



Elicitation of solid callus cultures of *Salvia miltiorrhiza* Bunge with salicylic acid and a synthetic auxin (1-naphthaleneacetic acid)

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Abstract

The presented study analyses the influence of salicylic acid (SA) and the synthetic auxin 1-naphthaleneacetic acid (NAA) on total tanshinone level and on dihydrotanshinone (DHT), cryptotanshinone (CT), tanshinone I (TI) and tanshinone IIA (TIIA) level in *Salvia miltiorrhiza* callus cultures growing on solid Murashige and Skoog (MS) medium. The influence of SA and NAA was evaluated at 10-day intervals throughout a 80-day treatment period. SA was applied at 0.1, 0.2 and 0.4 mM, and NAA at 2.69, 13.43, 26.85 and 40.28 μM . DHT, CT, TI and TIIA concentrations were measured using HPLC. NAA did not increase the concentration of any tanshinone. SA increased content in a concentration- and time-dependent manner; however, the yields were relatively low, possibly due to the metabolic specificity of *S. miltiorrhiza* cultivars in Poland. Total tanshinone concentration reached $226.38 \pm 37.33 \mu\text{g g}^{-1} \text{DW}$ after 50 days of 0.4 mM SA elicitation. After 50 days of SA elicitation, the following maximum tanshinone concentrations were observed for 0.4 mM SA: DHT ($71.58 \pm 12.72 \mu\text{g g}^{-1} \text{DW}$), CT ($108.54 \pm 18.29 \mu\text{g g}^{-1} \text{DW}$), TI ($29.50 \pm 4.13 \mu\text{g g}^{-1} \text{DW}$) and TIIA ($16.75 \pm 2.74 \mu\text{g g}^{-1} \text{DW}$). To account for these observed differences in tanshinone biosynthesis, the distribution of SA and auxin responsive *cis*-active motif in the proximal promoters of the mevalonic acid, methylerythritol-4-phosphate and tanshinone-precursor biosynthesis pathway genes was evaluated in *A. thaliana* and *S. miltiorrhiza*. Our findings indicate that the SA-responsive *cis*-active elements have a much broader distribution than those recognized by auxin-responsive transcription factors.

Key message

Tanshinone concentration in *S. miltiorrhiza* callus growing on solid, modified MS medium may be increased by salicylic acid elicitation.

Keywords Callus culture · Elicitation · Promoter · *Cis*-active element · Tanshinone · Phytohormone

Abbreviations

NAA 1-Naphthaleneacetic acid
IAA Indole-3-acetic acid
BA N⁶-benzyladenine
SA Salicylic acid
2,4-D 2,4-Dichlorophenoxyacetic acid

MEP Methylerythritol-4-phosphate
MVA Mevalonic acid

Introduction

Salvia miltiorrhiza, also known as Danshen/Tanshen, is a medicinal plant that has been used in Chinese medicine since ancient times (Su et al. 2015). It is applied to treat ischemic cardiovascular disease, coronary heart disease, chronic heart failure, hypertension and hyperlipidemia (Su et al. 2015; Zhou et al. 2005). The medicinal properties of Danshen are mediated mainly by two groups of active components: the tanshinones, a group of lipophilic diterpenoid derivatives with an intense orange-brown color, e.g. tanshinone I (TI), tanshinone IIA (TIIA), cryptotanshinone (CT) and dihydrotanshinone (DHT), and the hydrophilic phenolic

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acids, including caffeic acid, ferulic acid, rosmarinic acid, salvianolic acid A-G, I-L and N (Wang et al. 2017).

The diterpene tanshinones are synthesized from two five-carbon precursors, isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), mainly by the plastidial methylerythritol-4-phosphate (MEP) pathway. The IDP and DMADP moiety is used for the stepwise biosynthesis of a 20-carbon geranylgeranyl diphosphate (GGPP), which then serves as a substrate for copalyl diphosphate synthase 1 and 2, producing (+)-copalyl diphosphate. The (+)-copalyl diphosphate is transformed into miltiradiene, the first committed tanshinone biosynthesis pathway intermediate, by the kaurene-like synthase 1 (Bai et al 2018; Guo and Ma 2019; Szymczyk et al. 2016).

The typical source of *S. miltiorrhiza* active ingredients is the root, which demonstrates the highest tanshinone concentration (Li et al. 2010). However, the most efficient alternative sources of *S. miltiorrhiza* plant material are hairy roots and cell cultures (Krajewska-Patan et al. 2007). Although the concentration of tanshinones in these sources is generally lower or at best comparable to roots of field plants, these cultures provide a continuous, season-independent supply of plant material (Wang and Wu 2010). The concentration of tanshinones can also be increased by application of chemical or physical components known as elicitors (Cheng et al. 2013; Hao et al. 2015). Although previous results suggest that salicylic acid positively influences tanshinone biosynthesis in hairy roots or cell cultures, little is known of the content in *S. miltiorrhiza* calluses growing on solid medium and exclusively elicited by SA; particularly for long culture durations (80 days), allowing for the accumulation of secondary metabolites (Wu et al. 2003). Previous studies related to callus culture elicitation used SA in relatively short tests that lasted for up to 18 days (Li et al. 2003; Chen and Chen 1999). A prompt metabolic response was observed after SA treatment (Hao et al., 2015) suggesting that the total tanshinone concentration in *S. miltiorrhiza* hairy root culture starts to increase 36 h after 0.1 mM SA treatment. Yu et al. (2016) report that 0.2 mM SA elicitation of suspension cell culture resulted in DHT and CT elevation after 3 days, TIIA augmentation after 4 days, and a reduction in TI concentration. However, Li et al. (2003) report that SA 0.2 mM did not increase the tanshinone concentration in Ti-transformed *S. miltiorrhiza* cell culture for up to 18 days.

Chen and Chen (1999) report that 50–500 μ M SA applied alone is insufficient to induce CT biosynthesis in *S. miltiorrhiza* suspension cell cultures. SA concentrations higher than 0.2 mM were found to have no effect on CT biosynthesis rate, although it was tested for only a very short period of 5 days (Chen and Chen 1999). Therefore, little is known of the influence of exclusive SA elicitation on callus cultures for longer periods, i.e. up to 80 days, and relatively high concentrations, i.e. up to 0.4 mM. In addition, the influence of auxin

phytohormone on callus cultures has not been studied. Only few studies have examined the effect of auxin phytohormones, such as indole-3-acetic acid (IAA) and NAA, on tanshinone concentration in *S. miltiorrhiza*; however, existing data suggests they do not stimulate tanshinone biosynthesis in hairy root cultures (Gupta et al. 2011). No such study has been performed on callus cultures of *S. miltiorrhiza*.

Callus culture has significant commercial potential, particularly with regard to the production of plant secondary metabolites with medicinal activity, as well as therapeutic antibodies and other recombinant proteins, in addition to preparing ornamental or agricultural plants by regeneration from calli. Active ingredients may be purified directly from callus culture to relieve the pressure on plants in their natural habitats (Park and Wi 2016; Top et al. 2019; Efferth 2019). Also, callus cultures may be easily converted into single cell cultures growing as suspension in bioreactors. Such culture may be subjected to scale-up and process optimization, similarly to large-scale animal cell cultures (Georgiev et al., 2009, 2018). Some commercially-available drugs are produced by large-scale callus cultures, such as Paclitaxel from yew (*Taxus*. sp.) or taliglucerase alpha (Elelyso) from carrot *Daucus carota* (Tabata 2004; Grabowski et al. 2014).

SA is a phytohormone produced by plants during their response to abiotic stress, including drought, heavy metal, heat, osmotic and chilling stress. However, SA seems to play a central role in the response to biotrophic pathogens, which colonize between plant cells to absorb host nutrients (Li et al. 2019). SA-responsive TGACG *cis*-active elements have been found in numerous plant promoters (Fonseca et al. 2010; Sun et al. 2018), and SA is known to induce the expression of the SmWRKY1 *trans*-factor, which strongly induce genes associated with tanshinone biosynthesis through interaction with the W-box TTGAC(C/T) *cis*-active element (Cao et al. 2018).

Contrary to SA, the influence of auxin on gene expression is not clearly activatory. Among 25 Auxin Response Factors (ARFs) identified in *S. miltiorrhiza* only seven (*SmARF* 2, 10, 19, 20, 22, 24, 25) act as transcription activators while the other 18 *SmARFs* may function as transcription repressors (Xu et al. 2016). Moreover, the binding affinity of the ARFs to DNA and strength of transcription interference depends not only on the presence of *cis*-active elements recognized by ARFs but also on the spatial organization of these elements allowing ARFs dimerization (Berendzen et al. 2012). Therefore, efficient transcription activation or inhibition of target gene expression by ARFs requires the presence of TGTCGG inverted repeats and TGTCTC or TGTCGG direct repeats (Freire-Rios et al. 2020). Also, the presence of consensus sequence TGTSTSBC (B=C or G or T; S=C or G) in the promoter region is positively associated with auxin-dependent gene expression regulation (Mironova et al. 2014).

The present paper compares the effects of elicitation by the synthetic auxin NAA and SA on the concentrations of four tanshinones CT, DHT, TI and TIIA, as well as total tanshinone content, in *S. miltiorrhiza* callus cultures grown for up to 80 days on solid MS medium. To explain the metabolic effects indicated by both phytohormones on *S. miltiorrhiza* callus, the study examined the distribution frequency of SA and auxin-responsive *cis*-active elements in promoters of selected genes associated with tanshinone precursor biosynthesis.

Materials and methods

Plant material

Salvia miltiorrhiza plants were cultivated from seeds provided by the Medicinal Garden of the Department of Pharmacognosy of the Medical University of Lodz (Poland). The plants were grown in pots of 0.5 L (12 cm in diameter) containing composite soil, at 22 ± 2 °C under natural light. Young plants at the age of 3 months were used for experiments.

Callus induction, salicylic acid and NAA treatment

The induction and proliferation of *S. miltiorrhiza* callus was performed according to Wu et al. (2003) with modifications: callus induction was performed with 10×10 mm leaf pieces from the *S. miltiorrhiza* plants, compared to 5×5 mm described in the original method, and 100 mm glass Petri dishes were used instead of the original 22×160 mm glass tubes. The explant surface was disinfected with 70% ethanol for 30 s, followed by treatment with 0.5% sodium hypochlorite with Tween 20 (two drops of Tween 20 per 100 mL) for 10 min; following this, the surface was rinsed five times with sterile distilled water. The explants were placed in 100 mm diameter glass Petri dishes containing 20 mL of solid medium to induce growth of primary callus. The medium consisted of Murashige and Skoog (1962) basal medium known as MS, supplemented with 3% sucrose, 1% Difco Bacto agar (Difco Laboratories, Detroit, Michigan, US) and 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The Petri dishes with explants were sealed with two layers of Parafilm (Pechiney Plastic Packaging, Chicago, USA). All procedures were performed under a laminar hood to avoid microbiological contamination. The cultures were incubated at 26 ± 2 °C in darkness for a period of 1 month. To avoid light, the glass Petri dishes were covered with two layers of aluminum foil. The induced primary callus was allowed to proliferate on a solid MS basal medium supplemented with 3% sucrose, 1% Difco Bacto agar (Difco Laboratories, Detroit, Michigan, US), 1 mg L^{-1} 2,4-D and 0.5 mg L^{-1} N⁶-benzyladenine

(BA). The proliferating callus was cultured three times at 20-day intervals in darkness in 100 mm diameter glass Petri dishes containing 20 mL of solid medium. Then, the proliferated callus was used for experiments with SA and NAA elicitation.

The solid medium consisted of MS basal medium supplemented with 3% sucrose, 1% Difco Bacto agar (Difco Laboratories, Detroit, Michigan, US) and 0.1, 0.2 and 0.4 mM SA sodium salt or 2.69, 13.43, 26.85 and 40.28 μM NAA. The callus cultures were maintained for up to 80 days. Fresh solid medium was provided every 20 days. Callus samples for HPLC experiments were harvested each 10 days, up to 80 days; however samples for growth index calculation were collected after 20, 40, 60 and 80 days. Callus samples harvested after 10 days were grown on the same medium they started growth. Callus samples grown longer than 20 days were transferred to new, fresh medium. For example, callus expected to grow for 30 days was nourished by the initial medium for the first 20 days and a fresh, exchanged portion for the next 10 days.

All the experiments were repeated three times, and growth measurements were performed with three replicates per harvesting.

Before the phytohormones were added, the pH of each medium was adjusted to 5.7 ± 0.1 with 1 N NaOH or HCl, before autoclaving at 121 °C, 105 kPa, 20 min. Stock solutions of phytohormones 2,4-D (0.4 mg/mL), NAA (0.2 mg/mL) and SA sodium salt (100 mM) in 70% ethanol were sterilized with a syringe filter (0.4 μm pore size). They were then added to the warm (50 °C) freshly-autoclaved MS medium under a laminar hood. The control group was the NAA and SA untreated callus.

The callus mass was evaluated on electronic scales localized under a laminar hood to avoid microbiological contamination of the callus. The electronic scales and working surface of the laminar hood were initially sterilized with UV irradiation (30 min) and then with 70% ethanol. The glass material and steel forceps used to manipulate the calluses were sterilized at 200 °C, 30 min before use in the laminar hood. Plastic tips were autoclaved as liquid media.

Growth index (GI) was calculated according to the formula described by Godoy-Hernández and Vázquez-Flota (2006). The GI of fresh calls weight is defined as follows: $(GI_F) = \text{final callus fresh weight (FW)} - \text{initial callus fresh weight (FW)} / \text{initial callus fresh weight (FW)}$. The same definition was used to calculate the growth index of dry callus— GI_D .

Preparation of callus extract

The extraction of callus was performed according to Wan et al. (2009). Methanol or 80% methanol solution is now commonly used to extract tanshinones from *S. miltiorrhiza*

plant material (Wei et al. 2019; Zhao et al. 2015; Liu et al. 2017). By using methanol, it is possible to avoid low pressure evaporation of additional solvents, such as chloroform before starting the HPLC procedure (Liu et al. 2006). Removing the evaporation stage is particularly important for experiments based on a small amount of dried callus measured in tens of mg (Zhao et al. 2015). Such very small dried samples produce a tiny amount of extracted tanshinones in the glass vessel that may be not completely solubilized in the added methanol, resulting in relatively high error rates. The callus was harvested carefully and freeze-dried for a 3 days in a lyophilizer Alpha 1-2 LD (Martin Christ, Osterlode, Germany) connected to a GVD1.5 vacuum pump (Atlas Copco, Stockholm, Sweden). The pressure was maintained at 0.1 mbar by a vacuum pump.

The 30 mg dry callus was finely ground with a mortar and a pestle and extracted with 1.5 mL 100% methanol under 90 min sonication (UM1 disintegrator, Unimal, Olsztyn, Poland) at room temperature. The liquid/solid ratio was 50:1 as in the original method (Wan et al. 2009). The samples were then centrifuged at 12,000× rpm for 10 min at room temperature, filtered through 0.45 µm Chromafil membrane (Machery-Nagel, Duren, Germany) and used for HPLC analysis. The samples were stored in darkness at – 25 °C to avoid tanshinone decomposition.

HPLC analysis

Standard HPLC-grade substances such as TI, TIIA, CT and DHT were provided by Sigma Aldrich Poland (Poznan, Poland). Methanol, acetonitrile and water (J.T. Baker HPLC Analyzed) were received from Avantor Performance Materials (Gliwice, Poland). All analyses were performed on the Agilent HPLC System 1200 (Agilent, Palo Alto, US), equipped with a quaternary-pump delivery system, an on-line degasser, an auto sampler, a column temperature controller and a UV–VIS DAD detector. The system was connected with the Agilent ChemStation 2001–2010 software.

Chromatographic separation was performed on an Agilent Zorbax Extend C18 reversed phase column (5 µm, 250×4.6 mm) with an Agilent Zorbax Extend C18 guard column (5 µm, 10×4.6 mm). The detection was performed at 270 nm, the time of analysis was 20 min, the flow rate was 1.2 mL min⁻¹ and the column temperature was maintained at 20 °C; the sample volume was 20 µL. The mobile phase consisted of A (water for HPLC) and B (acetonitrile). The following gradient program was applied: initially 45% B at 0 min, linearly increasing to 60% B at 2 min, maintaining 60% B from 2 to 9 min, linearly increasing B to 80% at 10 min, linearly increasing B to 82% at 13 min and finally linearly decreasing B to 45% at 20 min. After each analysis, 45% B was pumped and

held for 10 min to re-equilibrate the system for baseline stability. The procedure is a modified method of Liu et al. (2006).

Preparation of calibration curves and standard solutions

The starting methanol stock solutions of 0.1 mg mL⁻¹ TI, TIIA, CT and DHT were prepared and then stored in dark, Parafilm-sealed HPLC vials at – 25 °C; storage at 5 °C may cause decomposition. To prepare standard curves, the initial standard solution was appropriately diluted. The retention time for DHT was 7.1 min. The calibration curve is described by the following equation $y = 2.9630x + 1.1634$, the correlation coefficient is 0.9997, LOD 0.9624 and LOQ 2.9165. The retention time for CT was 10.7 min. The calibration curve for CT is $y = 4.3133x + 1.5136$, the correlation coefficient 0.9995, the LOD 1.3153 and LOQ 3.986. The retention time for TI was 11.5 min, the calibration curve $y = 2.4617x + 2.2342$, the correlation coefficient 0.9995, LOD 1.3325 and LOQ 4.077. For TIIA the retention time was 13.7 min, the standard curve $y = 5.3145x + 1.3120$, the correlation coefficient 0.9997, LOD 1.0337 and LOQ 3.1325. The linearity range of all calibration curves appeared to be 1.25–50 ng.

Promoter analysis

The findings regarding the *A. thaliana* and *S. miltiorrhiza* genes used in the study are presented below. They were checked against the PlantPAN3.0, Arabidopsis org-TAIR, NCBI (Nucleotide) and Uniprot databases (Chow et al. 2019; Rhee et al. 2003; NCBI Resource Coordinators 2016; The UniProt Consortium 2021; Majewska et al. 2018). The TSSP software was used to characterize transcription start sites in *S. miltiorrhiza* promoters (Shahmuradov et al. 2005). The precise description of genes used in the study is presented in Supplement text file 1.

Although the promoter region in *A. thaliana* is approximately 0.5–1.0 kb long, the in silico searches were concentrated on proximal promoters within 300 bp of the transcription start site (Kristiansson et al. 2009). The decision to decrease the length of studied promoter is based on the higher biological relevance of *cis*-active motifs found within proximal promoters (Mironova et al. 2014; Keilwagen et al. 2011; Yu et al. 2016).

The gene proximal promoters were searched for the following *cis*-active elements: SA-responsive TGACG and W-boxes TTGAC(C/T), auxin-responsive TGTCGG, TGT CTC and TGTSTSBC (B=C or G or T, S=C or G) (Fonseca

et al. 2010; Sun et al. 2018; Cao et al. 2018; Freire-Rios et al. 2020; Mironova et al. 2014).

Statistical analysis

The results of the HPLC analysis were evaluated by the Kruskal–Wallis test with STATISTICA (StatSoft Inc. 2013, version 13.1). Data concerning wet or dry callus samples mass were compared using the Student's t-test. Values of $p < 0.05$ were considered statistically significant.

Results

Changes in tanshinone concentration after SA treatment

Generally, SA elicitation showed time- and concentration-dependent effects. The four tanshinones (TI, THIA, CT and DHT) were not induced simultaneously, and their elicitation occurred stepwise.

After 50 days of SA elicitation, all tanshinones demonstrated induction for all three SA concentrations, reaching the highest values for 0.4 mM SA DHT ($71.58 \pm 12.72 \mu\text{g g}^{-1}$ DW), CT ($108.54 \pm 18.29 \mu\text{g g}^{-1}$ DW), TI ($29.50 \pm 4.13 \mu\text{g g}^{-1}$ DW) and THIA ($16.75 \pm 2.74 \mu\text{g g}^{-1}$ DW) (Fig. 1). Lower values were observed than 0.1 mM SA elicitation after 50 days: DHT ($19.96 \pm 1.16 \mu\text{g g}^{-1}$ DW), CT ($29.50 \pm 1.97 \mu\text{g g}^{-1}$ DW), TI ($13.00 \pm 0.97 \mu\text{g g}^{-1}$ DW) and THIA ($5.75 \pm 1.24 \mu\text{g g}^{-1}$ DW). Also, 0.2 mM SA elicitation demonstrated lower levels for 50 days of treatment: DHT ($28.17 \pm 2.06 \mu\text{g g}^{-1}$ DW), CT ($44.33 \pm 2.96 \mu\text{g g}^{-1}$ DW), TI ($15.21 \pm 5.68 \mu\text{g g}^{-1}$ DW) and THIA ($9.63 \pm 0.72 \mu\text{g g}^{-1}$ DW). After this time, the elicitation effects of all three SA concentrations were decreasing, and the concentrations of all tanshinones decreased stepwise (Fig. 1).

Comparing the 40-day and 50-day elicitation by 0.4 mM SA, the concentrations of all four tanshinones could be organized in the following manner $\text{CT} > \text{DHT} > \text{TI} > \text{THIA}$ (Fig. 1).

The presented changes are associated with fluctuations of total tanshinone concentration as compared to control samples (Fig. 2). The maximum total tanshinone concentration observed after day 50 (Fig. 2). All SA concentrations reached maximum elicitation after this period, viz. 0.1 mM ($68.21 \pm 4.80 \mu\text{g g}^{-1}$ DW), 0.2 mM ($97.33 \pm 5.67 \mu\text{g g}^{-1}$ DW) and 0.4 mM SA ($226.38 \pm 37.30 \mu\text{g g}^{-1}$ DW) (Fig. 2). Longer SA elicitation resulted in a significant decrease in the total tanshinone concentration.

Tanshinones have a well-known and strong orange-red coloration (Wu et al. 2003). Appropriate pigmentation changes in callus colors are induced by SA treatment. These changes are well visible after 50 days of elicitation. Danshen

calluses treated with 0.1 or 0.2 mM SA demonstrated a yellow to pale-orange pigmentation as compared to control samples (Fig S1D). In addition, *S. miltiorrhiza* calluses treated with 0.4 mM SA appeared to be darker, which may be related to higher total tanshinone concentration.

Changes of callus growth index induced by SA treatment

The growth index (GI) of the final biomass is calculated by dividing harvested fresh weight or dry weight by the inoculum fresh weight or dry weight. The GI for both fresh (GI_F) and dry (GI_D) callus weight was analysed for 80 days in a 20-day intervals. The GI_F for control callus cultures was 3.47–71.15 for the 20 to 80-day interval (Fig. 4), with the GI_F for callus cultures being 2.00–27.03 (0.1 mM SA) and 2.12–27.23 (0.2 mM SA) and only 1.59–5.12 at 0.4 mM SA (Fig. 4). The GI_D values were generally similar to GI_F , these being 3.77–80.79 for controls for the 20 to 80-day interval, but 2.42–24.63 (0.1 mM), 2.19–26.42 (0.2 mM SA) and 1.64–5.58 (0.4 mM SA) (Fig. 3).

Changes of tanshinone productivity mediated by SA elicitation

As the highest total tanshinone concentration ($226.38 \pm 37.30 \mu\text{g g}^{-1}$ DW) was observed after day 50 of the 0.4 mM SA elicitation it could be expected that this system should be the best source of tanshinones. In addition, the total tanshinone level observed for 0.2 mM SA, was 2.33 times lower than for 0.4 mM SA.

Treatment with 0.2 mM SA yielded $4.65 \pm 0.78 \text{GI}_F$ for day 40 and $11.21 \pm 0.93 \text{GI}_F$ for day 60, encompassing the time point (50 days) of the highest total tanshinone concentration. These values were approximately 2.03–2.99 times higher for cultures induced with 0.4 mM SA (GI_F 2.29 ± 0.20 at day 40 and 3.75 ± 0.52 for day 60). Therefore, on day 50, the lower concentration of total tanshinones observed for 0.2 mM SA than 0.4 mM SA may be compensated by the higher GI_F of the callus growing on 0.2 mM SA, i.e. approximately 2.03–2.99 times higher.

Distribution of SA responsive cis-active elements within proximal promoters of *A. thaliana* and *S. miltiorrhiza*

The SA-responsive *cis*-elements were found only in one MEP pathway gene, 4-(cytidine 5'-diphospho)-2-C-methyl-d-erythritol kinase (CMK), and one MVA route gene: diphosphomevalonate decarboxylase 1 (PMD1) (Tables S1–2). Furthermore, eight of the 18 tested *A. thaliana* genes encoding enzymes participating in GGPP biosynthesis indicate an SA-responsive *cis*-active motif (Table S3). Therefore, the *A.*

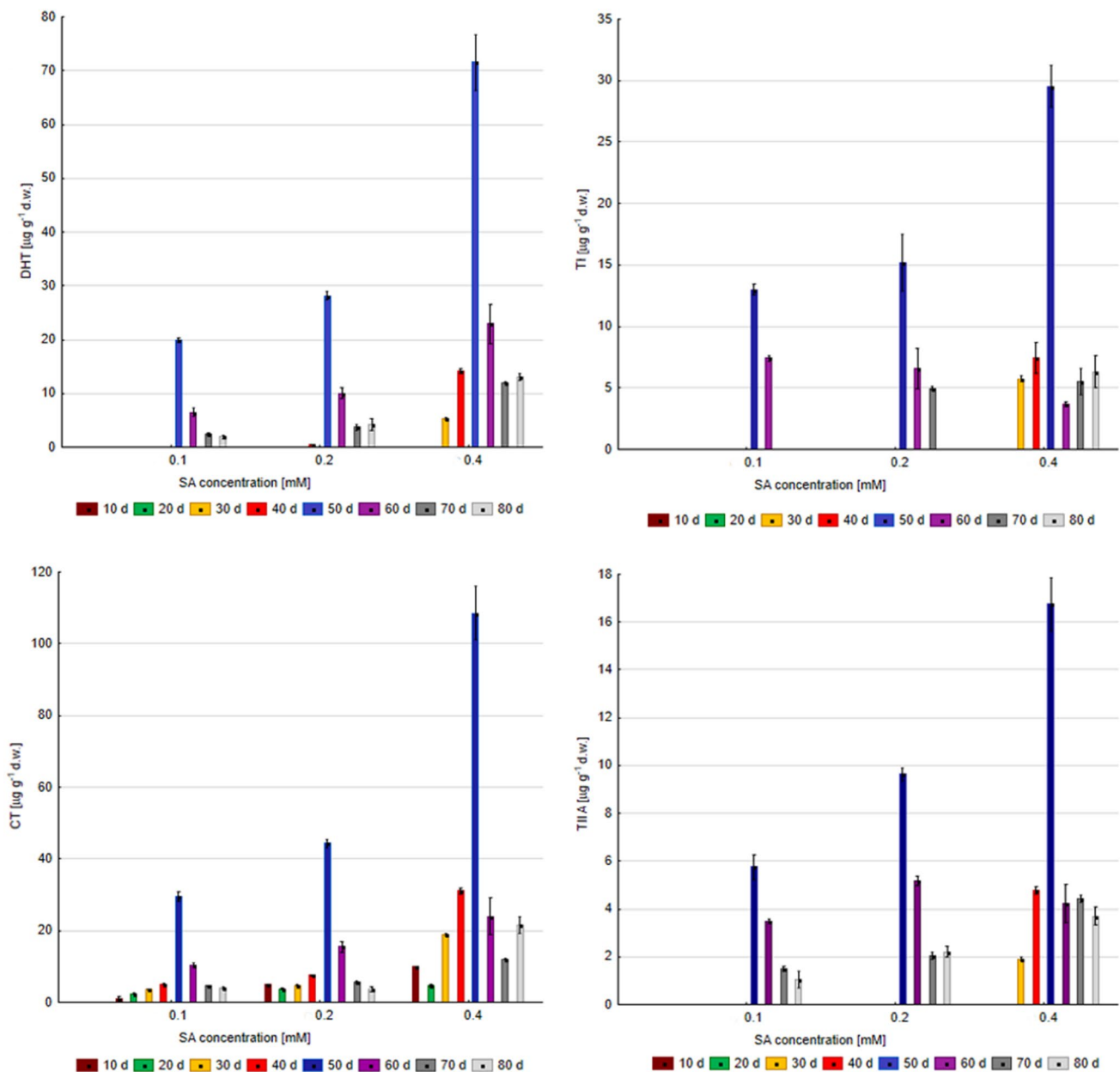


Fig. 1 Concentration of four tanshinones cryptotanshinone (CT), dihydrotanshinone (DHT), tanshinone I (TI) and tanshinone IIA (TIIA) in *S. miltiorrhiza* callus presented as a function of salicylic

acid (SA) concentration (0.1, 0.2 and 0.4 mM) and elicitation time (10–80 days). Control values were not presented as tanshinone concentration was not detectable

thaliana and *S. miltiorrhiza* genes associated with isoprenoid biosynthesis demonstrate much better representation of SA-responsive motifs than auxin-responsive *cis*-elements. Among 35 tested *A. thaliana* genes, as much as ten (28.57%) indicated SA-responsive elements (Fig. 4).

In addition, three among seven (42.86%) *S. miltiorrhiza* genes present these *cis*-active motifs, which may explain the increased tanshinone concentration in response to SA treatment observed in the present study (Table S4) (Fig. 4). Among these three, the *SmCPS* and *SmHMGR2* genes were

verified experimentally by RT-PCR studies in transgenic plants (Szymczyk et al. 2016, 2018). Similarly, the *S. miltiorrhiza* genes *SmIDI*, *SmGGPPS*, *SmHMGS*, *SmHDR* and *SmDXR* have also been experimentally-confirmed to respond to SA treatment (Hua et al. 2012; Hao et al. 2013, 2015; Jiang et al. 2019; Yan et al. 2009; Zhang et al. 2010, 2016). *SmDXS2* gene responded positively to overexpression of *SmWRKY1*, that is stimulated by SA treatment (Cao et al. 2018).

Fig. 2 Concentration of total tanshinone as a function of salicylic acid (SA) concentration (0.1, 0.2 and 0.4 mM) and elicitation time 10–80 days. Control values were not presented as tanshinone concentration was not detectable

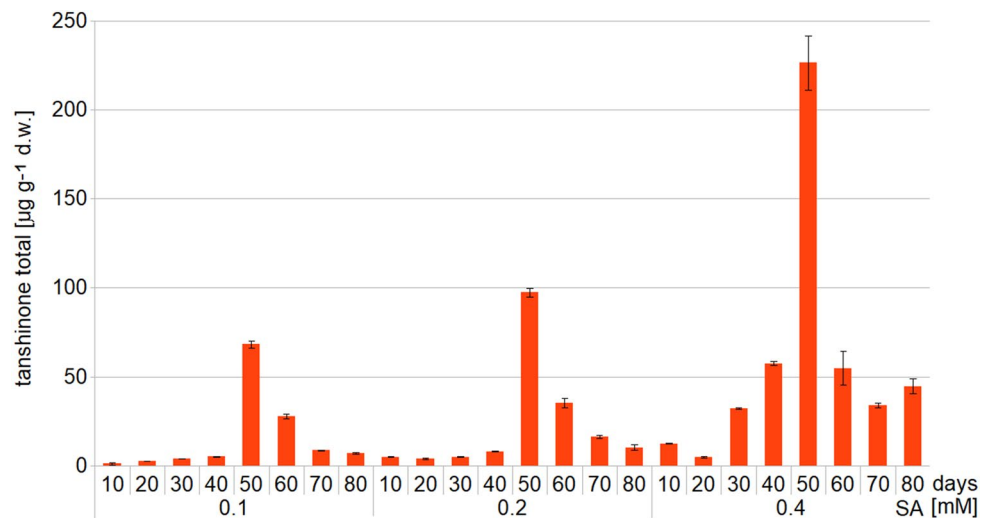
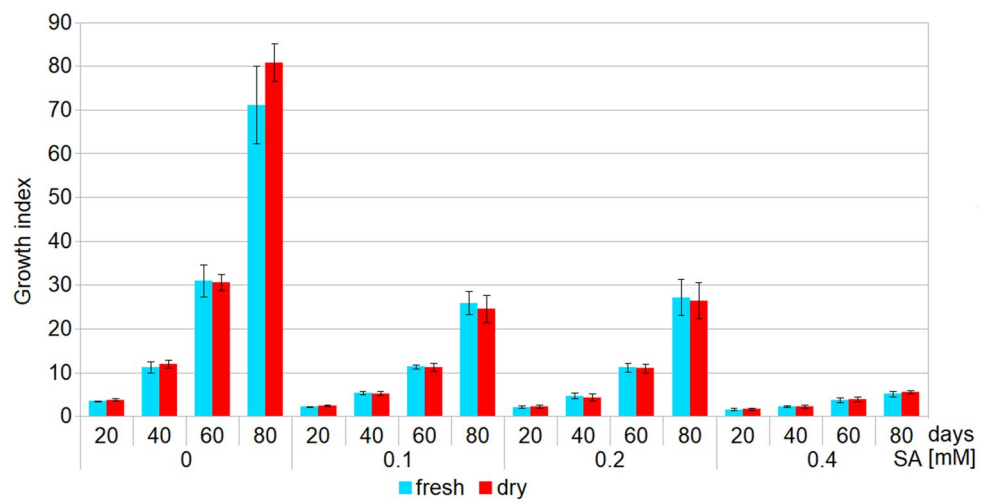


Fig. 3 Growth index for fresh (blue) and dry (red) *S. miltiorrhiza* callus presented for control and three SA concentrations 0.1, 0.2 and 0.4 mM. (Color figure online)



In addition, closely-spaced SA-responsive elements were observed within two *A. thaliana* genes: *isopentenyl-diphosphate Delta-isomerase* and *geranylgeranyl diphosphate synthase 9* (Table S5). Such closely-spaced repetitions of *cis*-active motifs allow the formation of hetero- or homodimers of *trans*-factors to precisely regulate gene expression.

Callus response to NAA treatment

The elicitation of *S. miltiorrhiza* callus cultures by 2.69, 13.43, 26.85 and 40.28 µM NAA did not increase the concentration of four tanshinones TI, TIIA, CT and DHT over the 80 days. The values of TI, TIIA, CT and DHT were so low in the treated samples and the untreated controls that they could not be detected by HPLC.

Changes of callus growth index after NAA treatment

The GI_F for control callus cultures was 3.42–70.63 for the 20–80 days interval (Fig. 5). Similar GI_F values were obtained for callus cultures growing on 2.69 µM NAA (3.19–67.35), 13.43 µM NAA (3.66–75.34) and 26.85 µM NAA (3.62–83.03). The 40.28 µM NAA treatment inhibited callus growth rate (GI_F 2.32–21.69). The values of GI_D for control and tested samples were similar to GI_F (Fig. 5). The color of callus growing on medium containing NAA within the range 2.69–40.28 µM was related to control samples (Fig S1D).

Distribution of auxin responsive *cis*-active elements within proximal promoters of *A. thaliana* and *S. miltiorrhiza*

Analysis of *A. thaliana* MVA pathway genes showed no auxin-responsive elements in the proximal promoter region

Fig. 4 Enzymatic reactions participating in tanshinone biosynthesis (Guo and Ma 2019)

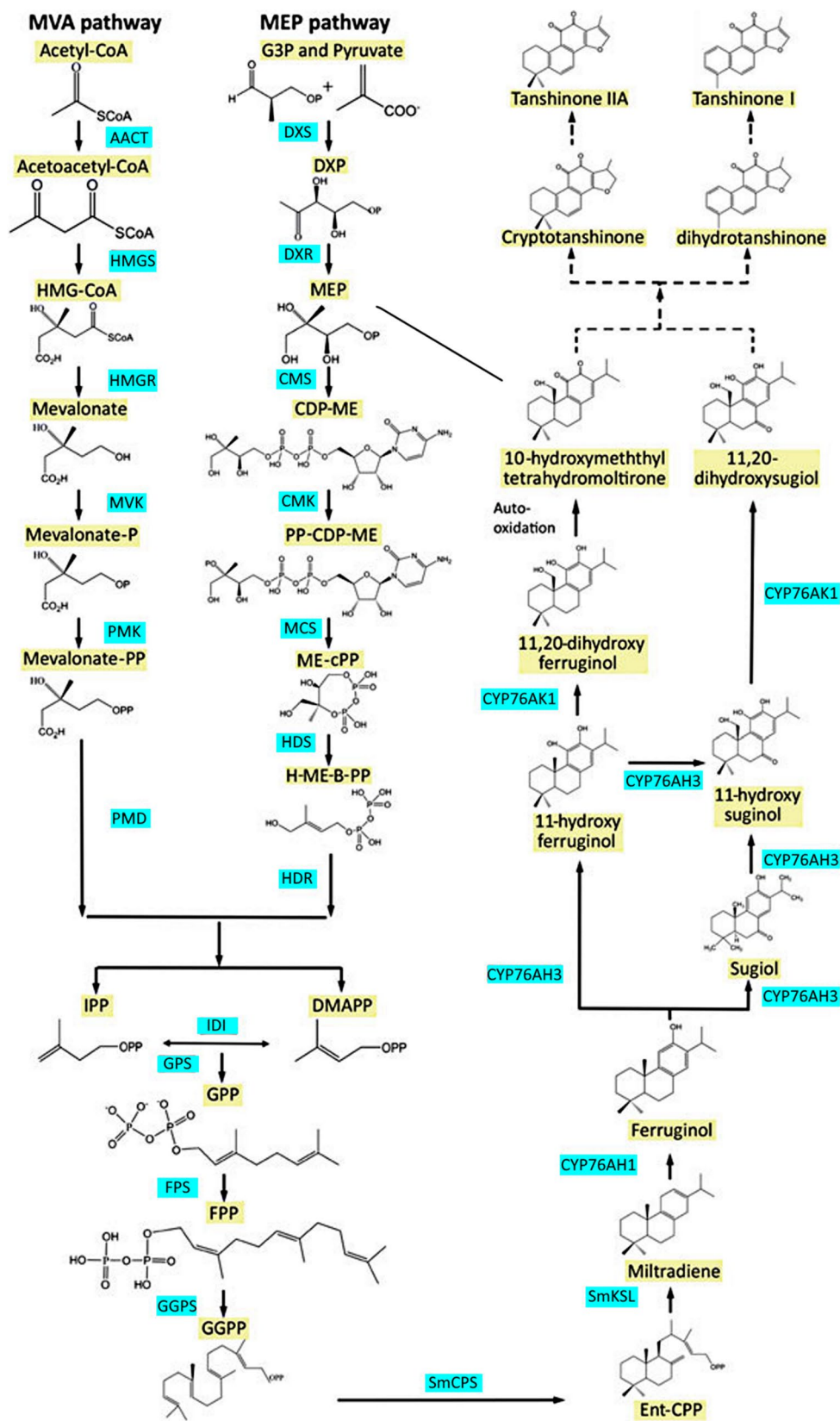
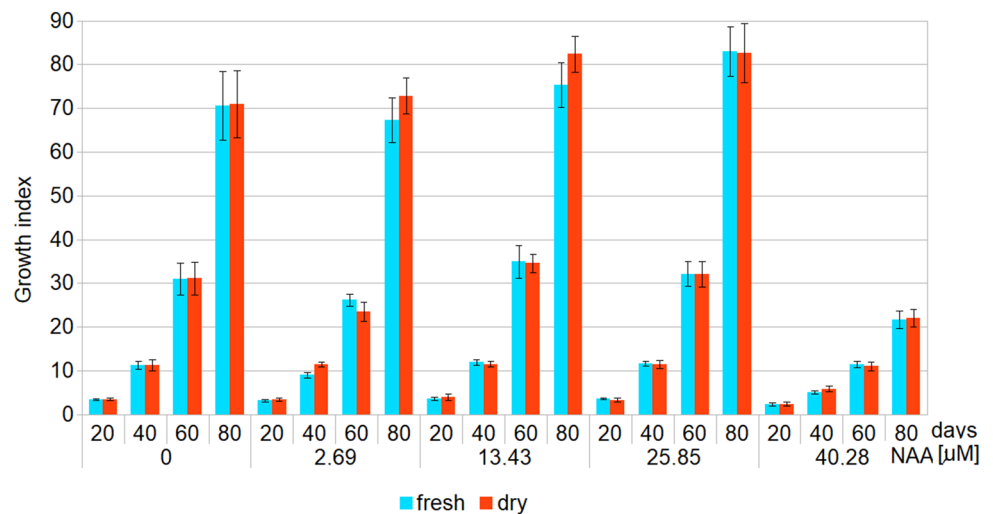


Fig. 5 Growth index for fresh (blue) and dry (orange) *S. miltiorrhiza* callus presented for control and four 1-naphthaleneacetic acid (NAA) concentration 2.69, 13.43, 26.85 and 40.28 μM . (Color figure online)



localized up to 300 bp upstream from TSS (Table S2). The auxin-responsive elements were found in two genes of the *A. thaliana* MEP pathway: *2-C-methyl-d-erythritol 4-phosphate cytidyltransferase* and *4-hydroxy-3-methylbut-2-enyl diphosphate reductase* (Table S1). Moreover only one among 18 studied *A. thaliana* genes participating in GGPP biosynthesis, known as *geranylgeranyl diphosphate synthase 12 (GGPPS12)* displays an auxin-responsive *cis*-active motif in the proximal promoter (Table S3). The lack of these sequences in the seven tested *S. miltiorrhiza* genes may be related to the observed scarcity of metabolic response to auxin treatment (Table S4). Previous studies suggest that IAA 2.85 μM (0.5 mg L^{-1}) treatment does not induce of *SmHMGR2* gene expression (Szymczyk et al. 2018); this is consistent with the lack of tested auxin-responsive *cis*-active motifs in *SmHMGR2* or its *A. thaliana* homolog (Table S4) (Fig. 4).

No closely-spaced, tandem or inverted repeats of auxin-responsive elements in proximal promoters were observed, suggesting that the phytohormone has low functional importance in regulating the tested genes (Freire-Rios et al. 2020).

Discussion

Assuming that some plant biosynthetic routes are tissue or organ specific, like the tanshinone or ginsenoside biosynthesis pathway observed in the rhizome, callus cultures offer generally less efficient product biosynthesis (Hao et al. 2015; Szymanska et al. 2013). Genes encoding enzymes of critical importance for late stages of tanshinone biosynthesis, such as *SmCPS1*, *SmKSL1* and *SmCYP76AH1* are predominantly expressed in taproot of *S. miltiorrhiza* (Hao et al. 2020). Therefore the callus cultures could be expected to indicate a lower biosynthesis rate than roots or *S. miltiorrhiza* plants.

Indeed, previous studies on *S. miltiorrhiza* calluses show a generally low total tanshinone concentration of 0.077% (Gryszczynska et al. 2015). Similar values 0.06–0.08% were observed in Ti-transformed suspension cultures of *S. miltiorrhiza* (Chen et al. 1997). This is considerably lower than in roots regenerated in vitro (0.269–1.137%) (Li et al. 2010) or in native plant roots growing in China (0.260–0.388%) (Cheng et al. 2013). The cultivar dependency is a significant factor, as *S. miltiorrhiza* plants in Poland are characterized by a lower tanshinone concentration of 0.01–0.26% (Buchwald et al. 2006).

Our results confirm SA-dependent tanshinone elicitation in callus cultures growing on solid medium. Low total tanshinone concentrations of 0.023, 0.010 and 0.007% was observed after 50-day elicitation of 0.4, 0.2 and 0.1 mM SA, respectively, which was consistent with previous observations. Therefore, only the highest SA concentration of 0.4 mM was able to increase the total tanshinone concentration within the lower range observed in native plant roots growing in Poland. However, such high SA concentrations inhibit the callus growth rate and is twice higher than 0.2 mM commonly applied in plant elicitation experiments.

Evaluation of tanshinone types induced by SA indicates that the *S. miltiorrhiza* callus growing on solid MS medium is dominated by CT with a lower concentration of DHT observed. Similar results suggesting predominant concentration of DHT and CT were provided by other authors in callus or hairy root cultures (Hao et al., 2015; Cheng et al. 2013). Also, Krajewska-Patan et al. (2007) showed that DHT had an advantage (0.27%) over the CT (0.12%) in Danshen callus cultures. Zhao et al. (2010) found that CT was significantly stronger when stimulated by several elicitors as Co^{2+} , Ag^+ , SA, yeast elicitor, sorbitol and chitosan in *S. miltiorrhiza* cell cultures. Only results of Cao et al. (2018) show the dominant concentration of TI over CT and DHT in transformed *S. miltiorrhiza* hairy roots overexpressing

SmWRKY1 transcription factor. Similarly, the predominant tanshinone in native *S. miltiorrhiza* plant roots is TIIA.

These differences may be explained by the tanshinone biosynthesis pathway structure, where the CT is further modified to TIIA, whereas DHT serves as a substrate to produce TI (Guo and Ma 2019). Alternatively, CT plays a role in phytoalexin function, concentrated in cells and directly involved in response to yeast extract elicitation (Chen and Chen 2000).

The time course of tanshinone accumulation in our solid callus culture SA-induced system was slower and not as stable as in previous studies using cytokinin elicitation (Wu et al. 2003). Also *S. miltiorrhiza* cell suspension or hairy root cultures show a relatively short (24-h) response time to SA treatment (Cheng et al. 2013). In addition, Hao et al. (2015) indicate that the total tanshinone concentration in *S. miltiorrhiza* hairy root culture starts to increase 36 h after 0.1 mM SA treatment. Our findings show generally lower tanshinone accumulation dynamics; this may be related to the fact they were grown on solid rather than liquid medium, or the generally lower initial tanshinone productivity demonstrated by plants growing in Poland (Buchwald et al. 2006; Cheng et al. 2013).

Although 0.4 mM SA induced 2.33-times higher total tanshinone concentration than 0.2 mM SA after 50 days of induction, the decreased tanshinone concentration is compensated by GI_F , which was 2.03–2.99 times higher for a lower SA value. Similar values of GI were observed for dry weight (GI_D). The GI noted for callus cultures on solid medium was generally lower than GI for callus cultures on liquid medium. Li et al. (2003) observed GI values of 11.44 in controls and 9.93 for samples treated with 0.2 mM SA for 18 days. Our data show much slower GI of 3.47 ± 0.15 for the control group and 2.12 ± 0.27 for 0.2 mM SA after 20 days of culture on solid medium.

Elicitation of callus by auxin (NAA) did not show increased tanshinone concentration nor any significant change in GI_F or GI_D for 2.69, 13.43, 26.85 and 40.28 μM NAA. Only the highest NAA concentration 40.28 μM (7.5 mg L^{-1}) NAA slowed the callus growth rate. It is possible that SA and NAA elicitation may influence the tanshinone biosynthesis rate though inhibiting the transcription activity of most ARFs in *S. miltiorrhiza* (Xu et al. 2016). Also, the expression of certain genes, such as *SmHMGR2*, in *S. miltiorrhiza* is not induced by auxin IAA (Szymczyk et al. 2018).

In contrast, SA induces the expression of genes associated with tanshinone biosynthesis (Hua et al. 2012; Hao et al. 2013, 2015; Yan et al. 2009; Zhang et al. 2010, 2016). Most of these genes or their *A. thaliana* homologs indicate SA-responsive *cis*-elements in proximal promoters. In contrast, compared to SA-responsive elements, the auxin-responsive *cis*-active elements are much rarer in the tested proximal promoters of *A. thaliana* and *S. miltiorrhiza* genes participating in isoprenoid biosynthesis.

Conclusion

The presented study compares the influence of SA and NAA elicitation on tanshinone concentration in *S. miltiorrhiza* callus cultures growing for up to 80 days on a solid MS medium. The SA indicated clear but relatively low induction of four tanshinones, viz. CT, DHT, TI and TIIA, as well as total concentration, which may be due to the relatively low tanshinone concentration in *S. miltiorrhiza* plants growing in Poland. However, NAA elicitation did not increase tanshinone concentration. The influence of either phytohormone on tanshinone concentration seems to be proportional to the distribution frequency of auxin and SA-responsive *cis*-active elements in proximal promoters of *S. miltiorrhiza* or *A. thaliana* homologous genes, encoding for the enzymes of the MEP and MVA pathways or the later stages of tanshinone precursor (miltiradiene) biosynthesis.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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