Plant Cell Rep
DOI 10.1007/s00299-016-2076-x

ORIGINAL ARTICLE

Increasing the synthesis of bioactive abietane diterpenes in *Salvia sclarea* hairy roots by elicited transcriptional reprogramming

M. C. Vaccaro[1] · M. E. Alfieri[1] · N. Malafronte[1] · N. De Tommasi[1] · A. Leone[1]

Received: 5 July 2016 / Accepted: 8 November 2016

Abstract
Key message **Transcriptional activation of genes belonging to the plastidial MEP-derived isoprenoid pathway by elicitation with methyl jasmonate and coronatine enhanced the content of bioactive abietane diterpenes in *Salvia sclarea* hairy roots.**
Abstract We have shown that aethiopinone, an abietane diterpene synthesized in *Salvia sclarea* roots is cytotoxic and induces apoptosis in human melanoma cells. To develop a production platform for this compound and other abietane diterpenes, hairy root technology was combined with the elicitation of methyl jasmonate (MeJA) or the phytotoxin coronatine (Cor). Both MeJA and Cor induced a significant accumulation of aethiopinone, but prolonged exposure to MeJA irremediably caused inhibition of hairy root growth, which was unaffected by Cor treatment. Considering together the fold increase in aethiopinone content and the final hairy root biomass, the best combination was a Cor treatment for 28 days, which allowed to obtain up to 105.34 ± 2.30 mg L^{-1} of this compound to be obtained, corresponding to a 24-fold increase above the basal content in untreated hairy roots. MeJA or Cor elicitation also enhanced the synthesis of other bioactive abietane–quinone diterpenes. The elicitor-dependent steering effect was due to a coordinated transcriptional activation of several biosynthetic genes belonging to the plastidial MEP-derived isoprenoid pathway. High correlations between aethiopinone content and MeJA or Cor-elicited level of gene transcripts were found for *DXS2* ($r^2 = 0.99$), *DXR* ($r^2 = 0.99$), and *GGPPS* ($r^2 = 0.98$), encoding enzymes acting upstream of GGPP, the common precursor of diterpenes and other plastidial-derived terpenes, as well as *CPPS* ($r^2 = 0.99$), encoding the enzyme involved in the first cyclization steps leading to copalyl-diphosphate, the precursor of abietane-like diterpenes. These results point to these genes as possible targets of metabolic engineering approaches to establish a more efficient production platform for such promising anti-proliferative plant-derived compounds.

Keywords Bioactive abietane diterpenes · *Salvia sclarea* hairy roots · Elicitation · Transcriptional reprogramming

Introduction

Plant diterpenes are one of the largest and most diverse classes of plant metabolites, with more than 10,000 different structures already described. Diterpenes are characterized by a C-20 hydrocarbon backbone and derived from the universal substrate C-20 geranylgeranyl pyrophosphate (GGPP), which is synthesized through the plastidial MEP-derived isoprenoid pathway. Several plant diterpenoids have well-established antitumor activities, such as the most widely used breast cancer drug taxol (Weaver 2014) or ingenol-3-angelate from *Euphorbia peplus*, a promising skin cancer chemotherapeutic agent, used in a topically applied formulation and evaluated in clinical trials (Song et al. 2013). Recently, plant-derived abietane diterpenoids

Communicated by E. Benvenuto.

Electronic supplementary material The online version of this article (doi:10.1007/s00299-016-2076-x) contains supplementary material, which is available to authorized users.

✉ A. Leone
aleone@unisa.it

[1] Department of Pharmacy, University of Salerno, Via Giovanni Paolo II 134D, 80084 Fisciano, Italy

Published online: 16 November 2016

with a quinone moiety have also raised great attention for their interesting cytotoxic effects on different cancer cell lines (González 2015 for a recent review). More than 400 diterpenoids with different abietane skeletons have so far been characterized and several are synthesized in different organs and tissues of *Salvia* species (Kabouche and Kabouche 2008; Bonito et al. 2011). Aethiopinone from *Salvia aethiopis* L. has been proved to be cytotoxic against the KB human carcinoma cell line, with an IC_{50} value of 2 μM (Hernández-Pérez et al. 1999). High cytotoxicity against HL-60 and NALM-6 leukemia cells was also reported for salvipisone and aethiopinone, isolated from *Salvia sclarea*, due to caspase-3 activity and induction of apoptosis in a time- and concentration-dependent manner (Rózalski et al. 2006). We recently demonstrated that aethiopinone is also cytotoxic against different solid tumor cell lines, i.e., MCF7 (breast adenocarcinoma), HeLa (epithelial carcinoma), PC3 (prostate adenocarcinoma), and A375 (human melanoma) and induced apoptosis in A375 melanoma cells at a concentration not cytotoxic to control cells (Vaccaro et al. 2014). These results point to this abietane-type diterpene as a promising molecule or lead compound for treating melanoma, the most aggressive skin cancer, which is notoriously resistant to all current modalities of cancer therapy (Holohan et al. 2013).

However, a more comprehensive analysis of the mechanism of action of aethiopinone is hampered by the minute amounts produced in roots of different *Salvia* species. Insufficient availability of bioactive plant secondary metabolites, coupled with the recurrent impossibility or high cost of their chemical synthesis, is a general drawback for the industrial development of bioactive plant-derived drugs. This has prompted major efforts to develop plant cell and tissue cultures as suitable systems for their production (Kuzma et al. 2006; Baque et al. 2012; Marchev et al. 2014). Besides optimization in growth media and systems, selection of high-yielding lines (Hussain et al. 2012), and metabolic engineering strategies (Dudareva and Della Penna 2013; Atanasov et al. 2015), elicitation of plant cells, and/or tissues with molecules of different origin and chemical structures has been widely used to enhance the synthesis of interesting plant-specialized products. Elicitors are molecules of different natures, capable of stimulating plant defense response, widely conserved in the plant kingdom, from angiosperms to gymnosperms (Wasternack and Hause 2013; Pauwels et al. 2009; Zhao et al. 2005). One of the most characterized elicitors is jasmonic acid (JA) and derivatives, which, when applied to plant cells and tissues of a variety of plant species, in the range of 100–200 μM, generally induce the synthesis of molecules belonging to the three main classes of plant secondary metabolites (terpenoids, alkaloids, and phenylpropanoids) (Zhao et al. 2005; Qian et al. 2005; Hu et al. 2006; Baenas et al. 2014). Endogenous JAs, synthesized from linolenic acid, or the synthetic methyl jasmonate (MeJA), are converted into the biologically active form (+)-7-iso-jasmonoyl-L-isoleucine (JA-Ile) (Fonseca et al. 2009). This binds to the CORONATINE INSENSITIVE1 (COI1) F-box protein receptor (Sheard et al. 2010), which operates as an E3 ubiquitin ligase and targets the JAZ repressor proteins for degradation by the 26S proteasome (Zhang et al. 2015). Multiple transcription factors (MYC and others) are depressed, which in turn activate JA-Ile-dependent transcriptional reprogramming, as established by several genome-wide transcript profiling studies in model plants and medicinal plants (Pauwels et al. 2009; De Geyter et al. 2012; Yang et al. 2013). MeJA has been used to stimulate accumulation of bioactive diterpenoids in *S. sclarea* hairy roots in both shake flasks and sprinkle bioreactors (Kuzma et al. 2009), but it is well known that different elicitors may exert a distinctive induction pattern on secondary metabolism and/or differentially affect final plant cell or tissue growth. Coronatine (Cor), a polyketide effector molecule produced by *Pseudomonas syringae* pv tomato strain DC3000 (Pst DC3000), binds the same receptor and acts as a structural agonist of JA-Ile (Yan et al. 2013). Few studies have focused on the effects of Cor on boosting the synthesis of specific secondary metabolites in plants, e.g., increased flavonoid content in rice leaves (Tamogami and Kodama 2000), increased production of alkaloids in *Eschscholzia californica* (Haider et al. 2005), the accumulation of glyceollins in soybean cell cultures (Fliegmann et al. 2003), paclitaxel and related taxanes in cell suspensions of *Taxus media* (Onrubia et al. 2013), and trans-resveratrol in grape cells (Taurino et al. 2015). Compared to natural and synthetic JAs, Cor may offer several advantages in eliciting plant secondary metabolites, due to a higher chemical stability compared to JA-Ile and no need of a previous conversion into JA-Ile to bind the COI1 receptor (Feng et al. 2003).

Here, we report a comparative analysis of the effects of MeJA and Cor on aethiopinone content of *S. sclarea* hairy roots in promoting the accumulation of aethiopinone as well as other interesting tricyclic abietane-type diterpenes (salvipisone, ferruginol, and carnosic acid), by transcriptional activation of several genes encoding enzymes belonging to the plastidial MEP-derived isoprenoid pathway.

Methods

Salvia sclarea hairy root cultures

Hairy roots were initiated after infection of *S. sclarea* axenic leaf sections with *A. rhizogenes* strain ATCC15834,

as described in Vaccaro et al. (2014). Hairy root independent lines, with no bacterial contamination, were selected and grown at 23 °C in the dark in 250 ml flasks with 0.5X MS (Murashige and Skoog 1962) liquid medium, supplemented with 30 g L^{-1} of sucrose on a rotary shaker at 120 rpm, and maintained by sub-culturing at the end of the exponential stage every 21 days (d). The hairy root line used in this study was maintained for about 20 cycles before being used in the elicitation experiments.

Elicitor treatment and hairy root growth

Salvia sclarea hairy roots, grown into 500 mL flasks, containing 100 mL of 0.5X MS liquid medium, were elicited with methyl jasmonate (MeJA) or coronatine (Cor) (Sigma-Aldrich Co, St Louis, MO, USA). In a first set of experiments, hairy roots (1 g), harvested at their late exponential stage, were grown into 0.5X MS liquid fresh medium for 21 days and then treated for 7 days with microfiltered (0.22 mm sterile PES filters, Millipore, Billerica, MA, USA) MeJA, dissolved in 96% ethanol, or Cor, dissolved in bidistilled water, and added to the growth medium to a final concentration of 100 and 0.1 μM, respectively. In a second set of experiments, the same amount of hairy roots was inoculated in fresh growth medium, as described above, and continuously elicited for 28d with the same MeJA or Cor concentration. As mock control, the hairy roots, grown under the same growth conditions, received equal volumes of 96% ethanol (0.005%, final concentration) or an equal volume of H_2O. Control and elicited hairy roots were maintained on a rotary shaker at 120 rpm at 23 °C in the dark. Hairy root growth was determined as described previously (Vaccaro et al. 2014) by monitoring the dry weight of control and elicited hairy roots at 7-day intervals during 28 days.

Amplification and sequencing of partial cDNAs encoding *S. sclarea* MEP-pathway biosynthetic genes

Hairy roots were homogenized in liquid nitrogen and total RNA extracted with the plant RNA purification kit (Norgen Biotek Corporation Ontario, Canada) according to the manufacturer's protocol. The complementary DNA (cDNA) was synthesized from 1 μg of total RNA, previously treated with RNase-free DNAse I (Invitrogen, Carlsbad, CA, USA), using random hexamers and the Superscript III Reverse transcriptase (Invitrogen) at 50 °C for 50 min. The partial sequences of *S. sclarea* biosynthetic genes belonging to the MEP biosynthetic pathway were obtained by RT-PCR amplification with high-fidelity Taq polymerase (Invitrogen), using different combinations of degenerate primers, designed on the basis of alignment of sequences available in GenBank database Amplicons (200–300 bp). After purification and cloning into pcR2.1 vector (TOPO TA Cloning kit, Invitrogen), the obtained partial cDNAs were sequenced (PRIMM Biotech, Milan, Italy) and alignment to publically available plant sequences revealed homology with gene encoding different isoform of known plant biosynthetic genes of the MEP-derived isoprenoid pathway. Sequences of genes analyzed in this study were published in dbEST/GenBank database and used to design specific oligonucleotides at 5′ and 3′ for transcriptional analysis by quantitative PCR analysis.

Quantitative reverse transcription-PCR (q-RT-PCR)

Total RNA was extracted from hairy roots treated with MeJA 100 μM or Cor 0.1 μM at different times after elicitation (12, 24, and 48 h) or from a control mock-treated hairy root line, using the plant RNA purification kit (Norgen Biotek Corporation Ontario, Canada). cDNA was synthesized from 1 μg of total RNA, previously treated with RNase-free DNAse I (Invitrogen, Carlsbad, CA, USA), and retro-transcribed by Superscript III RT, as described above. Quantitative RT-PCR was performed with SYBR Green PCR Mastermix (Roche, USA) in a LightCycler 480 system (Roche Diagnostics Ltd, Lewes, UK), according to the manufacturer's instruction, using 0.5 μM oligonucleotides designed on the partial sequences of *S. sclarea* MEP-pathway biosynthetic gene sequences, identified as reported above. The primer pairs were designed by primer 3 software and analyzed with *UNAFold, Integrated DNA Technologies* and *Two-state melting* (*folding*) software information and listed in Table 1S. Transcript levels of targeted genes in control hairy roots and MeJA- or Cor-elicited hairy roots were quantified using SYBR green detection and the comparative threshold cycle (Ct) method (Schmittgen and Livak 2008). The Ct values for *S. sclarea* 18S RNA and actin mRNA (the internal standard) were subtracted from that of the gene of interest to obtain a ΔCt value. The Ct value of the control sample was subtracted from the ΔCt value to obtain a ΔΔCt value. Each fold change in the expression level relative to that of the control was expressed as 2–ΔΔC. All real-time PCR reactions were performed in triplicate and in three independent experiments.

Extraction and quantitative analysis of abietane diterpenes

Lyophilized and powdered *S. sclarea* hairy roots (0.5 g) were extracted with acetone for 48 h at room temperature, and targeted analysis of the abietane diterpenes was carried out by HPLC–DAD, on a C8 column (Agilent, Zorbax Eclipse C8 250 × 4.6 mm), as described in Vaccaro et al.

(2014). The different diterpenes were detected at 280 nm and contents of abietane diterpenoids were calculated by interpolating the peak areas with calibration curves, obtained with standard purified compounds in the range 10–200 μg mL^{-1}, and expressed as mg g^{-1} of hairy root dry weight or mg L^{-1} of liquid culture.

Statistical analysis

All reported data for growth, metabolic, or transcriptional analysis represent the mean ± SD of three biological experiments and three independent technical replicas. The statistical significance of transcript levels was analyzed by one-way analysis of variance (ANOVA), with Bonferroni's post-test (***$P \leq 0001$, **$P \leq 0.01$, *$P \leq 0.05$). The statistical significance of variation in abietane–diterpene content and hairy root growth was examined by one-way analysis of variance (ANOVA). Means followed by different letters differ significantly from one another at $P \leq 0.05$, according to Tukey's multiple comparison test. Correlation analysis of gene transcript level and aethiopinone content was calculated using Pearson test (r) analysis at the statistical significance ($P \leq 0.01$ or $P \leq 0.05$). All statistical analyses were conducted using GraphPad Prism 5 software.

Results

Biomass growth and aethiopinone content in elicited hairy roots

Salvia sclarea hairy roots grown in liquid batches were cultured for 21 days (d) and then elicited continuously for 7 days with MeJA or Cor added to a final concentration of 100 or 0.1 μM, respectively, on the basis of a preliminary combined metabolic and growth kinetics analysis (data not shown). Compared to control untreated hairy roots, elicitation with MeJA or Cor both induced a significant increase in aethiopinone content, conferring a typical red color to the hairy roots (Fig. 1). However, MeJA appeared to be more effective in triggering aethiopinone accumulation (9.72 ± 0.08 mg g^{-1} DW) than Cor (2.57 ± 0.15 mg g^{-1} DW), corresponding to a 25-fold increase over the content of untreated hairy roots (0.38 ± 0.07 mg g^{-1} DW), against the approx. sevenfold increase attained in Cor-elicited hairy roots. Since the final biomass of hairy roots after MeJA and Cor elicitation for 7 days was substantially the same and did not differ from that of control hairy roots, the total amount of aethiopinone extracted from 1 l of MeJA hairy root liquid culture (92.34 ± 1.21 mg L^{-1}) was significantly higher than the amount in Cor-elicited hairy roots (24.08 ± 0.79 mg g^{-1} DW) (Fig. 1).

The effect of a longer elicitation (28 days) on aethiopinone content was also tested by adding MeJA or Cor to freshly inoculated hairy roots (1 g FW). Again, the highest accumulation in aethiopinone was observed in MeJA-elicited hairy roots (20.36 ± 0.18 mg g^{-1} DW). However, long-term elicitation with MeJA caused a severe hairy root growth inhibition, whereas no detrimental effects on final biomass were observed in hairy roots treated with Cor for 28 days (Fig. 1; Fig. S1). Considering the final biomass in 1 L of hairy root culture, a Cor treatment for 28 days allowed to extract 103.32 ± 2.10 mg L^{-1} of aethiopinone, corresponding approximatively to a 24-fold increase over the basal content of control hairy roots (4.40 ± 0.13 mg L^{-1}). This content was higher than the 16-fold increase

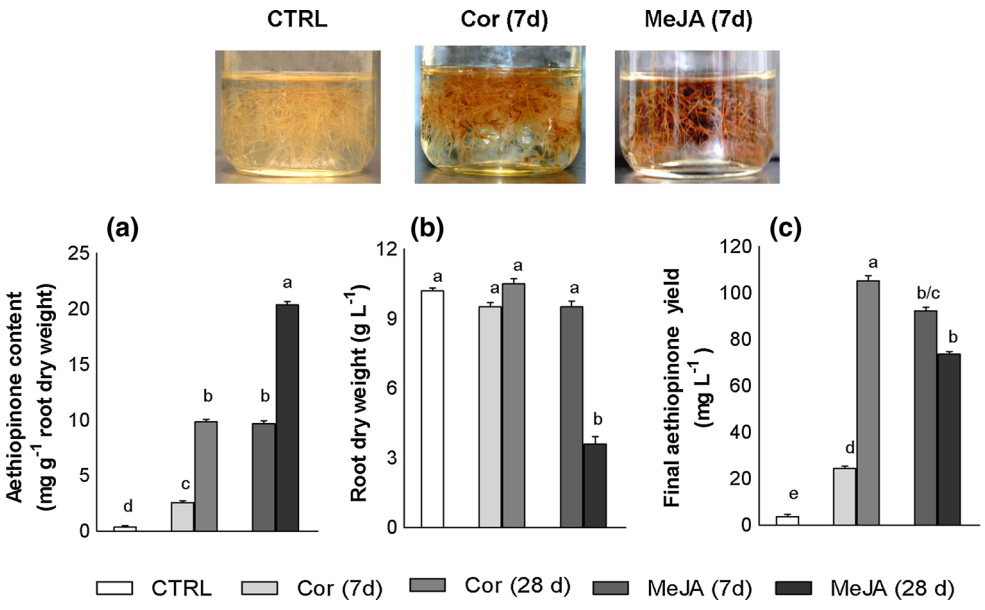

Fig. 1 Aethiopinone content (**a**), root dry weight (**b**), and final aethiopinone yield (**c**) in control and elicited *S. sclarea* hairy roots. The same amount of freshly inoculated hairy roots, harvested at their late exponential growth stage, were treated with MeJA (final concentration 100 μM) or Cor (final concentration 0.1 μM) for 7 or 28 days. Data represent mean values ± SD of three biological replicates. Means followed by the same letter do not differ significantly from one another at $P \leq 0.05$, according to Tukey's multiple comparison test

(73.29 ± 0.11 mg L^{-1}) induced by elicitation with MeJA for the same elicitation time and greater than the final yield obtained after 7 days with this elicitor.

MeJA- and Cor-elicited transcript gene profiling

To gain a more comprehensive understanding of the mode of action of the two elicitors in enhancing the accumulation of aethiopinone in *S. sclarea* hairy roots, expression levels of genes encoding enzymes belonging to the plastidial MEP-derived isoprenoid pathway to GGPP were monitored by quantitative RT-PCR during 48 h after elicitation. Based on the delta–delta Ct (2-ΔΔCt) method, relative expression levels of the selected biosynthetic genes above the basal level of control hairy roots were calculated and compared with those measured upon the different elicitor treatments. The studied genes were *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), and both involved in early biosynthetic steps of this pathway; *CMS* (encoding 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol synthase), *CMK* (encoding 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase), *MCS* (encoding 2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase), *HDS* (encoding 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), *HDR* (encoding 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase), and *GGPPS* (encoding geranyl–geranyl-diphosphate synthase), acting upstream of GGPP, the general precursor of diterpenes, as well as *CPPS1* (encoding the copalyl-diphosphate synthase), the first committed enzyme that diverts part of the plastidial GGPP pool toward cyclic diterpenes (Fig. 2). A preliminary analysis indicated that genes encoding the isoform DXR2, HDR1, GGPPS1, and CPPS1 were the most expressed in *S. sclarea* hairy roots, and, therefore, specific primers were used for qRT-PCR amplification (Table 1S).

Both elicitors steered a significant change in the transcript level of the genes encoding biosynthetic enzymes acting upstream of GGPP (Fig. 2). The most activated genes were *DXS2* (approximately 60- and 20-fold increase, after MeJA or Cor elicitation, respectively) and *DXR* (20-fold in MeJA-elicited roots *versus* approximately 15-fold increase upon Cor treatment). About a 6- and 12-fold increase in transcript levels of the *CMK* and *MCS* genes, respectively, was also found upon MeJA or Cor treatment, while the transcript level of the *HDS* gene was induced at a higher level by MeJA (approx 35-fold increase) compared to Cor treatment (fivefold increase). Transcription of the *HDR1* gene was also enhanced, with a higher effect of MeJA (about 30-fold increase) than Cor (about eightfold increase) upon elicitation. The less inducible gene was CMS, acting upstream of GGPP, whose transcript level increased only two times higher than the basal level of *S. sclarea* control hairy roots after MeJA or Cor elicitation.

MeJA elicitation also activated the transcription of the *GGPPS1* gene, catalyzing the condensation of three IPP molecules and one molecules of DMAPP to yield GGPP, the common precursor of diterpenes. An approximate 20-fold increase in *GGPPS1* transcript level was triggered after 24 h of MeJA, much higher than the threefold increase obtained upon Cor elicitation.

MeJA and Cor also enhanced the transcript level of the *CPPS1* gene, encoding a copalyl disphosphate synthase, a class II diterpene synthase responsible for catalyzing the cyclization of linear isoprenoid precursors to form the complex hydrocarbon skeletons of cyclic diterpenes, again with a stronger effect of MeJA elicitation (approximately 60-fold increase) compared to Cor treatment (eightfold increase).

Taken together, the transcriptional data indicated that, in general, the maximum peak in the transcript level was observed for all the analyzed genes after 24 h of treatment with both elicitors, followed by a decline in the next 24 h. In addition, MeJA treatment was more effective than Cor elicitation in inducing a higher expression level of most of the analyzed genes.

Correlation analysis of gene expression and aethiopinone accumulation

Elicitation studies are also useful in elucidating possible bottleneck in the biosynthesis of these specific abietane diterpenoids. As expected, no correlations were found between gene expression level and aethiopinone content after 24, 48, and 72 h of elicitation, since it is widely reported that the gene changes in gene expression precede MeJA-induced changes in secondary metabolite content. Therefore, correlations were calculated between gene expression level after 24 h of treatment, corresponding to the maximum expression peak of all the biosynthetic genes considered in this study (Fig. 2) and the final aethiopinone content upon MeJA or Cor elicitation for 7 days in 1L of hairy root liquid culture. Although a positive correlation was found for all the analyzed genes, in MeJA-treated hairy roots, the highest r^2 values ($P \leq 0.01$) were identified for *DXS2* ($r^2 = 0.9982$), *DXR* ($r^2 = 0.9965$), *GGPPS1* ($r^2 = 0.9632$), *HDR1* ($r^2 = 0.9960$), and *CPPS1* ($r^2 = 0.9975$). Similarly, the highest correlation values ($P \leq 0.05$) were obtained for Cor-elicited hairy roots for *DXS2* ($r^2 = 0.9975$), *DXR* ($r^2 = 0.9758$), and *CPPS* ($r^2 = 0.9804$), whereas for *GGPPS1* and *HDR1,* the r^2 value was 0.7059 and ($r^2 = 0.8950$), respectively.

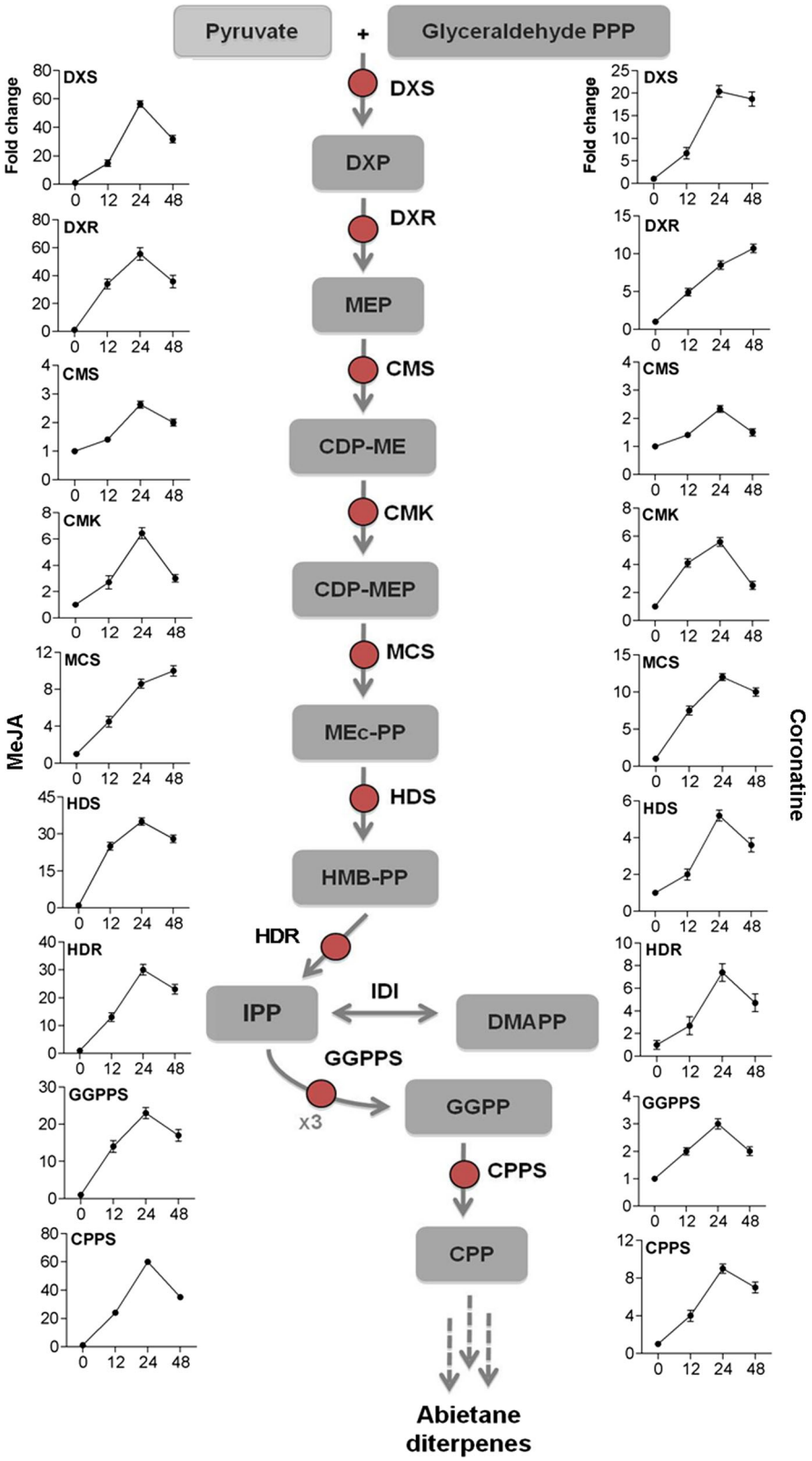

Fig. 2 MeJA- or Cor-induced expression profiles for the genes belonging to plastidial 2C-methyl-D-erythritol 4-phosphate (MEP) isoprenoid pathway, from which abietane diterpenes derive, during 48 h from elicitation. Enzyme abbreviations are presented in *bold capital letters*, next to the corresponding catalyzed reaction (*plain arrow*). *Dotted arrows* indicate multiple enzymatic reactions. The relative gene expression pattern is reported as a graphic, where the *X*-axis represents hours post elicitation (h) and the *Y*-axis represents the fold change in transcript levels above the basal level of non-elicited control hairy roots, calculated by the CT method. Values represent mean ± SD of three biological replicates for each elicitation treatment. *, **, *** Indicate significant differences at $P \leq 0.05$, 0.01, and 0.001, respectively, in the fold increase of transcript level for each gene between *S. sclarea* elicited and control hairy roots. *DXS* (1-deoxy-D-xylulose 5-phosphate synthase), *DXR* (1-deoxy-D-xylulose 5-phosphate reductoisomerase), *CMS* (4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol synthase), *CMK* (4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase), *MCS* (2-C-methyl-D-erythritol-2,4-cyclodiphosphate synthase), *HDS* (1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase), *HDR* (encoding 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase), *GGPPS* (geranyl–geranyl-diphosphate synthase), *CPPS* (copalyl-diphosphate-synthase)

MeJA or Cor elicitation affects accumulation of other tryciclic abietane-type diterpenes

Although maximum elicited induction was observed for aethiopinone, MeJA or Cor elicitation triggered accumulation of additional trycyclic abietane-type diterpenes (Fig. 3). The final yield of each compound, expressed as mg L^{-1}, was elicitor- and time-dependent, as for aethiopinone. Upon MeJA-elicitation for 7 days, the content of ferruginol increased from 1.93 ± 0.35 to 56.13 ± 1.66 mg L^{-1}, not differing significantly from the increase triggered by a long-term exposure (55.48 ± 1.2 mg L^{-1}). Considering the final yield, 103.01 ± 1.80 mg L^{-1} of ferruginol were extracted from hairy roots treated with Cor for 28 days, corresponding to an approximately 54-fold increase over the content of control hairy roots. MeJA- or Cor-elicited hairy roots also accumulated salvipisone and oxo-derivatives of either aethiopinone (1-oxo-aethiopinone) or ferruginol (1-oxo-ferruginol).

Interestingly, both elicitors enhanced the content of carnosic acid, a benzenediol abietane diterpene which, to the best of our knowledge, has never before been identified in *S. sclarea* roots. The maximum yield of this compound was obtained after elicitation with Cor for 28 days (36.75 ± 2.20 mg L^{-1}), corresponding to about an 18-fold increase above the basal content of control hairy roots.

These data indicate that MeJA or Cor is able to push the metabolic flux of *S. sclarea* hairy roots toward a higher total content of abietane diterpenes. Cor elicitation at very low concentrations for 28 days did not cause any growth inhibition and it was possible to extract 410.97 ± 2.50 mg L^{-1} as the sum of diterpenoids, significantly higher than the content either after short or prolonged elicitation with MeJA (Table 1).

Discussion

Plant cell and tissue cultures hold great promise as a valid alternative to cultivation to produce a stable biomass for the extraction of valuable plant-derived bioactive compounds (Leone et al. 2007; Marchev et al. 2014). For secondary metabolites that are synthesized only in differentiated cells, hairy roots have also been proved to be an ideal bio-production platform for diverse classes of plant-specialized metabolites (Baque et al. 2012). However, product yield and productivity are major bottlenecks limiting a wider industrial application of cell/tissue cultures aimed at producing large quantities of bioactivity secondary metabolites. Yield limitations imposed by endogenous metabolic control might be overcome by metabolic engineering when sufficient information on biosynthetic and regulatory genes is available or via elicitation, which may also be useful for predictive identification of unknown key limiting biosynthetic steps as well as regulatory proteins of the entire metabolic pathway (Atanasov et al. 2015; O'Connor 2015).

In the present study, we provided evidence that the content of aethiopinone, an abietane diterpene which was shown to induce G_2/S arrest in human melanoma cells

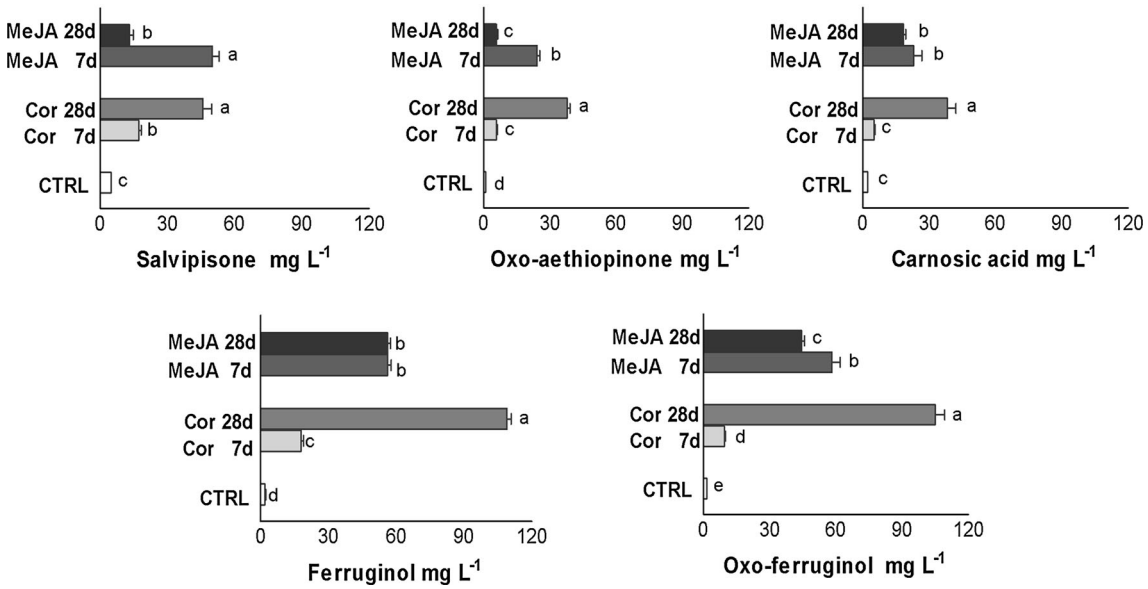

Fig. 3 Content of other abietane diterpenes in *S. sclarea* hairy roots upon elicitation with MeJA (100 μM) or Cor (0.1 μM), compared to control hairy roots. Elicitation treatment conditions were the same as those reported in Fig. 1. Values represent mean values ± SD of three biological replicates. Means followed by different letters differ significantly from one another at $P \leq 0.05$, according to Tukey's multiple comparison post-test

Table 1 Total content of abietane diterpenes as a sum of aethiopinone, oxo-aethiopinone salvipisone, ferruginol, oxo-ferruginol, and carnosic acid in *S. sclarea* hairy roots control or elicited with (MeJA (100 uM) or Cor (0.1 uM)

	Total abietane diterpene content (mg g^{-1} dry weight)	Root dry weight (g L^{-1})	Total abietane diterpene production (mg L^{-1})
CTRL 1	1.48 ± 0.11^{c}	9.8 ± 0.10^{a}	14.50 ± 0.31^{e}
MeJA (7 days)	$32.01 \pm 1.10^{b/c}$	9.5 ± 0.13^{a}	304.01 ± 2.21^{b}
Cor (7 days)	8.27 ± 0.25^{d}	9.5 ± 0.12^{a}	78.56 ± 1.12^{d}
CTRL 2	1.40 ± 0.21^{e}	10.5 ± 0.10^{a}	14.70 ± 0.13^{e}
MeJA (28 days)	58.54 ± 1.25^{a}	3.6 ± 0.13^{b}	210.74 ± 1.26^{c}
Cor (28 days)	39.14 ± 0.82^{b}	10.5 ± 0.11^{a}	410.97 ± 2.50^{a}

Elicitation treatment conditions were the same as those reported in Fig. 1

Different letters differ significantly from one another at $P \leq 0.05$, according to Tukey's multiple comparison post-test

(Vaccaro et al. 2014), can be enhanced in hairy roots of *S. sclarea* by elicitation with MeJA or Cor. Under our experimental conditions, elicitation of freshly hairy roots inoculated at their exponential phase with MeJA for 7 days, and enhanced accumulation in aethiopinone yield (about 20-fold increase) above the basal level in control hairy roots) a steering effect higher than that reported previously in *S. sclarea* hairy root culture either in shake culture or sprinkle bioreactor (approximatively a ninefold increase) (Kuzma et al. 2009). A longer MeJA treatment (28 days) arrested hairy root growth almost totally, as also reported by the same group. This inhibitory effect of MeJA on cells and hairy root growth, presumably due to the reported G_2 arrest of cell cycle progression that MeJA caused even at very low concentrations (Pauwels et al. 2008), is well documented and poses a major problem for its application at the industrial level (Patil et al. 2014).

In seeking to optimize the production of aethiopinone in *S. sclarea* hairy roots, we also tested elicitation with coronatine (Cor), a toxin produced by *P. syringae*, which binds with the same COI1 receptor, acting as an agonist of MeJA, and to a certain extent, mimics its biological activities. In addition, Cor is more stable than MeJA and does not require chemical conversion to JA-Ile to bind to the COI1 receptor (Feng et al. 2003). A concentration as low as 0.1 μM of Cor was sufficient to trigger considerable changes in aethiopinone and in the total abietane diterpene content of *S. sclarea* hairy roots. This concentration was even lower than that used in the few studies in which Cor was used as elicitor of plant secondary metabolites, generally in the range 1–10 μM (Onrubia et al. 2013; Taurino et al. 2015; Haider et al. 2005; Gallego et al. 2015). Compared to MeJA, Cor elicitation for 28 days did not affect hairy root growth, and as a consequence, the final yield of aethiopinone obtained from 1 L of hairy root culture after 28 days of continuous Cor treatment was on average 32% higher than the content after prolonged elicitation with MeJA and even higher than the content in hairy roots elicited with MeJA for 7d.

The elicitation studies were also very informative to establish potential key limiting steps in the biosynthetic pathway of abietane diterpenes in *S. sclarea.* According to the current prevailing view, this class of compounds originates from GGPP through the plastidial MEP-derived terpenoid pathway, and the enzymatic steps to GGPP as well as the corresponding encoding genes are well characterized in different plant species (Zhao et al. 2013). Gene expression analysis demonstrated that MeJA- or Cor-induced accumulation of aethiopinone in hairy roots of *S. sclarea* was associated with upregulation of all the genes acting upstream GGPP. Interestingly, the most induced genes were *DXS2*, highly expressed in *Salvia miltiorrhiza* roots compared to the other *DXS* genes identified in this species (Ma et al. 2012), and *DXR*, encoding two enzymes which have been reported to be limiting for the synthesis of different plastidial terpenes in a variety of plant species (Cordoba et al. 2009). The expression level of these two genes was highly correlated with the aethiopinone content in MeJA- or Cor-elicited hairy roots. Such results are consistent with our recent findings obtained by overexpressing *Arabidopsis DXS* and *DXR* genes in *S. sclarea* hairy roots, which triggered a 2.5 and 4.4-fold increase in aethiopinone, respectively (Vaccaro et al. 2014). Similar results were reported in *S. miltiorrhiza* hairy roots, for which DXS expression is MeJA-induced, and overexpression of DXS was also critical to induce accumulation of tanshionone and derivatives in *S. miltiorrhiza* hairy roots (Kai et al. 2011). In addition, the level of expression of *GGPPS*, encoding the enzyme converting geranyl diphosphate into GGPP, was highly correlated to the aethiopinone content. Focusing only on *Salvia* species, *GGPPS1* is also reported to have been induced by MeJA in hairy roots of *S. miltiorrhiza* (Ma et al. 2012). However, elicitation was more effective in enhancing the aethiopinone content than overexpressing either DXS or DXR gene in *S. sclarea* hairy roots (Vaccaro et al. 2014). It has also been reported that co-expression of *DXS* and *GGPPS* genes *S. miltiorrhiza* in hairy roots of this species has additive effects on

tanshinone content (Kai et al. 2011). These data indicate that DXS, DXR and GGPPS contribute only partially to the overall boosting effects of MeJA on the biosynthesis of diterpenes. MeJA and Cor elicitation were effective in activating also other biosynthetic genes acting upstream of GGPP, such as *CMK, MCS* genes. Especially, *HDR1* was highly induced by MeJA, indicating an important role of this enzyme in the biosynthesis of diterpenes, as corroborated also by enhanced production of tanshinones in hairy roots of *S. miltiorrhiza* overexpressing *SmHDR1* (Hao et al. 2013). Collectively, these results indicate a tight transcriptional coordination of the entire MEP-derived pathway, mediated by MeJA- or Cor-inducible regulatory proteins. Several recent studies have revealed the role of transcription factors, such as myc-2 and some WRKY (Zhou et al. 2016; Yang et al. 2013; Gao et al. 2014; Legrand et al. 2010) which, together with negative regulatory proteins SmJAZ3 and SmJAZ9 identified in *S. miltiorrhiza* (Shi et al. 2016), might contribute to the overall MeJA-mediated regulation of diterpene biosynthesis.

Recent studies have indicated a crosstalk between the MVA and MEP pathways for several terpenes and in different species (Vranovà et al. 2013), as also supported by the increased content in tanshinones in *S. miltiorrhyza* hairy roots overexpressing the HMGR gene (Kai et al. 2011). It would be interesting to ascertain also the potential contribution of the MVA pathway to the increased content in abietane diterpenes in elicited *S. sclarea* hairy roots.

Recent advancements in metabolomics, coupled with RNA sequencing in Lamiaceae species, such as *S. sclarea* calix (Legrand et al. 2010) and roots (Hao et al. 2015b), *S. miltiorrhiza* hairy roots (Wenping et al. 2011; Yang et al. 2013), *Salvia fruticosa* (Chatzopoulou et al. 2010), and *Rosmarinus officinalis* (Božić et al. 2015), are contributing to filling the gaps in the enzymatic reactions involved in the biosynthesis of abietane diterpenes acting downstream GGPP. Interestingly, we found an elevated expression level of the *CPPS1* gene, belonging to class II diTPS, responsible of the enzymatic reaction which produce CPP from GGPP, and in MeJA- and Cor-elicited *S. sclarea,* hairy roots were highly correlated with the aethiopinone content. A significant increase in the expression of *SmCPPS* gene was also reported in MeJA-elicited *S. miltiorrhiza* hairy roots (Cheng et al. 2013; Hao et al. 2015a), and tanshinone accumulation was positively correlated to the expression of this gene (Hao et al. 2015a). CPPS is also emerging as another crucial step in the synthesis of different diterpenes, e.g., carnosic acid and other phenolic diterpenes in *S. fruticosa* and *R. officinalis* (Božić et al. 2015). However, thorough enzymatic and precursor studies are necessary to better define the role of the CPPS in aethiopinone biosynthesis. In addition, to fill the gap in the aethiopinone biosynthetic pathway, it is necessary to define the contribution of other class II diTPS, as well as class I diTPSs, which convert class II diTPS products via ionization of the diphosphate group and subsequent cyclization, oxygenation, and/or rearrangement (Zerbe and Bohlmann 2015).

Interestingly, in *S. sclarea* hairy roots, MeJA and Cor elicitation also triggered the accumulation of other abietane diterpenes, such as ferruginol, as well as carnosic acid, a phenolic diterpene hitherto unidentified in *S. sclarea* roots and hairy roots, probably because it was present at trace levels. Both compounds are derived from CPP through enzymatic reaction catalyzed by kaurene synthase-like (KSL) and CYP76H, as reported recently in *R. officinalis* (Božić et al. 2015) and *S. miltiorrhiza* (Guo et al. 2013). These data indicate indirectly that such enzymes acting downstream of CPP might be operative in *S. sclarea* and their genes might also be MeJA- and Cor inducible. Despite of their interesting biological activities (González 2015), the increased content in ferruginol and carnosic acid and other abietane-type diterpenes in elicited *S. sclarea* hairy roots have to be seen as a constraint in enhancing further the content of aethiopinone because of a potential competition for CPP. Identification and silencing of the *S. sclarea* KSL, coupled to overexpression with CPPS in hairy roots, might be one possibility to produce higher quantities of aethiopinone in *S. sclarea* hairy roots.

Although the two elicitors bind to the same COII receptor and probably trigger the same intracellular signal pathway, MeJA proved to be a more powerful transcriptional inducer of DXS, DXR, GGPPS, HDR, and CPPS genes than Cor, at least at the concentration used in our experiments. However, no substantial differences were found in the expression pattern of taxane biosynthetic genes in *Taxus* cells upon MeJa or Cor elicitation (Onrubia et al. 2013). We can simply speculate that in different plant species or tissues, the two elicitors may trigger distinct but as yet uncharacterized regulatory mechanisms, which need further studies to be elucidated. Importantly, however, a very low concentration of Cor (0.1 μm) was able to activate a coordinated transcription of several biosynthetic genes of the MEP-derived iosprenod pathway at a sufficient level to push the metabolic flux toward a higher content of aethiopinone in *S. sclarea* hairy roots, without interfering with active hairy root growth. It is widely reported that MeJA has to be applied at comparatively higher concentrations ($\geq$100 μM) to trigger the secondary metabolism, with recurrently reported pleotropic negative effects on growth whether in vivo or in vitro (Fliegmann et al. 2003; Geng et al. 2014; Ramirez-Estrada et al. 2016).

Conclusions

The results presented herein show that both MeJA and Cor induce a reprogramming of gene expression in *S. sclarea* hairy roots, which accounts for the enhanced production of

aethiopinone and other abietane-type diterpenes. Although a lower transcriptional activation of the studied genes was induced by Cor, a positive correlation between transcript level of *DXS2*, *DXR*, *GGPPS1*, *CPPS1,* and *HDR1* genes was found using a low concentration of Cor (0.1 μm), which does not interfere with active growth of *S. sclarea* hairy roots. This results in a balanced trade-off between final hairy root biomass and yield in aethiopinone and provides sufficient quantities for gaining insights into the molecular mechanisms underlying their promising anti-proliferative action in melanoma cells and other cancer cell lines. Finally, by targeted transcriptional profiling, it was possible to identify additional potential key limiting steps (HDR, GGPPS, and CPPS) for the biosynthesis of abietane diterpenes in *S. sclarea* which, together with DXS or DXR, might be co-expressed in different combinations to enhance the content of bioactive abietane diterpenes in *S. sclarea* hairy roots.

Author contribution statement MV and AL conceived and designed the study. MV, MA, and NM performed the experiments. MA performed the bioinformatics work to design degenerate primers and cDNA cloning. MV sampled plant material generated hairy root cultures and performed elicitor treatments. MV and MA conducted q-RT-PCR experiments. MV conducted the hairy roots extraction of metabolites. NM and ND performed the HPLC–DAD quantitative analysis and analyzed data. MV did the statistical analyses. AL and MV wrote the manuscript. All authors revised and approved the final manuscript.

Acknowledgements This work was supported by FARB 2014 funds of the University of Salerno to AL. MV is also grateful for the short-term mobility funds of the COST Action FA 1006 "Plant Metabolic Engineering for High Value Products".

Compliance with ethical standards

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

References

Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P (2015) Discovery and resupply of pharmacologically active plant-derived natural products: a review. Biotechnol Adv 33:1582–1614. doi:10.1016/j.biotechadv.2015.08.001

Baenas N, García-Viguera C, Moreno DA (2014) Biotic elicitors effectively increase the glucosinolates content in Brassicaceae sprouts. J Agr Food Chem 62:1881–1889. doi:10.1021/jf404876z

Baque MA, Moh SH, Lee EJ, Zhong JJ, Paek KY (2012) Production of biomass and useful compounds from adventitious roots of high-value added medicinal plants using bioreactor. Biotechnol Adv 30:1255–1264. doi:10.1016/j.biotechadv.2011.11.004

Bonito MC, Cicala C, Marcotullio MC, Maione F, Mascolo N (2011) Biological activity of bicyclic and tricyclic diterpenoids from *Salvia* species of immediate pharmacological and pharmaceutical interest. Nat Prod Commun 6:1205–1215

Božić D, Papaefthimiou D, Brückner K, de Vos RC, Tsoleridis CA, Katsarou D, Papanikolaou A, Pateraki I, Chatzopoulou FM, Dimitriadou E, Kostas S, Manzano D, Scheler U, Ferrer A, Tissier A, Makris AM, Kampranis SC, Kanellis AK (2015) Towards elucidating carnosic acid biosynthesis in Lamiaceae: functional characterization of the three first steps of the pathway in *Salvia fruticosa* and Rosmarinus officinalis. PLoS One 10:e0124106. doi:10.1371/journal.pone.0124106

Chatzopoulou FM, Makris AM, Argiriou A, Degenhardt J, Kanellis AK (2010) EST analysis and annotation of transcripts derived from a trichome-specific cDNA library from *Salvia fruticosa*. Plant Cell Rep 29:523–534. doi:10.1007/s00299-010-0841-9

Cheng Q, He Y, Li G, Liu Y, Gao W, Huang L (2013) Effects of combined elicitors on tanshinone metabolic profiling and SmCPS expression in *Salvia miltiorrhiza* hairy root cultures. Molecules 18:7473–7485. doi:10.3390/molecules18077473

Cordoba E, Salmi M, León P (2009) Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. J Exp Bot 60:2933–2943. doi:10.1093/jxb/erp190

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

Dudareva N, Della Penna D (2013) Plant metabolic engineering: future prospects and challenges. Curr Opin Biotechnol 24:226–228. doi:10.1016/j.copbio.2013.02.002

Feng S, Ma L, Wang X, Xie D, Dinesh-Kumar SP, Wei N, Deng XW (2003) The COP9 signalosome interacts physically with SCF COI1 and modulates jasmonate responses. Plant Cell 15:1083–1094. doi:10.1105/tpc.010207

Fliegmann J, Schüler G, Boland W, Ebel J, Mithöfer A (2003) The role of octadecanoids and functional mimics in soybean defense responses. Biol Chem 384:437–446. doi:10.1515/BC.2003.049

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol 5:344–350. doi:10.1038/nchembio.161

Gallego A, Imseng N, Bonfill M, Cusido RM, Palazon J, Eibl R, Moyano E (2015) Development of a hazel cell culture-based paclitaxel and baccatin III production process on a benchtop scale. J Biotechnol 195:93–102. doi:10.1016/j.jbiotec.2014

Gao W, Sun HX, Xiao H, Cui G, Hillwig ML, Jackson A, Wang X, Shen Y, Zhao N, Zhang L, Wang XJ, Peters RJ, Huang L (2014) Combining metabolomics and transcriptomics to characterize tanshinone biosynthesis in *Salvia miltiorrhiza*. BMC Genom 28:15–73. doi:10.1186/1471-2164-15-73

Geng X, Jin L, Shimada M, Kim MG, Mackey D (2014) The phytotoxin coronatine is a multifunctional component of the virulence armament of *Pseudomonas syringae*. Planta 240:1149–1165. doi:10.1007/s00425-014-2151-x

González MA (2015) Aromatic abietane diterpenoids: their biological activity and synthesis. Nat Prod Rep 32:684–704. doi:10.1039/c4np00110a

Guo J, Zhou YJ, Hillwig ML, Shen Y, Yang L, Wang Y, Zhang X, Liu W, Peters RJ, Chen X, Zhao ZK, Huang L (2013) CYP76AH1 catalyzes turnover of miltiradiene in tanshinones biosynthesis and enables heterologous production of ferruginol in yeasts. Proc Natl Acad Sci USA 110:12108–12113. doi:10.1073/pnas.1218061110

Haider G, von Schrader T, Füsslein M, Blechert S, Kutchan TM (2005) Structure-Activity relationships of synthetic analogs of jasmonic acid and coronatine on induction of

benzophenanthridine alkaloid accumulation in *Eschscholzia californica* cell cultures. Biol Chem 381:741–748. doi:10.1515/BC.2000.094

Hao G, Shi R, Tao R, Fang Q, Jiang X, Ji H, Feng L, Huang L (2013) Cloning, molecular characterization and functional analysis of 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase (HDR) gene for diterpenoid tanshinone biosynthesis in Salvia miltiorrhiza Bge. f. alba. Plant Physiol Biochem 70:21–32. doi:10.1016/j.plaphy.2013.05.010

Hao X, Shi M, Cui L, Xu C, Zhang Y, Kai G (2015a) Effects of methyl jasmonate and salicylic acid on tanshinone production and biosynthetic gene expression in transgenic *Salvia miltiorrhiza* hairy roots. Biotechnol Appl Bioch. 62:24–31. doi:10.1002/bab.1236

Hao C, Chen SL, Osbourn A, Kontogianni VG, Liu LW, Jordán MJ (2015b) Temporal transcriptome changes induced by methyl jasmonate in *Salvia sclarea*. Gene 558:41–53. doi:10.1016/j.gene.2014.12.043

Holohan C, Van Schaeybroeck S, Longle DB, Johnston PG (2013) Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 13:714–726. doi:10.1038/nrc3599

Hu F, Huang J, Xu Y, Qian X, Zhong JJ (2006) Responses of defense signals, biosynthetic gene transcription and taxoid biosynthesis to elicitation by a novel synthetic jasmonate in cell cultures of Taxus chinensis. Biotechnol Bioeng 94:1064–1071. doi:10.1002/bit.20921

Hussain MS, Fareed S, Ansari S, Rahman MA, Ahmad IZ, Saeed M (2012) Current approaches toward production of secondary plant metabolites. J Pharm Bioallied Sci 4:10–20. doi:10.4103/0975-7406.92725

Kabouche A, Kabouche Z (2008) Bioactive diterpenoids of Salvia species. In: Atta-u-Rahman (ed) Studies in natural products chemistry, vol 35. Elsevier, Amsterdam, pp 753–833 doi:10.1016/S1572-5995(08)80017-8

Kai G, Xu H, Zhou C, Liao P, Xiao J, Luo X, You L, Zhang L (2011) Metabolic engineering tanshinone biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. Metab Eng 13:319–327. doi:10.1016/j.ymben.2011.02.003

Kuzma L, Skrypek Z, Wysokinska H (2006) Diterpenoid and triterpenoids in hairy root of *Salvia sclarea*. Plant Cell Tissue Org Cult 84:171–179. doi:10.1007/s11240-005-9018-6

Kuzma L, Bruchajzer E, Wysokinska H (2009) Methyl jasmonate effect on diterpenoid accumulation in *Salvia sclarea* hairy root culture in shake flasks and sprinkle bioreactor. Enz Microb Tech 44:406–410. doi:10.1016/j.enzmictec.2009.01.005

Legrand S, Valot N, Nicolé F, Moja S, Baudino S, Jullien F, Magnard JL, Caissard JC, Legendre L (2010) One-step identification of conserved miRNAs, their targets, potential transcription factors and effector genes of complete secondary metabolism pathways after 454 pyrosequencing of calyx cDNAs from the Labiate *Salvia sclarea* L. Gene 450:55–62. doi:10.1016/j.gene.2009.10.004

Leone A, Grillo S, Monti L, Cardi T (2007) Molecular tailoring and boosting of bioactive secondary metabolites in medicinal plant. In: Ranalli P (ed) Improvement of crop plants for industrial end uses. IX: 471–507. Springer, Netherlands. ISBN: 978-1-4020-5486-0

Ma Y, Yuan L, Wu B, Li X, Chen S, Lu S (2012) Genome-wide identification and characterization of novel genes involved in terpenoid biosynthesis in *Salvia miltiorrhiza*. J Exp Bot 63:2809–2823. doi:10.1093/jxb/err466

Marchev A, Haas C, Schulz S, Georgiev V, Steingroewer J, Bley T, Pavlov A (2014) Sage in vitro cultures: a promising tool for the production of bioactive terpenes and phenolic substances. Biotechnol Lett 36:211–221

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Plant Physiol 15:473–497. doi:10.1111/j.1399-3054.1962.tb08052

O'Connor SE (2015) Engineering of secondary metabolism. Ann Rev Genet 49:71–94. doi:10.1146/annurev-genet-120213-092053

Onrubia M, Moyano E, Bonfill M, Cusidó RM, Goossens A, Palazón J (2013) Coronatine, a more powerful elicitor for inducing taxane biosynthesis in Taxus media cell cultures than methyl jasmonate. J Plant Physiol 170:211–219. doi:10.1016/j.jplph.2012.09.004

Patil RA, Lenka SK, Normanly J, Walker EL, Roberts SC (2014) Methyl jasmonate represses growth and affects cell cycle progression in cultured Taxus cells. Plant Cell Rep 33:1479–1492. doi:10.1007/s00299-014-1632-5

Pauwels L, Inzé D, Goossens A (2009) Jasmonate-inducible gene: what does it mean? Trends Plant Sci 14:87–91. doi:10.1016/j.tplants.2008.11.005

Pauwels L, Morreel K, De Witte E, Lammertyn F, Van Montagu M, Boerjan W, Inzé D, Goossens A (2008) Mapping methyl jasmonate-mediated transcriptional reprogramming of metabolism and cell cycle progression in cultured Arabidopsis cells. Proc Natl Acad Sci USA 105:1380–1385. doi:10.1073/pnas.0711203105

Qian ZG, Zhao ZJ, Xu Y, Qian X, Zhong JJ (2005) Highly efficient strategy for enhancing taxoid production by repeated elicitation with a newly synthesized jasmonate in fed-batch cultivation of *Taxus chinensis* cells. Biotechnol Bioeng 90:516–521. doi:10.1002/bit.20460

Ramirez-Estrada K, Vidal-Limon H, Hidalgo D, Moyano E, Golenioswki M, Cusidó RM, Palazon J (2016) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. Molecules 21:2–24. doi:10.3390/molecules21020182

Rózalski M, Kuźma L, Krajewska U, Wysokinska H (2006) Cytotoxic and proapoptotic activity of diterpenoids from in vitro cultivated *Salvia sclarea* roots. Studies on the leukemia cell lines. Z Naturforsch C 61:483–488. doi:10.1515/znc-2006-7-804

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3:1101–1108. doi:10.1038/nprot.2008.73

Sheard LB, Tan X, Mao H, Withers J, Ben-Nissan G, Hinds TR, Kobayashi Y, Hsu FF, Sharon M, Browse J, He SY, Rizo J, Howe GA, Zheng N (2010) Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature 468:400–405. doi:10.1038/nature09430

Shi M, Zhou W, Zhang J, Huang S, Wang H, Kai G (2016) Methyl jasmonate induction of tanshinone biosynthesis in *Salvia miltiorrhiza* hairy roots is mediated by JASMONATE ZIM-DOMAIN repressor proteins. Sci Rep 6:20919. doi:10.1038/srep20919

Song X, Lopez-Campistrous A, Sun L, Dower NA, Kedei N, Yang J et al (2013) RasGRPs are targets of the anti-Cancer agent Ingenol-3-Angelate. PLoS One 8:e72331. doi:10.1371/journal.pone.0072331

Tamogami S, Kodama O (2000) Coronatine elicits phytoalexin production in rice leaves (*Oryza sativa* L.) in the same manner as jasmonic acid. Phytochemistry 54:689–694. doi:10.1016/S0031-9422(00)00190-4

Taurino M, Ingrosso I, D'amico L, De Domenico S, Nicoletti I, Corradini D, Santino A, Giovinazzo G (2015) Jasmonates elicit different sets of stilbenes in *Vitis vinifera* cv. Negramaro cell cultures. SpringerPlus 4:49. doi:10.1186/s40064-015-0831-z

Vaccaro MC, Malafronte N, Alfieri M, De Tommasi N, Leone A (2014) Enhanced biosynthesis of bioactive abietane diterpenes by overexpressing AtDXS or AtDXR genes in *Salvia sclarea* hairy roots. Plant Cell Tissue Org Cult 119:65–77. doi:10.1007/s11240-014-0514-4

Vranovà E, Coman D, Gruissem W (2013) Network analysis of the MVA and MEP pathways for isoprenoid synthesis. Annu Rev Plant Biol 64:665–700. doi:10.1146/annurev-arplant-050312-120116

Wasternack C, Hause B (2013) Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. An update to the 2007 review in Annals of Botany. Ann Bot 111:1021–1058. doi:10.1093/aob/mct067

Weaver BA (2014) How Taxol/paclitaxel kills cancer cells. Mol Biol Cell 25:2677–2681. doi:10.1091/mbcE14-04-0916

Wenping H, Yuan Z, Jie S, Lijun Z, Zhezhi W (2011) De novo transcriptome sequencing in *Salvia miltiorrhiza* to identify genes involved in the biosynthesis of active ingredients. Genomics 98:272–279. doi:10.1016/j.ygeno.2011.03.012

Yan J, Li H, Li S, Yao R, Deng H, Xie Q, Xie D (2013) The Arabidopsis F-box protein CORONATINE INSENSITIVE1 is stabilized by SCFCOI1 and degraded via the 26S proteasome pathway. Plant Cell 25:486–498. doi:10.1105/tpc.112.105486

Yang L, Ding G, Lin H, Cheng H, Kong Y, Wei Y, Fang X, Liu R, Wang L, Chen X, Yang C (2013) Transcriptome analysis of medicinal plant *Salvia miltiorrhiza* and identification of genes related to tanshinone biosynthesis. PLoS One 8:e80464. doi:10.1371/journal.pone.0080464

Zerbe P, Bohlmann J (2015) Plant diterpene synthases: exploring modularity and metabolic diversity for bioengineering. Trends Biotechnol 33:419–428. doi:10.1016/j.tibtech.2015.04.006

Zhang L, Yao J, Withers J, Xin XF, Banerjee R, Fariduddin Q, Nakamura Y, Nomura K, Howe GA, Bolandf Wilhelm, Yang H, He SY (2015) Host target modification as a strategy to counter pathogen hijacking of the jasmonate hormone receptor. Proc Natl Acad Sci USA 112:14354–14359. doi:10.1073/pnas.1510745112

Zhao J, Davis LC, Verpoorte R (2005) Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv 23:283–333. doi:10.1016/j.biotechadv.2005.01.003

Zhao L, Chang WC, Xiao Y, Liu HW, Liu P (2013) Methylerythritol phosphate pathway of isoprenoid biosynthesis. Ann Rev Biochem 82:497–530. doi:10.1146/annurev-biochem-052010-100934

Zhou H, Li Y, Zhang Q, Ren S, Shen Y, Qin L, Xing Y (2016) Genome-wide analysis of the expression of WRKY family genes in different developmental stages of wild strawberry (*Fragaria vesca*) fruit. PLoS One 11(5):e0154312. doi:10.1371/journal.pone.0154312