as the core of the regulating network, involved in the transmission of this environmental signal? Could EO-induced changes in phytohormonal status lead to enhanced defense of neighboring plants? These are still burning questions to which this research attempts to find answers.

Having in mind nutritional and economic importance, as one of the main reasons for the popularity of potato (*Solanum tuberosum* L.) as a research model in plant and agricultural biology on one side, and French marigold (*Tagetes patula* L.) as an important companion plant on the other, our team has previously established a laboratory system in which potato plants were exposed to French marigold essential oil (Stupar et al. 2021). This controlled experimental setup was used to better understand the molecular background and other aspects of "communication" between crop plants and EO-emitting companion plants. Our previous research has shown that

French marigold EO triggered significant transcriptional changes in exposed potato plants after 8 hours exposure, with α-linolenic acid metabolism and plant hormone signal transduction as one of the most affected pathways (Stupar et al. 2021). These results indicated that phytohormones have an important role in response to EO. It will be interesting to see how French marigold EO affects the phytohormonal response of exposed potato plants – does phytohormone status change in a stress response manner, and if so, does it lead to phytotoxicity or could the plant benefit from it by, for example, inducing or priming plant defenses.

Similarly, several studies investigating the effects of volatiles on plant metabolism have shown that volatiles affect phytohormonal signaling pathways (Ameye et al. 2018; Ye et al. 2019; Dani and Loreto 2022). For example, green leaf volatiles have been shown to enhance jasmonic acid (JA)-induced defense responses in *Arabidopsis* (Hirao et al. 2012). A common herbivore-induced plant volatile, indole, has been shown to induce herbivore resistance in exposed rice plants by modulating JA signaling pathway (Ye et al. 2019). There is also evidence that some volatiles activate defense responses in a JA-independent manner, such as monoterpenes which promote systemic acquired resistance (SAR) in *Arabidopsis* through salicylic acid (SA) and azelaic acid (AzA) signaling (Riedlmeier et al. 2017).

The above-mentioned examples not only demonstrate that phytohormonal signaling pathways can be altered upon receiving volatile signal, but also argue that plant defense responses are affected by the activation of various phytohormonal signaling cascades. Therefore, to gain a better insight into the control and coordination of plant defense mechanisms, it is important to understand the levels of individual phytohormones.

Until recently, most studies on the role of phytohormones in plant defense focused only on "stress-related" phytohormones, particularly JA and SA, while other phytohormones were neglected. However, emerging information on some growth-related phytohormones suggests that they also play a role in defense and susceptibility to pathogens and herbivores (Akhtar et al. 2020). The balance between "stress-" and "growth-related" phytohormones is essential for both plant development and defense responses. Phytohormonal crosstalk is what enables plants to adjust their defenses while optimizing their growth and development (Vos et al. 2015). The importance of the interplay between phytohormones in regulating every aspect of plant physiology, including defense, is one of the reasons why a comprehensive phytohormone study has been of such great interest.

Due to our knowledge, there are no data in the literature focusing on the phytohormonal status of plants exposed to complex EO volatile mixtures, only reports referring to the effects of some individual EO components, such as citral and farnesene, on certain plant hormones (Grana et al. 2013; Araniti et al. 2017; Werrie et al. 2020). However, not only that complex volatile blends such as EOs can have very different effects than their specific components they could also provide more accurate information about environmental stressors and enable plants to better manage their responses. Thus, this research could not only provide important insight into the fluctuations in plant endogenous phytohormone levels triggered by EOs and volatiles in general, but also contribute to future understanding of the integrated effects of multiple volatile signals specific to complex volatile mixtures such as EOs.

Hence, the aim of the present study is to reveal, for the first time, a comprehensive phytohormonal profile of potato plants upon interaction with French marigold EO. Changes in the expression levels of genes involved in phytohormone metabolism are shown in order to provide a more vivid picture of the phytohormonal status of the EO-exposed plant by presenting occurrences on the level of phytohormone biosynthesis and catabolism.

Materials and Methods

Experimental design

The experimental setup and plant growth conditions were previously described by Stupar et al. (2021). Shortly, 18-day-old potato plants (*Solanum tuberosum* L.) germinated from chemically-untreated tubers and cultivated individually in 5 L glass jars containing soil mixture, were exposed to French marigold (*Tagetes patula* L.) essential oil (EO) of known composition for 4, 8, and 12 hours. Detailed list of compounds contributing to the EO composition is published in Stupar et al. (2021), and herein a graphical presentation of volatiles' chemical groups is presented (Fig. 1). The essential oil (10 µL) was applied to the filter paper and placed in the jar on the metal holder without any physical contact with the potato plants. Subsequently, the jars were tightly closed with lids and sealed with parafilm. Jars containing the control plants were maintained under the same conditions, but without

exposure to EO. Plant material (potato leaves) was collected from 3 or 4 plants per treatment, homogenized in liquid nitrogen, and kept at -80°C for further analysis.

Analysis of cDNA microarray data

Complementary DNA (cDNA) microarray analysis, reported by Stupar et al. (2021), was performed on RNA isolated from potato samples exposed for 8 h to EO and untreated control plants, using *de novo* assembled Agilent® SurePrint G3 CustomPotato GE 8x60 K Array platform. Expression of genes involved in biosynthesis and catabolism of stress-related phytohormones was extracted from microarray data processed in R 3.6 (R Core Team, 2019; Stupar et al. 2021), which was for the purposes of this study subjected to additional bioinformatic analyses which included cross-referencing of microarray data with the KEGG Pathways Database (Kyoto Encyclopedia of Genes and Genomes). Results are presented as color-coded heatmaps. Data shown correspond to $\log_2$ of fold change ($\log_2$FC) values (with FC≥2 cut off) of differentially expressed transcripts ($p \leq 0.05$, n=4).

Microarray results related to phytohormonal metabolism are available at the trusted digital repository RADaR, at https://hdl.handle.net/21.15107/rcub_ibiss_5388.

Phytohormone analysis

The analysis of phytohormones was performed as described in Dobrev and Vankova (2012) and Djilianov et al. (2013). Approximately 100 mg of homogenized frozen plant material of each biological sample was lyophilised and cold extraction buffer consisting of methanol/formic acid/water (15/1/4; v/v/v) was added to the plant homogenates along with a mixture of stable isotope-labeled internal standards (10 pmol). The list of internal standards used was as follows: [$^{13}C_6$]indol-3-acetic acid (IAA; Cambridge Isotope Laboratories, Tewksbury, MA); [$^2H_4$]salicylic acid (SA; Sigma-Aldrich); [$^2H_3$]phaseic acid (PA; NRC-PBI, Saskatoon, Canada); [$^2H_5$]jasmonic acid (JA; C-D-N Isotopes Inc., Pointe-Claire, Canada); [$^2H_6$]abscisic acid (ABA; NRCPBI); [$^2H_5$]*trans*-zeatin (*t*Z); [$^2H_5$]*trans*Z-9-riboside (*t*ZR); [$^2H_5$]*trans*Z-7-glucoside (*t*Z7G); [$^2H_5$]*trans*Z-9-glucoside (*t*Z9G); [$^2H_5$]*trans*Z-*O*–glucoside (*t*ZOG); [$^2H_5$]*trans*ZR-*O*–glucoside (*t*ZROG); [$^2H_5$]*trans*ZR-5´-monophosphate (*t*ZRMP); [$^2H_3$]dihydrozeatin (DHZ); [$^2H_3$]DHZ-9-riboside (DHZR); [$^2H_3$]DHZ-9-glucoside (DHZ9G); [$^2H_6$]N^6-(Δ^2-isopentenyl)adenine (iP); [$^2H_6$]N^6-(Δ^2-isopentenyl)adenosine (iPR); [$^2H_6$]iP-7-glucoside (iP7G); [$^2H_6$]iP-9-glucoside (iP9G); [$^2H_6$]iPR-5´-monophosphate (iPRMP); and [$^2H_4$]1-aminocyclopropane-1-carboxylic acid (ACC) (*t*Z, *t*ZR, *t*Z7G, *t*Z9G, *t*ZOG, *t*ZROG, *t*ZRMP, DHZ, DHZR, DHZ9G, iP, iPR, iP7G, iP9G, iPRMP and ACC standards were from OlChemlm, Olomouc, Czech Republic). The concentration of *cis*-zeatin (*cis*Z) derivatives was established based on the retention times and mass spectra of the unlabeled standards and the response ratio of their *trans*Z counterparts. The system of cytokinin (CK) abbreviations was accepted and adapted according to Kamínek et al. (2000).

Detection and quantification of phytohormones were performed by HPLC (Ultimate 3000, Dionex, Sunnyvale, CA, USA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (3200 Q TRAP, Applied Biosystems, Foster City, CA, USA) set in the selected reaction-monitoring mode, negative for fraction A and positive for fraction B (Representative chromatograms from fraction A and fraction B of one sample of potato leaf extract analyzed are located in Supplementary material-Online Resource 2). Fractions were obtained by reversed phase and ion-exchange chromatography (Oasis-MCX, Waters, Milford, MA, USA). Fraction A, eluted with methanol, contained phytohormones of acidic and neutral character such as auxins, ABA, SA, JA and their derivatives, while fraction B, eluted with 0.35 M NH_4OH in 70% methanol contained the phytohormones of basic character (CKs) and ACC.

The mass spectrometer was set at electrospray ionisation mode with the following ion source parameters: ion source voltage −4000 V (negative mode) or +4500 V (positive mode); nebuliser gas 50 psi; heater gas 60 psi; curtain gas 20 psi; heater gas temperature 500°C. The phytohormones were quantified using the isotope dilution method with multilevel calibration curves. All gathered data were processed with Analyst 1.5 software (Applied Biosystems). The concentrations of analyzed phytohormones were calculated as the amount (pmol) per 1 g fresh weight (FW) of plant material.

To evaluate endogenous levels of phytohormones, three biological replicates (n=3) were used for each time point for both EO-exposed potato plants (4, 8, and 12 h) and untreated control. The analyses were repeated three times with comparable results.

Statistical analysis was performed using SAS software (SAS Institute, 2002. SAS/STAT, ver. 9.00. SAS Institute Inc., Cary, NC, USA). Results were expressed as mean values of three biological replicates ± standard error (SE). Statistical processing of the data included one-factorial analysis of variance (ANOVA) and comparison of the means using Fisher's least significant difference (LSD) post-hoc test with a significance level of 0.05 ($p \leq 0.05$).

Results

To identify the effects of French marigold essential oil (EO), with known volatiles composition (Fig. 1) on phytohormone metabolism of exposed potato plants, we examined the levels of major phytohormones, their precursors and metabolites, as well as the expression levels of genes involved in the biosynthesis and catabolism of stress-related phytohormones.

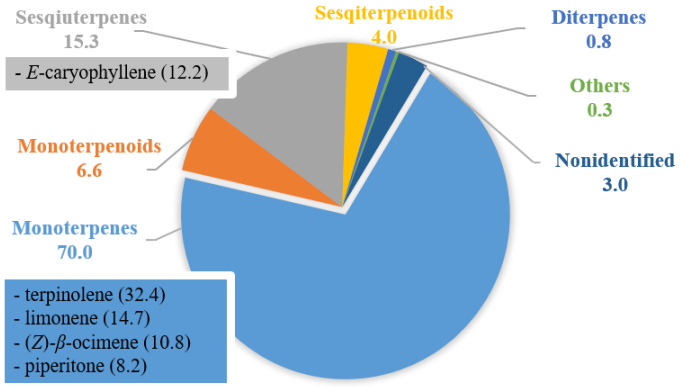

Fig. 1 Phytochemical composition (%) of French marigold essential oil used in the study. The complete list of compounds is presented in Stupar et al. 2021.

Stress-related phytohormones

The results of microarray gene expression analyses showed that French marigold EO induced jasmonic acid (JA) biosynthesis in potato plants after an 8 h exposure, as the majority of identified genes belonging to this metabolic pathway were upregulated (Fig. 2). Among the upregulated genes, the gene coding for 12-oxophytodienoate reductase 1-like (*OPR*) showed the highest change in expression levels with $\log_2$FC of 6.45. This is also the highest detected change in gene expression among all phytohormone-related genes analyzed in this study. Interestingly, we also detected a slight change in the expression of the gene encoding methyl jasmonate esterase (*MJE*), an enzyme responsible for the conversion of methyl jasmonate (MeJA) to JA, which was downregulated with $\log_2$FC of -1.56.

Analysis of potato leaves revealed significant increase in JA levels after a 4 h exposure to French marigold EO (Fig. 2). Potato plants exposed to EO for 4 h had approximately 2.5-fold higher levels of JA than non-exposed control plants. On the other hand, no statistically significant changes in JA levels were observed after prolonged exposure (8 and 12 h). Despite the similar response pattern with a maximum reached at 4 h, JA-Isoleucine, a bioactive metabolite of JA, and JA precursor *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) showed no significant changes in endogenous levels after exposure to EO compared to control plants. Conversely, for both metabolites and also JA, there was a statistically significant difference between the levels measured after 4 h on one side, and the levels measured after 8 and 12 h on the other side (Fig. 2b).

Microarray analysis of 8 h EO-exposed potato plants showed induction ($\log_2$FC=3.36) of phenylalanine ammonia lyase-like (*PAL*) and repression ($\log_2$FC=-2.14) of *AIM1*, both of which encode enzymes involved in the SA biosynthetic pathway that takes place in the cytosol, and repression ($\log_2$FC up to -2.14) of the gene for

isochorismate synthase (*ICS*), which is involved in the chloroplastic biosynthetic pathway (Fig. 2c). Only one of the genes involved in the catabolism of SA, coding for uridine diphosphate-glycosyltransferase 74F1-like (*UGT74F1*), was affected, with noticeably high $\log_2$FC value of 3.89 (Fig. 2c).

Values recorded for SA levels in potato plants exposed to EO were unchanged in comparison to the non-exposed control at all EO-exposure periods analyzed, however, a significant difference was observed between 4 h and 12 h EO treatments, with prolonged exposure leading to a decrease in SA levels (Fig. 2d).

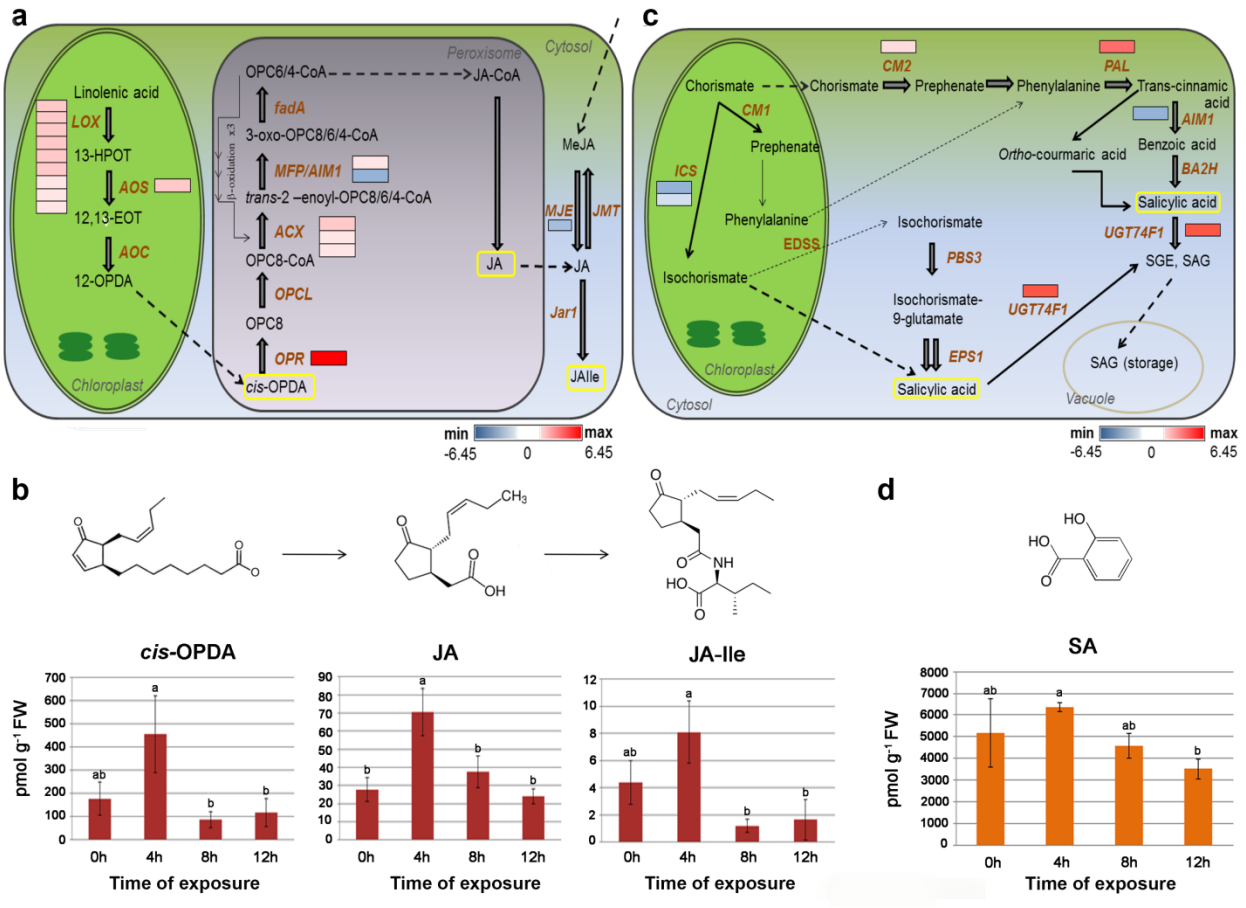

Fig. 2 Proposed **(a)** jasmonic acid (JA) and **(c)** salicylic acid (SA) metabolic pathways and associated genes expression in potato plants exposed to French marigold EO for 8 hours. Levels of differentially expressed genes ($p \leq 0.05$, n=4), corresponding to $\log_2$FC values obtained by cDNA microarray analysis, are presented as a single rectangle on color coded heat maps (blue, downregulated; red, upregulated). Content of **(b)** jasmonates (cis-OPDA, JA and JA-Ile) and **(d)** SA in potato plants exposed to French marigold EO for different time periods (4, 8, and 12 h). Values are presented as means ± standard errors (n=3) and expressed in pmol g^{-1} FW. Different letters denote values that are statistically different based on the LSD test, $p \leq 0.05$. For abbreviations, see the text and the Supplementary Table S1.

Although expression of genes from the abscisic acid (ABA) biosynthetic pathway was only slightly altered compared with control plants (Fig. 3a), an increased accumulation of ABA (Fig. 3b) and its metabolite phaseic acid (PA) and catabolic derivatives 9'-hydroxy ABA (9OH-ABA) and ABA-glucose ester (ABA-GE), was observed after 4 h long treatment with French marigold EO (Fig. 3c). However, this increase was significant only for ABA-GE which showed a considerable (9.34-fold) increase in level after a 4 h EO exposure (Fig. 3c). A significant decrease in levels was also observed for ABA and ABE-GE after prolonged exposure (12 h) compared with a short exposure (4 h) (Figs. 3b and c).

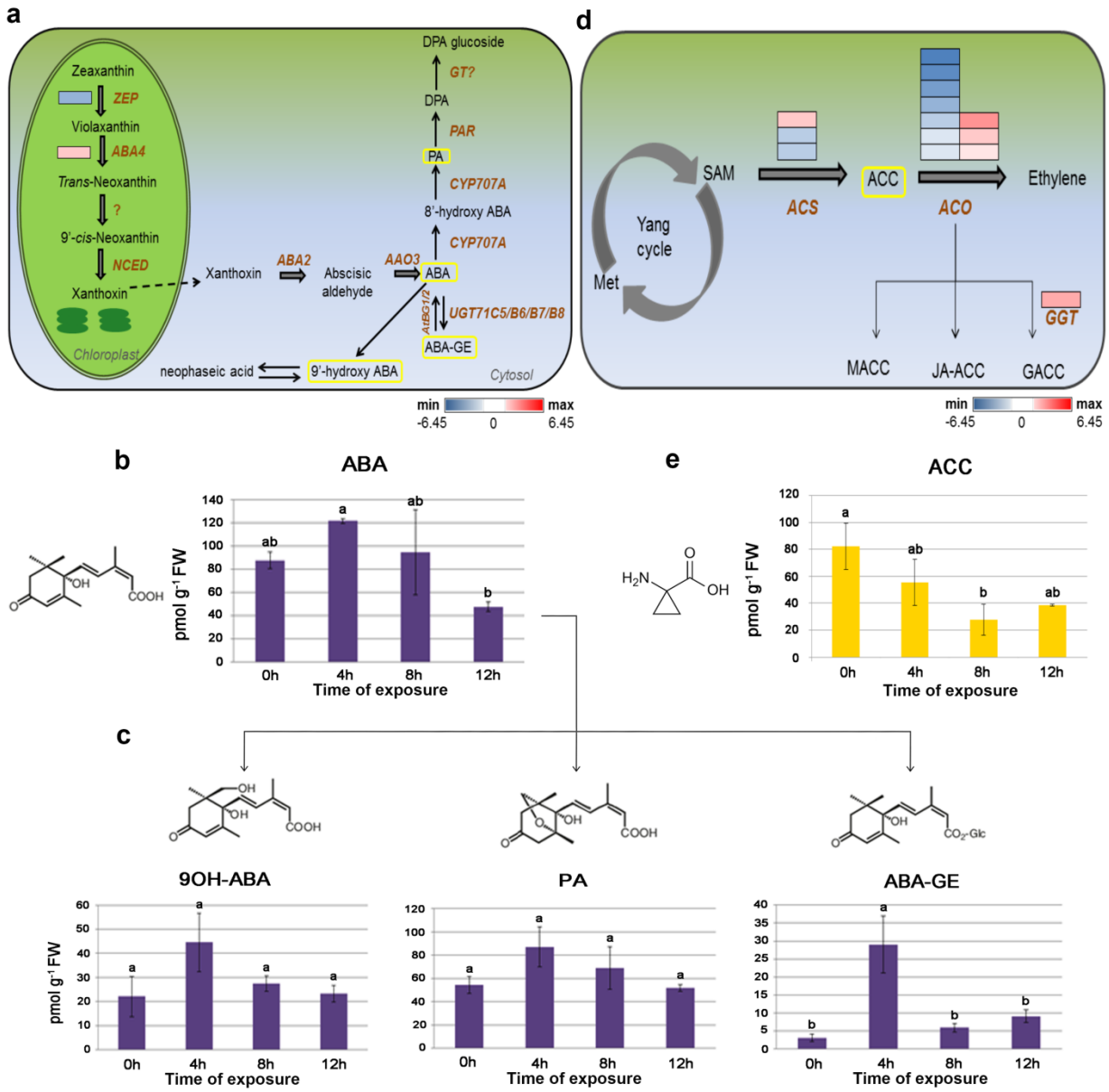

Fig. 3 Proposed **(a)** abscisic acid (ABA) and **(d)** ethylene metabolic pathways and associated genes expression in potato plants exposed to French marigold EO for 8 hours. Levels of differentially expressed genes ($p \leq 0.05$, n=4), corresponding to $\log_2$FC values obtained by cDNA microarray analysis, are presented as a single rectangle on color coded heat maps (blue, downregulated; red, upregulated). Content of **(b)** ABA and **(c)** ABA metabolites (9OH-ABA, PA and ABA-GE) and **(e)** ethylene precursor ACC in potato plants exposed to French marigold EO for different time periods (4, 8, and 12 h). Values are presented as means ± standard errors (n=3) and expressed in pmol g^{-1} FW. Different letters denote values that are statistically different based on the LSD test, $p \leq 0.05$. For abbreviations, see the text and the Supplementary Table S1.

The biosynthetic pathway of ethylene (ET) was altered after 8 h exposure to French marigold EO, as shown by the results of microarray analysis (Fig. 3d). The majority of differentially expressed transcript variants coding for 1-aminocyclopropane-1-carboxylate synthase (ACS), which is responsible for the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (ACC), and aminocyclopropane-1-carboxylate oxidase (ACO), which

converts ACC to ET, were downregulated, with a maximum change in expression levels of log_2FC=-3.56. Genes encoding γ-glutamyl transpeptidase 3 (GGT3), an enzyme responsible for the formation of derivatives of ACC and glutathione, exhibited slightly increased expression, with log_2FC=1.74 (Fig. 3d). Endogenous levels of ACC, the direct ET precursor, decreased 66.79% after 8 hours of exposure compared with control levels in untreated plants (Fig. 3e), confirming the observed change in gene expression.

Growth and development-related phytohormones

Content of auxin indol-3-acetic acid (IAA) showed a statistically significant decrease after 8 h (38.19%) and 12 h of EO exposure (30.22%) compared with control, whereas its precursor indole-3-acetamide (IAM) showed no changes in endogenous concentrations after exposure to EO, as well as IAA major primary catabolite 2-oxindole-3-acetic acid (OxIAA) (Fig. 4a). On the other hand, changes were observed in IAA-aspartate (IAA-Asp), one of the most common IAA-amino acid conjugates, which levels increased 2.37-fold after 12 h exposure to French marigold EO. Levels of the non-indole auxin analogue phenylacetic acid (PAA) were significantly lower after treatment with EO (all analyzed periods considered), reaching their lowest value after an 8 h exposure period, with a 58.32% decrease, while another non-indole auxin analogue, benzoic acid (BzA), showed no altered levels after exposure to EO (Fig. 4b).

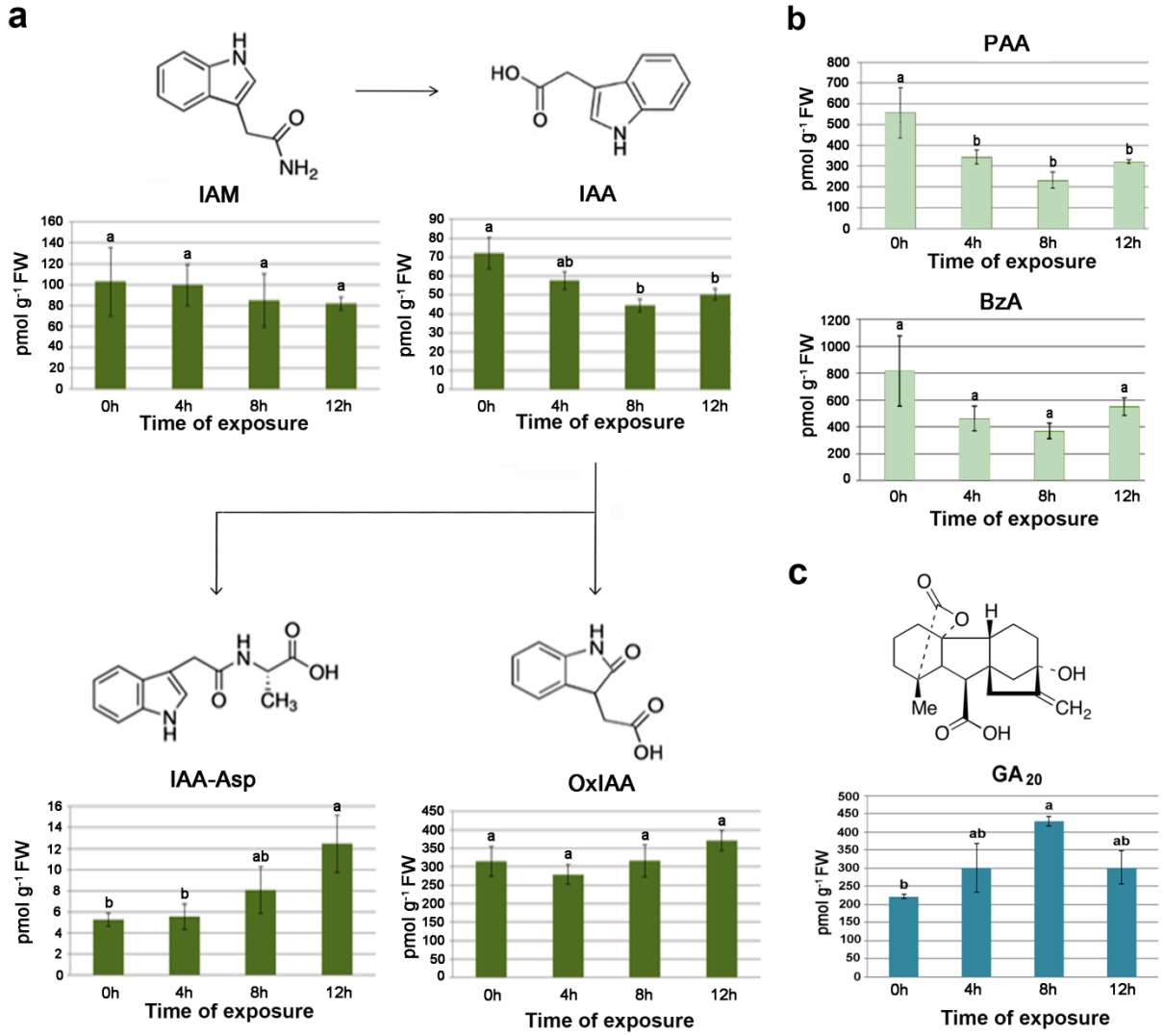

Fig. 4 Content of **(a)** indole-3-acetic acid (IAA), its precursor indole-3-acetamide (IAM) and metabolites IAA-aspartate (IAA-Asp) and oxo-IAA (OxIAA), **(b)** auxin analogues phenylacetic acid (PAA) and benzoic acid (BzA), and **(c)** gibberellin GA_{20} in potato plants exposed to French marigold EO for different time periods (4, 8, and 12 h). Values are presented as means ± standard errors (n=3) and expressed in pmol g^{-1} FW. Different letters denote values that are statistically different based on the LSD test, $p \leq 0.05$. For abbreviations, see the text and the Supplementary Table S1.

Gibberellin GA_{20}, precursor of the bioactive phytohormone GA_1, showed differences in levels only in the samples exposed for 8 hours. The measured levels were increased 1.93-fold compared to the non-exposed control (Fig. 4c).

The results of phytohormone analysis for endogenous cytokinin (CK) levels are arranged according to CK conjugation status and presented in five different groups: CK free nucleobases (*cis*Z, *trans*Z, DHZ, iP), CK ribosides (*cis*ZR, *trans*ZR, DHZR, iPR), *O*-glucosides (*cis*ZOG, DHZOG, *cis*ZROG, *trans*ZROG, DHZROG), *N*-glucosides (*cis*Z7G, *trans*Z7G, DHZ7G, iP7G, *cis*Z9G, *trans*Z9G, DHZ9G, iP9G), CK phosphates (*cis*ZRMP, *cis*ZRDP, *cis*ZRTP, *trans*ZRMP, *trans*ZRDP, *trans*ZRTP, DHZRMP, DHZRDP, DHZRTP, iPRMP, iPRDP, iPRTP).

Total CK levels were significantly increased (1.36-fold) after 4 h of exposure compared with control (Fig. 5a), as well as the levels of *N*-glucosides (1.33-fold) (Fig. 5b). A similar change was observed for CK free nucleobases, the bioactive CK forms, which also showed a significant increase after short exposure (1.91-fold), whereas the levels of CK ribosides, CK phosphates, and *O*-glucosides remained unchanged after all exposure periods.

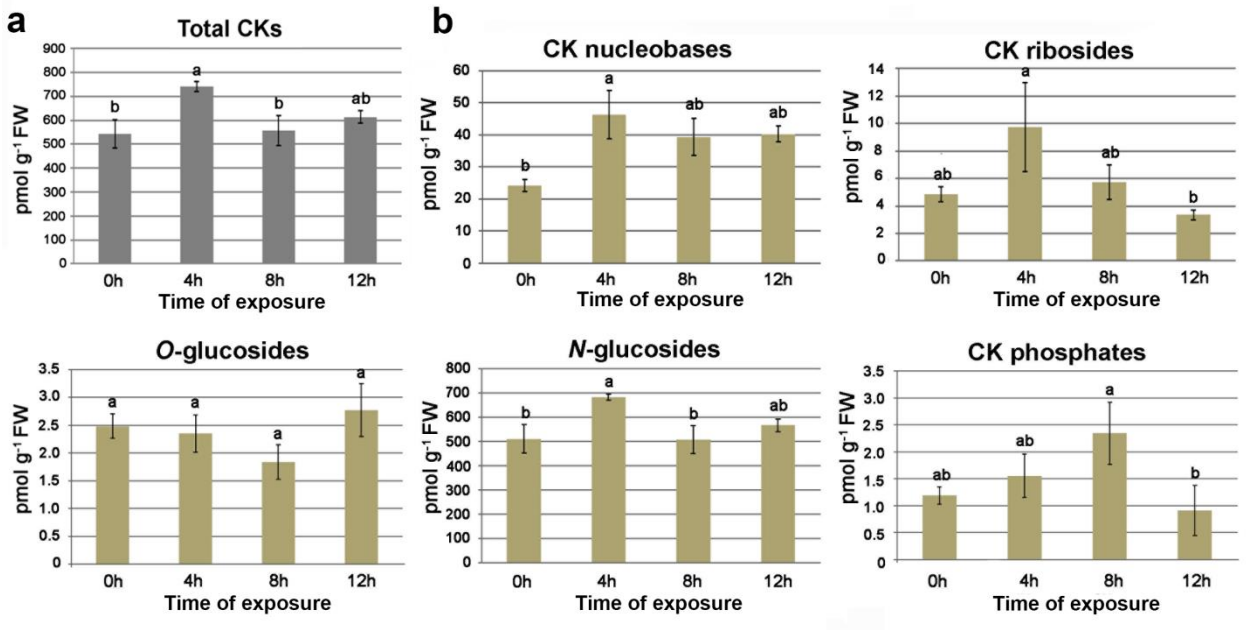

Fig. 5 Content of **(a)** total cytokinins (CKs) and **(b)** CK groups classified based on their conjugation status in potato plants exposed to French marigold EO for different time periods (4, 8, and 12 h). Values are presented as means ± standard errors (n=3), and expressed in pmol g^{-1} FW. Different letters denote values that are statistically different based on the LSD test, $p \leq 0.05$. For abbreviations, see the text and the Supplementary Table S1.

Analysis of individual CK conjugates (Supplementary Table S2), showed that the change in the content of CK free nucleobases reflected only the change in the *cis*Z levels, since all other nucleobases exhibited unchanged levels upon exposure to EO. The *cis*Z levels were increased after all exposure times, with the highest alteration rate occurring after 4 h exposure. More detailed analyses of *N*-glucoside content showed changes in the levels of

DHZ7G and iP7G, which were increased after 4 h EO exposure, and in the levels of *trans*Z9G, which were increased after all exposure times (Supplementary Table S3).

Analysis of the results in terms of the chemical structure of the CKs suggests that only DHZ- and iP-type CKs show statistically significant differences in their levels after 4 h of exposure to EO compared with the control. CKs of the *trans*Z- and *cis*Z-types showed no changes in their concentrations compared with the control, the only difference was observed when comparing the levels of *trans*Z-types from plants exposed to EO for 4 h and 12 h (Supplementary Table S4).

Discussion

Although the mechanisms underlying volatile-induced responses in plants are complex and not yet fully comprehended, it is becoming increasingly clear that phytohormones play an important role in plant responses to environmental volatiles.

Perception of different elicitor cues triggers different defense responses regulated by phytohormones, especially stress-related ones such as jasmonic acid (JA) and salicylic acid (SA), although others play important roles as well (Denancé et al. 2013; Akhtar et al. 2020). Volatiles have been shown to increase plant defense by enhancing JA signaling. For example, green leaf volatiles have been demonstrated to induce and prime the expression of JA biosynthesis genes in lima bean and poplar (Arimura et al. 2000; Frost et al. 2008) and enhance JA-induced responses in Arabidopsis (Hirao et al. 2012) and maize (Engelberth et al. 2004). It has also been shown that indole can prime the formation of bioactive jasmonate, JA-isoleucine (JA-Ile), in maize (Erb et al. 2015) and induce herbivore resistance in rice by increasing JA accumulation (Ye et al. 2019). Herbivore-induced volatile (E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) can trigger JA-dependent defense responses of neighboring tea plants (Jing et al. 2021).

Potato plants exposed to French marigold essential oil (EO) for 8 h exhibited induced expression of genes related to the biosynthesis of JA, however, an increased amount of endogenous JA was detected only after a 4 h treatment (Fig. 2a and b). This could be explained by a burst of synthesized JA upon recognizing the EO signal and subsequent return to baseline after prolonged exposure period. Tissue damage, e.g. by local wounding by herbivores, could lead to an immediate increase in JA levels within a few minutes (Glauser et al. 2008; Mielke et al. 2011), because enzymes involved in JA biosynthesis are constitutively present in leaf tissues (Stenzel et al. 2003b), whereas the transcriptional machinery for the expression of genes coding for linoleate lipoxygenase (*LOX*), allene oxide synthase (*AOS*), allene oxide cyclase (*AOC*), and 12-oxophytodienoate reductase 3 (*OPR3*) is activated later, after at least 15 min (Stenzel et al. 2003a, b; Chung et al. 2008; Koo and Howe 2009). If the EO signal is to be perceived as a stress warning signal, a similar scenario could be expected in our exposed potato plants. However, allocating resources to defense in absence of predators, among other things, negatively affects yield and is therefore not cost-efficient. For this reason, long-lasting activation of induced defense cannot be achieved by modifying phytohormone signaling, which could explain the need for decrease of JA levels after 8 and 12 h EO exposure and their subsequent return to control levels. However, this does not mean that our potato plants are not better fitted to protect themselves after prolonged period of exposure, since there is a possibility of achieved "memory" or so-called priming of exposed potato plants. In addition, an 8 h EO exposure resulted in a downregulation of methyl jasmonate esterase (*MJE*) (Fig. 2a), suggesting less conversion of methyl jasmonate (MeJA) to JA, which likely contributes to leveling JA content and enables more MeJA for intra- and inter-plant signaling. This is particularly interesting because MeJA has been suggested as one of the signals most likely responsible for "communication" between plants (Farmer and Ryan 1990; Arimura et al. 2000; Yamashita et al. 2021).

In plants, JA and SA are closely linked and influence each other through an intricate network of synergistic and antagonistic interactions (Yang et al. 2015; Li et al. 2019; Zhao and Li 2021). Microarray analysis has shown that isochorismate (IC) biosynthetic pathway, which is responsible for the synthesis of more than 95% of SA (Chen et al. 2009; Qi et al. 2018), was deactivated, and the phenylalanine ammonia-lyase (PAL) pathway was activated in the present study. *Trans*-cinnamic acid, produced by the activity of PAL, can be converted to SA via benzoic acid (BzA) or via *ortho*-coumaric acid (*o*-CA) (Maruri-López et al. 2019). Production of BzA was downregulated in

exposed potato plants (Fig. 2c), likely favoring the *o*-CA branch of SA production. Unchanged levels of BzA are consistent with these findings (Fig. 4b).

Since potato plants exposed to French marigold EO during our experiment did not show statistically significant changes in SA concentration (Fig. 2d), it is assumed that EO volatiles suppressed SA signaling and/or SA-dependent defenses by interacting with JA. A number of constituents have been shown to mediate antagonistic interactions between JA and SA, such as NONEXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and some transcription factors such as WRKY or TGA (Caarls et al. 2015; Verma et al. 2016); these types of relations affect phytohormone synthesis or signaling (Oka et al. 2013) as well as expression of defense genes (Li et al. 2019).

Inactivation of SA is performed in the cytosol by modification of SA with glucose, forming SA glucoside (SAG) or SA glucose ester (SGE), the storage forms of SA (Chong et al. 2001; Ruuhola and Julkunen-Tiitto 2003; Lefevere et al. 2020). The upregulated expression of *UGT74F1* (Fig. 2c), which encodes the enzyme involved in the conversion of SA to mainly SAG, speaks in favor of induced storage. This could explain the unchanged levels of SA in exposed potato plants (Fig. 2d). However, this also implies a better potential for a SA-related defense response upon future attacks, since more SA is likely to be present in the reservoir form waiting to be converted.

Although no significant changes in the metabolism of abscisic acid (ABA) were detected, another stress-related phytohormone, ABA catabolic derivative, ABA-glucose ester (ABA-GE), showed higher levels than the non-exposed control after a 4 h exposure to EO (Fig. 3c). In addition to its role in maintaining ABA homeostasis, this hydrolysable ABA conjugate is considered to be root-to-shoot signaling molecule, as well as ABA reservoir (Burla et al. 2013). It has been shown that ABA-GE concentrations increase significantly under drought, salt, and osmotic stress (Sauter et al. 2002; Brunetti et al. 2019). The elevated levels of ABA-GE in our experiment may indicate that our EO signal is recognized as something comparable to environmental stress, leading to the preparation of potato plant defenses, and in the event of pest attack, could likely cause an increase in the levels of ABA and consequent stomata closure. Since it has been observed that volatile signals can enter through stomata (Tani et al. 2010; Jiang et al. 2020), this potential stomata closure could be a kind of self-regulatory mechanism that eventually disrupts the perception of the EO signal.

A complex defense signaling network, primarily including JA, SA, and ABA, is often modulated by ethylene (ET) (Broekgaarden et al. 2015; Verma et al. 2016). The majority of transcripts encoding enzymes 1-aminocyclopropane-1-carboxylate synthase (ACS), which converts *S*-adenosyl-L-methionine (SAM) to the ET precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and aminocyclopropane-1-carboxylate oxidase (ACO), which is responsible for the conversion of ACC to ET, showed downregulated expression after an 8 h exposure to EO (Fig. 3d). Moreover, the upregulated expression of *GGT3* indicated that the production of the ACC derivative, glutamyl-ACC (GACC), was induced, which additionally contributed to reduction of the ACC and ethylene levels (Fig. 3d). Both the downregulated conversion of SAM to ACC and the upregulated conjugation of ACC speak in favor of decreased levels of ACC after 8 h exposure, which is reflected in our analysis of the endogenous phytohormones (Fig. 3e). MeJA has been shown to affect the downregulation of *ACO* (Lee et al. 2017), resulting in a reduction in ET levels, which is consistent with our previous presumption of increased MeJA levels due to the downregulation of *MJE* (Fig. 2a). ET and JA defense signaling are often mutually synergistic (Bürger and Chory 2019), hence the observed decrease in JA levels after 8 h of exposure compared to that after 4 h (Fig. 2b) could possibly be associated to decrease in ET levels.

The role of ET in plant defense is rather contentious, as it promotes resistance in the majority of interactions, but endorses disease development in others (Zhao and Li 2021). For example, cytokinins (CKs) enhanced disease resistance to *Stagonospora nodorum* in wheat by inhibiting ET signaling (Veselova et al. 2021). In addition, some pathogens have evolved mechanisms that allow them to force the host plant to produce ET (Cohn and Martin 2005; Bürger and Chory 2019), which means that elevated levels of ET do not always contribute to defense. This needs to be considered in explaining the decreased ACC and presumably decreased ET levels in the exposed potato. Interestingly, there have been some assumptions that GACC is involved in sensing early stress signals, even though this ACC derivative generally plays a role in regulating ET homeostasis (Pattyn et al. 2021).

Environmental volatiles have been shown to affect some of the growth-related phytohormone signaling pathways, as well. For instance, indole can inhibit TIR1 (transport inhibitor response)-dependent indole acetic acid (IAA) signaling in Arabidopsis roots (Bailly et al. 2014). Herbivore-induced plant volatile indole is also known to prime herbivore resistance by inducing levels of gibberellin (GA) and ABA and suppressing IAA levels (Ye et al. 2021). There is even evidence that EO volatile components such as citral and farnesene interact with auxins and affect their polar transport (Grana et al. 2013; Araniti et al. 2017).

IAA is considered to negatively affect plant defense by interfering with other phytohormone signaling pathways or some resistance responses such as pathogen-associated molecular patterns (PAMP)-triggered immunity (Robert-Seilaniantz et al. 2011; Wang and Wang 2014). Elevated levels of endogenous IAA increase the susceptibility of the Arabidopsis mutant with constitutive overproduction of the *YUC1* biosynthetic gene against the plant pathogen *Pseudomonas syringae* (Mutka et al. 2013*).* Moreover, inactivation of IAA-mediated processes, such as cell expansion and plant cell wall relaxation, has been shown to activate *Nicotiana attenuata* defense in response to oral secretion from the herbivore *Manduca sexta* (Onkokesung et al. 2010).

After prolonged exposure (8 h and 12 h) to French marigold EO, the endogenous IAA content in our exposed potato plants decreased, which could probably strengthen the defenses of the potato plants (Fig. 4a). However, the content of the auxin conjugate IAA-aspartate (IAA-Asp) increased after 12 h EO exposure (Fig. 4a). There are indications for the role of IAA-Asp in abiotic stress and ripening of henbane (*Hyoscyamus niger*) and grape (*Vitis vinifera*) (Oetiker and Aeschbacher 1997; Böttcher et al. 2010), but no direct biological function has been demonstrated. This inactive auxin is considered to be a precursor for auxin catabolism (Woodward and Bartel 2005; Ludwig-Muller 2011) with important role only in regulating auxin homeostasis. Therefore, these elevated IAA-Asp concentrations could be considered a possible cause for the subsequent decrease in IAA levels after prolonged exposure.

Endogenous levels of PAA also decreased after exposure to French marigold EO (Fig. 4b). PAA is a non-indole natural auxin that can bind to the same receptors and induce the same genes as IAA (Sugawara et al. 2015), but has lower auxin activity than IAA in most plants (Haagen-Smit and Went 1935; Muir et al. 1967), even though its endogenous concentrations are often much higher than those of IAA (Wightman and Lighty 1982; Sugawara et al. 2015), as is the case in our experiment (PAA concentrations are ten times higher than those of IAA). This phytohormone plays a role in maintaining auxin levels, which are required to maintain adequate cellular activity in plants (Morris and Johnson 1987). In addition, there has been some evidence that PAA has a role in plant-microbe interactions (Kunkel and Harper 2018), but nothing has been clearly demonstrated. Since the bioactive functions of IAA and PAA are known to overlap and our results show similar patterns in the responses of both phytohormones to French marigold EO (Figs. 4a and b), this may also suggest their similar roles in defense responses.

In contrast to auxins, elevated CK levels increase disease resistance to various pathogens such as *Pseudomonas syringae* (Choi et al. 2010; Grosskinsky et al. 2011; Grosskinsky et al. 2016), *Hyaloperonospora arabidopsis* (Argueso et al. 2012), tobacco mosaic virus (Sano et al. 1994), and other pests such as cyst- and root-knot nematodes (Shanks et al. 2016; Dowd et al. 2017). There is also evidence that CKs are able to prime the defense responses (Dervinis et al. 2010; Giron et al. 2013), however, the underlying molecular mechanisms are still very unclear.

Our results show an increase in total CKs and CK *N*-glucosides, as well as CK nucleobases after 4 h of treatment (Figs. 5b and c). Since nucleobases are considered the only true bioactive forms of CKs (Lomin et al. 2015), it is even more affirmative seeing elevated levels of this CK subgroup in terms of enhanced defense against pests and possible priming. On the other hand, the main role of CK *N*-glucosides is only to maintain CK homeostasis, as they are considered biologically non-active, irreversible CK forms (Hothorn et al. 2011; Lomin et al. 2015; Šmehilová et al. 2016). Accordingly, the maintenance of a constant CK level is the most probable explanation for the increase in CK *N*-glucoside levels. Recently, however, there have been indications of metabolization and/or possible biological role(s) of CK *N*-glucosides, which open doors for some other possible explanations (Hallmark et al. 2020; Hošek et al. 2020; Pokorná et al. 2021).

Although data from most studies suggest that increased protection against pests is the result of a positive interaction between CK and SA signaling (Choi et al. 2010; Argueso et al. 2012; Akhtar et al. 2020), CKs are also

involved in other defense responses. Recent data have shown that CKs can promote JA-mediated defense (Schäfer et al. 2015), which is consistent with our results, as both JA and CK levels increased after exposure to EO (Figs. 2b and 5a), in contrast to SA, whose levels remained unchanged (Fig. 2c).

Gibberellins have only recently emerged as complex modulators not only of plant development, but also of plant defense. Studies show that GA acts as both positive and negative regulator of defense responses (Zhu et al. 2005; De Bruyne et al. 2014; Moosavi 2017). Their effects on disease resistance may vary depending on the plant species, the type of pest attacking the plant or the type of plant-pest interaction. For example, GA increases resistance to necrotrophs and susceptibility to (hemi)biotrophs in rice, while experiments with Arabidopsis, wheat, and barley showed opposite results (Yang et al. 2008; Saville et al. 2012; Qin et al. 2013).

The balance of SA/JA signaling during plant immunity is affected by GAs by inducing the degradation of DELLA proteins that competitively bind with some other proteins, such as JAZ (Navarro et al. 2008; Hou et al. 2010; Ito et al. 2018). Consequently, GAs act as repressors of JA perception and signaling, while promoting biosynthesis and signaling of SA. This GA-mediated repression of JA signaling leads us to hypothesis that the increase in GA levels after an 8 h EO exposure of treated potato plants (Fig. 4c) could potentially induce a subsequent decrease in JA levels after prolonged exposure, thus participating in leveling of JA levels to the baseline values (Fig. 2a).

It is particularly interesting that most of the phytohormone groups examined in our study showed similar response patterns concerning their endogenous levels – there is an increase in levels after short-term exposure (4 h), followed by a decrease to control values after prolonged exposure (8 h and 12 h). These patterns are easily seen by looking at the graphs, although not all exhibited statistically significant differences between control and EO-exposed plants due to the high variability within the biological replicates. However, an exception was ET and auxin, which showed an opposite pattern, with a decrease in endogenous levels. This could possibly argue for enhanced defense only after a short-term treatment, considering that a 4 h exposure ”turns on” phytohormones potentially involved in the activation of defense responses, while prolonged exposure leads to a possible inactivation of these mechanisms by balancing these phytohormone levels. Short-term exposure likely led the plant into a defensive mode investing all capacity into survival, whereas prolonged stimuli allowed the plant to adapt and respond in ways that were more energy-efficient and did not disrupt plant primary metabolism, which could ultimately be detrimental to the plant. This hypothesis is consistent with our previous findings on how EO affects potato starch metabolism (Stupar et al. 2021). Although it is possible that different evaporation rates of different classes of volatile organic compounds present in EO may explain the variation in plant responses after different exposure periods, the previous scenario is the more likely chain of events.

The cause of the divergent ET role may be found in the crosstalk between ET and other phytohormones, for example, auxin has been shown to stabilize ACS2 proteins (Lee et al. 2017). Therefore, decreased auxin levels could reduce ACS activity. The delay in auxin (IAA) responses could be explained by the importance of the effects of this phytohormone on plant growth and development. Since it would not be in the plant’s best interest for growth and development to be compromised in order to enhance plant defenses, especially when there is no imminent danger for the plant, it is not surprising that auxin levels remain unchanged after a short exposure to EO, especially if the premise is that EO led to priming.

To summarize, French marigold EO affected homeostasis of many different phytohormones in exposed potato plants, confirming involvement of phytohormones in response to environmental signals such as EO volatiles. Data received from this comprehensive phytohormone analysis of potato plants exposed to French marigold EO may help in better understanding of influence observed alternations have on plant’s stress responses and inter-plant communication.

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Conflict of interest

Authors have no competing interests to declare that are relevant to the content of this article.

Author contributions

Conceptualization and Supervision: J.S.; Investigation (gene expression): S.S., N.D., Lj.T.; Formal analysis (gene expression): S.S., J.S.; Investigation (phytohormones): V.M., P.D.; Formal analysis (phytohormones): V.M., P.D., S.S., T.Ć.; Data curation: S.S.; Writing – original draft and visualization: S.S., T.Ć., V.M., J.S.; Writing – review & editing: all authors.

Data availability

The data supporting the findings of this study are available within the paper, its supplementary materials (Online Resource 1 and 2) and RADaR - Digital Repository **(**https://hdl.handle.net/21.15107/rcub_ibiss_5388) or on reasonable request from the corresponding author.

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