S-Carvone Suppresses Cellulase-Induced Capsidiol Production in Nicotiana tabacum by Interfering with Protein Isoprenylation

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S-Carvone has been described as a negative regulator of mevalonic acid (MVA) production by interfering with 3-hydroxy-3-methyl glutaryl coenzyme A reductase (HMGR) activity, a key player in isoprenoid biosynthesis. The impact of this monoterpene on the production of capsidiol in Nicotiana tabacum, an assumed MVA-derived sesquiterpenoid phytoalexin produced in response to elicitation by cellulase, was investigated. As expected, capsidiol production, as well as early stages of elicitation such as hydrogen peroxide production or stimulation of 5-epi-aristolochene synthase activity, were repressed. Despite the lack of capsidiol synthesis, apparent HMGR activity was boosted. Feeding experiments using (1-13C)Glc followed by analysis of labeling patterns by 13C-NMR, confirmed an MVA-dependent biosynthesis; however, treatments with fosfomycin, an inhibitor of the MVA-independent 2C-methylene-d-erythritol 4-phosphate (MEP) isoprenoid pathway, unexpectedly down-regulated the biosynthesis of this sesquiterpene as well. We postulated that S-carvone does not directly inhibit the production of MVA by inactivating HMGR, but possibly targets an MEP-derived isoprenoid involved in the early steps of the elicitation process. A new model is proposed in which the monoterpene blocks an MEP pathway–dependent protein geranylgeranylation necessary for the signaling cascade. The production of capsidiol was inhibited when plants were treated with some inhibitors of protein prenylation or by further monoterpenes. Moreover, S-carvone hindered isoprenylation of a prenylatable GFP indicator protein expressed in N. tabacum cell lines, which can be chemically complemented with geranylgeraniol. The model was further validated using N. tabacum cell extracts or recombinant N. tabacum protein prenyltransferases expressed in Escherichia coli. Our study endorsed a reevaluation of the effect of S-carvone on plant isoprenoid metabolism.

Isoprenoids (or terpenoids) constitute the most diverse family of natural products involved in numerous biological functions. In addition to being essential for growth and development of plants, isoprenoids are also effective as ecological mediators (Bouvier et al., 2005; Baldwin, 2010). Some species accumulate terpenoid phytoalexins in response to pest attack (Dixon, 2001; Nugroho and Verpoorte, 2002; Okada, 2011; Ahuja et al., 2012). The metabolic origin of these terpenoid phytoalexins is diverse (Dixon, 2001). They are either built-up from prenyl diphosphates derived from the cytosolic mevalonate (MVA) pathway (largely sesquiterpenes and triterpenes) or from the plastidial 2C-methylene-d-erythritol 4-phosphate (MEP) pathway (largely diterpenes). Phytoalexins interfere with the development of pathogens and thus participate in the active plant defense system. Signaling networks leading to the production of those terpenoid metabolites are initiated by elicitation with specific effectors (Zhao et al., 2005; Vasconsuelo and Boland, 2007). Among plants synthesizing terpenoid-based bioactive defense compounds, members of the Solanaceae family such as Nicotiana tabacum or pepper (Capsicum annuum) respond to elicitation by synthesizing the bicyclic dihydroxylated sesquiterpene capsidiol, an antifungal agent, as a main product (Stoesel et al., 1976). The synthesis of this sesquiterpene is initiated by cyclization of all-trans farnesyl diphosphate (FPP) into 5-epi-aristolochene (EA), a reaction catalyzed by EA synthase (EAS), a terpene cyclase (Back and Chappell, 1996; Starks et al., 1997). EA is then further oxidized by CYP71D20, a cytochrome P450 hydroxylase, to form the end product capsidiol in two steps (Kalsiton et al., 2001). Transcriptional expression of...
the gene family encoding the terpene cyclase EAS has been detected at its highest level 4 h after elicitation of *N. tabacum* cell cultures. It was correlated with an induction of not only protein synthesis but also EAS enzyme activity (Vögeli and Chappell, 1990; Facchinii and Chappell, 1992). It has been well accepted that in *N. tabacum* and pepper, capsidiol originates from isoprenoid units that are built via the MVA pathway (Chappell and Nable, 1987; Yoshizawa et al., 1994). In addition, the transcriptional induction of EAS expression is correlated with that of the gene coding for the up-stream enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), catalyzing the biosynthesis of MVA, more precisely the stress-induced isogene HMGR2 (Weissenborn et al., 1995; Dorey et al., 1997). As a result, the induction of capsidiol production in *N. tabacum* in response to elicitation is apparently regulated at a transcriptional level. It has been demonstrated that the activated phosphorylated WRKY8 transcription factor is involved in HMGR2 induction in *N. tabacum* and *Nicotiana benthamiana* (Kim and Zhang, 2004; Ishihama et al., 2011). The direct participation of WRKY8 in capsidiol production has not been established, and further experiments are required in which the expression of EAS is investigated under similar conditions as was done with that of HMGR.

Nevertheless, modulation of HMGR activity is not only essential in phytoalexin production, but this enzyme is also considered as one of the key enzymes of the whole isoprenoid biosynthesis pathway (Hemmerlin, 2013). Its activity is controlled at multiple levels, such as in response to environmental factors. Direct consequences of this rate-limiting function are that the activity of HMGR is tightly regulated in vivo (see references cited in Hemmerlin et al., 2012; Hemmerlin, 2013) and that the enzyme stands as a natural target for bioactive agents produced by microorganisms or even plants (Bach, 1988; Clark, 1996; Tobert, 2003; Di Donna et al., 2009). Carvone, a secondary metabolite of the monoterpane family, was described as interfering with HMGR activity (Oosterhaven et al., 1993). This C10 metabolite is naturally found at high concentrations in caraway (*Carum carvi*) as a (4S)-(−)-isomer or in spearmint (*Mentha spicata*) as a (4R)-(−)-isomer (Bouwmeester et al., 1998; de Carvalho and da Fonseca, 2006), and is utilized in agriculture as a potato (*Solanum tuberosum*) tuber sprout growth inhibitor (Meigh, 1969; Hartmans and Diepenhorst, 1994; Oosterhaven et al., 1995). Oosterhaven et al. (1993) previously investigated the physiological and molecular details underlying this sprouting inhibition and proposed that HMGR is inhibited at a posttranslational level. A model was developed in which this product may enhance HMGR proteolysis, in a similar manner to how farnesol behaves in mammalian cells (Oosterhaven, 1995). The molecular details are not clear, but it now appears unlikely that HMGR is the only molecular target of S-carvone. Our study aimed to provide a more comprehensive understanding of how S-carvone acts in plants, as well as to elucidate and characterize its true target. To clarify the mechanism by which S-carvone may interfere with the isoprenoid metabolism, we used *N. tabacum* cell suspensions and plants, a species closely related to potato, as a biological model producing inducible MVA-derived sesquiterpenoids. We focused particularly on the action endorsed on HMGR activities as well as on the production of the putatively MVA-derived metabolite capsidiol.

**RESULTS**

**Enhanced HMGR Activity in *N. tabacum* Plants Treated with S-Carvone**

The *N. tabacum* BY-2 cell line was used to assess the potential inhibition of HMGR activity by S-carvone. Compared with other plant systems, this fast-growing cell suspension is characterized by a notably elevated basic HMGR activity and a high capacity to respond to different chemical treatments (Hemmerlin et al., 2004). We previously showed that the inhibition of HMGR activity with mevinolin induces inhibition of cell cycle progression, but also causes partial cell death (Hemmerlin and Bach, 1998). In contrast, up to 2 mM S-carvone had no impact on *N. tabacum* BY-2 cell growth, nor did it induce cell death (Fig. 1). To investigate whether HMGR is negatively affected, apparent activities in microsomal fractions isolated from cells treated with increasing concentrations of S-carvone were compared with those found in untreated cell fractions (Fig. 1). *N. tabacum* cells treated with S-carvone behaved in an unexpected manner: No attenuation of the enzyme activity could be detected at nontoxic concentrations. The opposite reaction was observed: At concentrations between 0.1 and 2 mM, the apparent HMGR activity was found to be slightly stimulated compared with the control, yielding a 1.6-fold stimulation for S-carvone at 0.1 mM (Fig. 1). Values decreased at concentrations becoming generally toxic to the cells (>2 mM). Thus, we can conclude that S-carvone does not down-regulate the housekeeping HMGR activity necessary for cell growth and division in *N. tabacum* BY-2 cells.

Based on inconsistencies in the results observed with potato tuber sprouts, we postulated that the target is most likely stress related. Indeed, activity of HMGR in plants results from the simultaneous expression and operation of several isozymes. Expression of the corresponding genes is controlled by different endogenous and exogenous factors (for review, see Hemmerlin, 2013), and some of these isoforms are regulated in response to stress. In potato tubers, the expression of HMGR2 and HMGR3 is inducible by arachidonic acid, but transcripts also accumulate in young flowers (Korth et al., 1997); therefore, sprouting of potato tubers can possibly be assimilated to stress induction. In *N. tabacum*, as in other Solanaceae, the housekeeping enzyme is considered as corresponding to HMGR1 and the stress-inducible isoform to HMGR2. To note, HMGR1 and HMGR2 isoforms can be discriminated...
only at a transcriptional level using specific sets of primers. Different events can induce the expression of these specific stress-induced HMGR isoforms (for review, see Hemmerlin et al., 2012). We propose that S-carvone might specifically target such an inducible HMGR isoform (HMGR2), which would explain why this compound does not inhibit the apparent HMGR activity expressed in N. tabacum BY-2 cells growing under standard conditions.

**S-Carvone Blocks the Production of a Stress-Induced Secondary Metabolite in N. tabacum Leaves**

To clarify whether S-carvone contributes to the inhibition of the stress-induced HMGR2 isoform in N. tabacum, we analyzed the impact of the monoterpene on the production of the stress-related MVA-derived secondary metabolite capsidiol. The major compound found in medium used in floating leaf-disc assays was the constitutive α-cembratrienediol. The identity of this compound was characterized by gas chromatography-mass spectrometry (GC-MS; retention time = 19.3 min; Fig. 2; Supplemental Fig. S1A) and 1H-NMR and 13C-NMR (Supplemental Table S1). The accumulation of capsidiol emerged in samples treated with cellulase (retention time = 16.2 min; Fig. 2), with a strong accumulation in the medium after 18 h (Supplemental Fig. S2). The structural identification was confirmed by GC-MS (Supplemental Fig. S1B), ultra-high-performance liquid chromatography–mass spectrometry (Supplemental Fig. S2), and 1H-NMR and 13C-NMR (Supplemental Table S1). As expected, S-carvone abolished the production and accumulation of this phytoalexin in elicited N. tabacum leaf discs (Fig. 2). We noticed that the inhibition was correlated with an all-or-nothing type of response. Indeed, we were unable to reduce its production even if we decreased S-carvone concentrations.

The inhibition of capsidiol production by S-carvone points toward an impact on the stress-regulated HMGR activity. Accordingly, this result prompted us to evaluate HMGR activity in elicited N. tabacum leaves. Four
different conditions were established in which apparent HMGR activities contained in microsomal protein fractions isolated from control leaf discs were compared with those isolated from carvone-, cellulase-, or carvone/cellulase-treated leaf discs (Fig. 3). HMGR activity was determined at three different levels: (1) apparent enzyme activity was estimated using an HMGR enzyme radioassay, (2) protein production was evaluated by western-blot analysis using an antibody raised against the N. tabacum HMGR2 catalytic entity, and (3) mRNA levels were evaluated by quantitative real-time PCR (Fig. 3).

Just as we noticed using BY-2 cells, in N. tabacum leaves S-carvone stimulated HMGR activity as well, and this at all levels of regulation: transcriptional, translational and apparent enzyme activity (Fig. 3). The overall transcript levels were increased in treated leaves, as was the protein content. Enzyme activity was strongly stimulated (approximately 10-fold) under such conditions. S-Carvone induced the expression of the stress-correlated HMGR2 isoform by 8-fold, but reduced the level of the housekeeping HMGR1 by approximately 2-fold. Overall, activity remained stimulated compared with nontreated control leaves, but globally S-carvone decreased the cellulase-induced transcription of both HMGR1 and HMGR2 genes by 4-fold. These results suggested that S-carvone might induce a stress response in N. tabacum that is not correlated with capsidiol production. To test whether the specific HMGR2 might be down-regulated, we challenged the leaves to produce capsidiol and therefore stimulated HMGR activity as well. As anticipated, cellulase induced HMGR activity but also promoted the synthesis of the corresponding protein and the transcription of both HMGR1/HMGR2 isogenes. The expression of the transcripts was stimulated after 18-h exposure to cellulase (approximately 10-fold for HMGR1 and up to 35-fold for HMGR2). The monoterpene did not down-regulate the cellulase-induced HMGR apparent activity and protein levels, but the total transcript levels decreased. Furthermore, quantification of HMGR bands (western blot) with ImageJ software (http://rsb.info.nih.gov/ij/) revealed a 15-fold induction of the protein level after S-carvone treatment (Fig. 3). Interestingly, S-carvone interfered proportionally with the transcriptional induction of both HMGR isogenes, by maintaining the same 2-fold HMGR1/HMGR2 ratio (Fig. 3).

The impact of S-carvone on the HMGR2 isoform was quite surprising at first. As a single treatment, the transcription of HMGR2 was stimulated, but the stimulating effect of cellulase was overcome in combination with cellulase. Moreover, S-carvone did not reduce HMGR activity in N. tabacum, but rather stimulated it. It can thus be inferred that S-carvone does not inhibit the stress-related isoform HMGR2. This result is clearly in contradiction to the hypothesis that S-carvone somehow targets the stress-inducible HMGR isoform that is most likely involved in the accumulation of capsidiol. At this point, it appears as if the biosynthesis of the elicitor-inducible, MVA-derived capsidiol is down-regulated.
by S-carvone, but not the regulatory enzyme that is responsible for providing the pool of the metabolic precursor required for its biosynthesis. This unexpected outcome prompted us to fully rethink and reshape a new hypothesis, which should allow the elucidation of the true mode of action of S-carvone in *N. tabacum*.

**MVA-Derived Capsidiol Biosynthesis Is Inhibited by Fosmidomycin**

The stimulation of HMGR activity is a result of complex regulatory mechanisms. In addition, plants generate a second pool of isopentenyl diphosphate [IPP] and dimethylallyl diphosphate [DMAPP] in plastids, through the so-called MEP pathway, that are used as precursors for many isoprenoids, including even sesquiterpenoids under certain conditions (see literature cited by Hemmerlin et al., 2012). It was previously demonstrated that a deregulation of the MVA-independent MEP pathway causes an increase in HMGR activity in *N. tabacum* BY-2 cells (Hemmerlin et al., 2003). An inhibition of the MEP pathway and thereby the production of an MEP-derived metabolite can lead to this cellular response requiring the adjustment of HMGR activity. For that reason, we first proposed a different, at least partial biosynthetic origin of capsidiol: This sesquiterpenoid may not exclusively be synthesized starting from isoprene units generated through the MVA pathway.

To test this hypothesis, we treated elicited *N. tabacum* leaf discs with inhibitors specifically targeting the MVA or MEP pathways. To this end, mevinolin blocking HMGR activity was used to decrease the MVA-derived IPP/DMAPP pool, and fosmidomycin, inhibiting 1-deoxy-D-xylulose 5-phosphate reducto-isomerase (MEP synthase) activity, was utilized to reduce the MEP-derived IPP/DMAPP pool. Based on experimental conditions with *N. tabacum* cell lines as described by Chappell and Nable (1987), we used mevinolin (20 μM). In addition, we applied fosmidomycin (200 μM) to block metabolism in leaf discs that are probably more hermetic to inhibitor treatments than isolated cells might be. We also applied the phosphonate drug alendronate (100 μM), an inhibitor of farnesyl diphosphate synthase (Bergstrom et al., 2000). Quantification of capsidiol production was achieved by gas chromatography-flame ionization detector (GC-FID) analysis using *N. tabacum* leaf discs treated simultaneously with the inhibitors plus cellulase (Fig. 4). The peak corresponding to capsidiol was identified by mass spectrometry in each GC-analyzed sample. As expected, inhibition of MVA synthesis by mevinolin decreased capsidiol production. The inhibition by alendronate also resulted in the attenuation of capsidiol production. More surprisingly, inhibition of MEP biosynthesis by fosmidomycin decreased the production in the same way as alendronate did (Fig. 4). From these observations, it could be concluded that either an MEP-derived isoprenoid pool is involved in its biosynthesis or that an MEP-derived isoprenoid molecule is somehow essential for the regulation of capsidiol biosynthesis.

To solve this dilemma, we first had to decipher the biosynthetic origin of capsidiol. Depending on the plant species, the biosynthesis of the sesquiterpene precursor FPP follows either an MVA-derived labeling pattern, more occasionally an MEP-derived one, or even a combination between both, with a geranyl diphosphate unit derived from the MEP pathway reacting with an IPP unit derived from MVA (for review, see Hemmerlin et al., 2012). For this purpose, we used (1-13C)Glc for incorporation into *N. tabacum* leaves. The labeling pattern of capsidiol produced in response to elicitation with cellulase was analyzed by NMR. Extraction of the elicitation medium with ethyl acetate afforded a mixture of capsidiol and the diterpene α-cembranetrienediol, which was directly analyzed by 13C-NMR spectroscopy, because there was no overlapping of signals (Supplemental Tables S1 and S2). The expected patterns obtained from (1-13C)Glc incorporation are described in Figure 5. Basically, isoprene units derived from MEP become labeled on carbon atoms C-1 and C-5, whereas isoprene units derived from MVA are labeled at positions C-2, C-4, and C-5 (Fig. 5). The labeling pattern (Supplemental Table S2) clearly indicates an MVA-derived biosynthesis with an efficient incorporation of the labeled Glc into the sesquiterpene synthesized de novo during the elicitation (Fig. 5). A weak labeling indicating a contribution of the MEP pathway was
found for the constitutive diterpene \(\alpha\)-cembratrienediol (Supplemental Table S3). Thus, the MEP pathway is not involved in the biosynthesis of capsidiol, at least in cellulase-elicited \textit{N. tabacum} leaves, but as expected contributes to the biosynthesis of diterpenes such as \(\alpha\)-cembratrienediol.

**Signals Leading to Capsidiol Production Are Not Induced When Leaves Are Treated with \(S\)-Carvone**

It was previously shown that in elicited \textit{N. tabacum} cells, capsidiol biosynthesis is induced at the expense of sterol formation (Threlfall and Whitehead, 1988). Under such conditions, apparent EAS activity involved in capsidiol biosynthesis is stimulated and squalene synthase controlling sterol biosynthesis is down-regulated (Vögeli and Chappell, 1988). Consistent with these earlier studies, EAS activity was found stimulated in the cellulase-elicited leaf discs (Fig. 6A). Treatments with the monoterpene abolished the cellulase-induced EAS enzyme activity (Fig. 6A). From these findings, we can deduce that either \(S\)-carvone blocks EAS activity or that the elicitor-dependent signaling cascade leading to the final production of capsidiol is no longer induced. To test whether \(S\)-carvone interferes with the signaling pathway, we examined some aspects of signal transmission in early responses ultimately leading to capsidiol production. As illustrated in Figure 6B, \(S\)-carvone prevented the production of reactive oxygen species necessary for the elicitation process (Rusterucci et al., 1996). This result is consistent with the fact that \(S\)-carvone interferes with early steps of the signal response leading to the production of capsidiol, among others, through activation of HMGR transcription. However, little attention has been paid to the possibility that an MEP-derived metabolite might be involved in this early response. In pepper, the cellulase-induced production of capsidiol is mediated by the activation of a signaling cascade that involves at least one GTP-binding protein (Ma, 2008). These proteins, in particular members of the Rop/Rac GTPases, are active as coordinators of various extracellular stimuli, including defense responses (Moeder et al., 2005; Nibau et al., 2006). In addition, they are functionally active in the plasma membrane, a localization requiring a posttranslational modification.

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**Figure 5.** Predicted labeling patterns of capsidiol into \textit{N. tabacum} leaves fed with [1\(^{13}\)C]Glc, and then elicited with 0.5% cellulase. The predicted labeling patterns for capsidiol are illustrated. \(^{13}\)C-NMR analyses showed that capsidiol follows an MVA pathway-derived pattern (Supplemental Table S2). The diterpene \(\alpha\)-CBT derived from plastidial GGPP was taken as a reference compound for the MEP pathway and its labeling pattern (Supplemental Table S3) is specified. Ac-CoA, Acetyl coenzyme A; \(\alpha\)-CBT, \(\alpha\)-cembratrienediol; GAP, glyceroldehyde 3-phosphate.
with prenyl groups (Assmann, 2002; Crowell and Huizinga, 2009; Sorek et al., 2009). Some proteins are posttranslationally modified with an MEP-derived geranylgeranyl diphosphate that originates from plastids (Gerber et al., 2009). Monoterpenes are often contained in natural essential oils and some of them are known to block the activity of animal and human type I protein prenyltransferases (Crowell et al., 1991; Gelb et al., 1995; Hardcastle et al., 1999). It can be hypothesized that S-carvone might restrict the biosynthesis of capsidiol by interfering with such a putative GTP-binding protein, more precisely via the inhibition of its isoprenylation with the geranylgeranyl moiety.

Inhibition of Protein Prenylation Hinders Capsidiol Production in N. tabacum

With this mechanism of action in mind, we tested different inhibitors of protein isoprenylation. A similar inhibition was obtained when leaf discs were treated with S-perillyl alcohol (Supplemental Table S4), described to block protein isoprenylation in plant cell cultures (Morehead et al., 1995). Unlike S-perillyl alcohol, the monoterpenic limonene previously described as inefficient (Morehead et al., 1995) could not suppress capsidiol production (Supplemental Table S4). Non-terpenoid commercial inhibitors known to block protein farnesylation, protein geranylgeranylation, or the maturation steps after the posttranslational modification were also applied. To note, most of these inhibitors were designed to act on human protein prenyltransferases in vitro; thus, their efficiency in vivo and in plants cannot be guaranteed. Furthermore, efficient incorporation of these inhibitors into leaves cannot be considered certain and needs to be attested. We tested two inhibitors, GGti-2133 that was previously identified as an efficient inhibitor of protein geranylgeranylation in N. tabacum BY-2 cells (Gerber et al., 2009), and FPT-inhibitor I described as specifically inhibiting protein farnesylation. GGti-2133 was found to prevent capsidiol N. tabacum production in elicited leaf discs, but the FPT-inhibitor I did not (Supplemental Table S4). However, we currently do not have a way to test whether the import of this inhibitor into the cells is efficient enough. Next we tried to block capsidiol production by treating elicited leaf disc with two S-prenyl-L-Cys analogs reported to interfere with the carboxymethylation catalyzed by the isoprenyl-Cys carboxyl methyltransferase and needed for the maturation of the isoprenylated protein: N-acetyl-S-farnesyl-L-cysteine (AFC) and N-acetyl-S-geranylgeranyl-L-cysteine (AGGC) (Winter-Vann and Casey, 2005). AFC, but not AGGC, reduced the production of the phytoalexin (Supplemental Table S4). Caseyro, it can be concluded that an isoprenylated protein may be involved in the sesquiterpene production.

Protein Geranylgeranylation Is Down-Regulated in N. tabacum Cells Treated with S-Carvone

With the goal of providing further arguments for the indirect inhibition of S-carvone on capsidiol production, we tested whether the monoterpenic block the capacity to isoprenylate a protein in vivo. First, we evaluated its ability to inhibit incorporation of labeled isoprenoid precursors into proteins. Because both MVA and MEP pathways are involved in isoprenylation of proteins, [14C]MVA and [14C]deoxy-D-xylulose ([14C]DX) were used for incorporation experiments in the presence or absence of the monoterpenic. The labeling of proteins was considerably affected by S-carvone treatment. After 24-h treatments, radiolabeling of BY-2 protein extracts separated on a SDS-PAGE decreased when cells were treated with S-carvone (Fig. 7A). It could be concluded that S-carvone diminishes the incorporation of...
isoprenoid precursors into \textit{N. tabacum} proteins. To definitively resolve the issue of whether \textit{S}-carvone blocks protein isoprenylation in plants, an in vivo visualization system for protein geranylgeranylation was utilized (Hartmann et al., 2013). This system is based on transgenic \textit{N. tabacum} BY-2 cell lines expressing GFP-BD-CVIL bearing a basic domain (BD) and a prenylation motif (CVIL) from rice CaM61 (Gerber et al., 2009). When modified with a geranylgeranyl group, this chimeric protein becomes membrane localized. Inhibition of protein isoprenylation is characterized by the mislocalization of the GFP fusion protein into the nucleus. The more the nucleus is labeled, the more the inhibition is efficient. Cells were treated with \textit{1 mM} of \textit{S}-carvone, a

Figure 7. \textit{S}-Carvone induced inhibition of protein geranylgeranylation in \textit{N. tabacum} BY-2 cells expressing GFP-BD-CVIL. A, \textsuperscript{14}C\textsuperscript{MV}A and \textsuperscript{14}C\textsuperscript{DX} incorporation in \textit{N. tabacum} BY-2 cell suspension treated (Ca) or not (C) with \textit{S}-carvone. Total protein extracts were separated on a SDS-PAGE; they were either stained with Coomassie Brilliant Blue or used to complete a fluorography. B to D, Quantification of protein prenylation capacity in vivo using \textit{N. tabacum} BY-2 cells expressing GFP-BD-CVIL. The distribution of the fluorescent protein is indicative for protein prenylation. If prenylated, the protein associates to the plasma membrane (M: black color); if not, the protein is localized in the nucleus (N; gray color). Cells with double localization (plasma membrane + nucleus, M+N; white color) indicate an intermediate prenylation state. B, Five-fold diluted \textit{N. tabacum} cells were treated with \textit{S}-carvone (1 mM), FPT-inhibitor I (60 \textmu M), or GGti-2133 (30 \textmu M). Reversion experiments were performed by complementing the \textit{S}-carvone treatment through addition of prenols (20 \textmu M): farnesol (Fol) or geranylgeraniol (GGol). Subcellular distributions of the GFP-BD-CVIL protein (isoprenylated or not) observed in samples treated with the different inhibitors are depicted in Supplemental Figure S3. C, Chemical complementation with GGol of \textit{S}-carvone-induced inhibition of geranylgeranylation over a concentration range from 1 to 20 \textmu M. D, Effects of fosmidomycin treatments: Synergism between a combined treatment with \textit{S}-carvone and fosmidomycin on geranylgeranylation inhibition. A fosmidomycin dose-response is observed. To see the synergic effect, the concentrations were lowered: Transformed cells were treated with fosmidomycin (FOS; 10 up to 100 \textmu M) and \textit{S}-carvone (0.5 mM).
nontoxic concentration for BY-2 cells, during 24 h. The fluorescence of the GFP fusion protein shifted predominantly to a nuclear localization, but the plasma membrane was still partially labeled. (Fig. 7B; Supplemental Fig. S3). The inhibition was similar as efficient as observed with the commercial FPT inhibitor I, but was less efficient than with GGti-2133. To note, FPT inhibitor I also affects the cell-free extracts isolated from N. tabacum protein prenyltransferase (PPTase) inhibition induced by farnesol. The stimulation of gene expression might indicate a cell response to overcome the added to the medium. The stimulation of gene expression would require a nuclear localization, but the plasma membrane was still partially labeled. (Fig. 7B). This reaction was dose dependent, with 5 μM being sufficient to fully restore plasma membrane GFP localization, indicative of an efficient isoprenylation of the protein (Fig. 7C). These results led us to conclude that the substrate geranylgeranyl diphosphate (GGPP) can restore prenylation inhibition induced by S-carvone, which would reflect a reversible inhibitory effect. We next verified whether S-carvone acts in synergy with fosmidomycin to block protein isoprenylation (Fig. 7D). This experiment confirmed that combinations using both inhibitors block isoprenylation of a protein bearing a geranylgeranylation motif, which led to a synergistic effect. Altogether, these results corroborated our hypothesis that S-carvone interferes with isoprenylation of proteins in N. tabacum, more precisely with an essential MEP-derived geranylgeranylation of an unknown protein.

**Figure 8.** Effect of S-carvone on protein prenyltransferase activity in N. tabacum. A, Quantitative real-time PCR experiments on cDNA generated from leaf discs treated for 18 h. B, Protein prenyltransferase assays using radiolabeled prenyl diphosphates (0.25 μCi/assay), purified GFP-CaaX protein substrates (20 μM), and 15 μg of total protein extracts isolated from 24-h-old N. tabacum BY-2 cells treated (Ca) or not (control [C]) with S-carvone (1 mM). C, Quantification of band intensities with ImageJ software and calculation of the stimulation of inhibition rates by S-carvone. A ratio of 1 is indicative of no effect, whereas values >1 are indicative of activity stimulation. Values were calculated on the basis of two independent experiments. ND, activity not detected.

Activities of N. tabacum Protein Prenyltransferases Are Inhibited by S-Carvone

Next we evaluated the impact of S-carvone on protein prenyltransferase gene expression (Fig. 8). Protein prenyltransferases occur as heterodimers with a common α-subunit and β-subunits specific for each enzyme (Nguyen et al., 2010). The expression of the three genes encoding three subunits was evaluated by quantitative real-time PCR: α (Nt-α), β-farnesyl (Nt-βF), and β-geranylgeranyl (Nt-βGG). There is a clear difference in the expression of the three subunits, with Nt-α being most prevalent (Fig. 8A). Nt-βF is the most underrepresented among all species of transcripts. When leaf discs were treated for 18 h with S-carvone, the average expression for all three genes was uniformly doubled; however, when they were challenged with cellulase, their quantities decreased by approximately 2-fold. Changes in transcript expression induced by S-carpone were abolished when cellulase was simultaneously added to the medium. The stimulation of gene expression might indicate a cell response to overcome the protein prenyltransferase (PPTase) inhibition induced by S-carvone. We were not able to detect PPTase activity in cell-free extracts isolated from N. tabacum leaves. For that reason, we tested it in N. tabacum BY-2 cell extracts, in which activity can easily be estimated (Randall et al., 1993; Gerber et al., 2009). A protein extract isolated from a 24-h-old cell suspension was used as an enzyme source. Activities were determined in vitro using the highly sensitive radiolabeled enzyme assay, which allowed us to evaluate the capacities to modify a protein bearing a farnesylation motif (GFP-CVIM) or a geranylgeranylation motif (GFP-CVIL), using either radiolabeled FPP or GGPP (Fig. 8B). GFP-SVIL that cannot be isoprenylated was used as a control for each enzyme activity. Values were calculated on the basis of two independent experiments. ND, activity not detected.

**Inhibition of Isoprenoid Metabolism by S-Carvone**
negative control. Under our experimental conditions, *N. tabacum* BY-2 cell-free extracts were unable to modify GFP-CVIM using GGPP (Fig. 8B). Intensities of obtained signals were quantified using ImageJ and ratios (band intensity S-carvone/ control) were calculated. After S-carvone treatment, the capacity to modify any protein substrate with FPP was decreased by 2-fold, whereas that to modify GFP-CVIL using GGPP as a prenyl diphosphate substrate was increased by nearly 3-fold (Fig. 8C). Clearly, in response to inhibition of geranylgeranylation by S-carvone, the cell seems to react by some physiological counteractions, through increasing the capacity to modify proteins with geranylgeranyl groups. This could be observed at the level of gene expression (Fig. 8A), but also at the level of apparent enzyme activity in extracts from treated BY-2 cells (Fig. 8, B and C).

To clarify the mode of inhibition of protein isoprenylation, we tested whether the monoterpene directly interferes with the activity of protein prenyltransferases in vitro. The monoterpene inhibited recombinant *N. tabacum* PPTase activity in vitro, and a 50% inhibition of initial activity (IC$_{50}$) value of 1.4 mM for NtPFT could be determined (Supplemental Fig. S4). At first glance, it appears surprising that this monoterpene is a low millimolar inhibitor of PPTases, values that are quite modest compared with specific and efficient commercial inhibitors such as GGti or Fti acting in the nanomolar range. This can be explained by the rather lipophilic property of terpenoids forming micelles in aqueous solution, which is alleviated for oxygenated monoterpenses (Weidenhamer et al., 1993) and by their volatile properties. Therefore, the actual IC$_{50}$ values determined in our experimental conditions are presumably overestimated, but are consistent with values obtained with animal enzymes (Hardcastle et al., 1999). Furthermore, the value for NtPGGT-I could not be estimated, because initial velocities in the presence of the monoterpene were unexpectedly activated by concentrations <4 mM. It appears that this unstable enzyme behaves somehow in a different manner than NtPFT in the presence of the compound with such lipophilic properties. We also tested the capacity of the monoterpene to inhibit in vitro enzymes contained in cell-free extracts isolated from untreated *N. tabacum* BY-2 suspensions. Under those experimental conditions, the prenylation of both GFP-CVIM with FPP and GFP-CVIL with GGPP was inhibited by S-carvone in the low millimolar range (Supplemental Fig. S4). Contrary to results observed with cell-free extracts from S-carvone-treated cells (Fig. 8), this treatment does not result in any positive regulatory response but brings about a direct inhibitory effect in vitro.

**DISCUSSION**

**S-Carvone Does Not Target HMGR in *N. tabacum***

Regulation of HMGR enzyme activity is complex and controlled at multilevels. Moreover, this activity needs to be adjusted rapidly in response to diverse stimuli such as stress or defense reactions. As a consequence, the regulation of this enzyme most likely depends on various signaling pathways that adjust the activity, particularly through posttranslational regulation (Antolin-Llovera et al., 2011; Hemmerlin, 2013). A genetic approach recently led to the identification of SUPPRESSOR OF DRY2 DEFACTS1 as a regulator of HMGR activity in Arabidopsis (Doblas et al., 2013). SUPPRESSOR OF DRY2 DEFACTS1 is a putative E3 ubiquitin ligase potentially involved in the endoplasmic reticulum-associated protein degradation pathway. It is worth noting that regulated HMGR protein degradation or inhibition of its synthesis have been well described for yeast (*Saccharomyces cerevisiae*) and animals (Burg and Espenshade, 2011), but little is known regarding HMGR turnover in plants. However, some hints exist. Light intensity and light quality have been reported as key elements (Korth et al., 2000). In lesion-mimicking Arabidopsis mutants overexpressing farnesyl diphosphate synthase, HMGR activity was significantly reduced, suggesting a close correlation between the development of necrotic lesions and endogenous levels of HMGR activities (Manzano et al., 2004). S-carvone has also been described as a regulator of plant HMGR activity by promoting the degradation of the protein (Oosterhoven et al., 1993; Oosterhoven, 1995). The underlying mechanism remained unknown, because this was a single description of a natural product inducing such an effect in plants. Possible functions of plant-derived monoterpenes as hypocholesterolemic or as anticaner agents in animal cells were investigated in several studies. Loss of activity of rat liver HMGR by cineole and menthol treatment was the result of alteration in enzyme synthesis or degradation (Clegg et al., 1982). Alternatively, limonene, perillyl alcohol, and geraniol were found to suppress HMGR synthesis at a posttranscriptional level in hamster kidney cells (Peffley and Gayen, 2003).

In contrast, this study presents results indicating that S-carvone significantly increased HMGR protein and activity in *N. tabacum* cells and leaves. Depending on the level of regulation, discrepancies were observed regarding the response of HMGR to the monoterpene treatment. The monoterpene stimulated *N. tabacum* HMGR activity, but down-regulated cellulase-activated levels of both *HMGR1* and *HMGR2* transcripts. Protein levels, and moreover enzyme activities, remained at the level of cellulase-treated *N. tabacum* plants. It can be postulated that inhibition of the target of S-carvone induces an HMGR activity that would be required to build up the MVA-derived sesquiterpene capsidiol. How to explain that an early step of the metabolic pathway is stimulated (increase of HMGR activity), without a concomitant increase of capsidiol, the pathway end product? This contradictory result led us first to postulate that capsidiol might not be synthesized from IPP units derived from the MVA pathway but instead might use plastidial MEP-derived units. This hypothesis turned out to be wrong, but still fosmidomycin, an inhibitor of the MVA-independent MEP pathway,
Involvement of an MEP Pathway–Derived Isoprenylated Protein in the Biosynthesis of Cellulase-Induced Capsidiol Production

Based on the results presented in this article, we propose a model that explains to a large extent why capsidiol production is inhibited by S-carvone (Fig. 9). In this model, the cellulase-induced signaling cascade involves at least one protein modified with a geranylgeranyl group that is biosynthesized in plastids through the MEP pathway. The contribution to the elicitation process of proteins carrying the canonical CaaX motifs was reported for several species (Vasconsuelo and Boland, 2007), but direct proof for protein modification with a prenyl group is not yet available. This might largely be due to the technical difficulties of analyzing such under-represented hydrophobic proteins. As a consequence, the precise identity of the protein possibly involved in the control of capsidiol is still unknown; however, it appears unlikely that the signaling pathway engages only one protein. Nevertheless, further studies are required to identify these proteins, but it can be postulated that members of the Rop/Rac GTfases family are involved. These GTfases function as regulators of signaling pathways (Gu et al., 2004), and many of them are predicted as being isoprenylated. NtRac5/NTGP2 (AJ250174/U64923) in N. tabacum is currently considered to control the activity of NADPH oxidase responsible for the oxidative burst induced after elicitation (Morel et al., 2004). Both NtRac5 and the NADPH oxidase were shown to be associated with lipid rafts, some plasma membrane microdomains, which among other functions are involved in plant defense (Mongrand et al., 2004). Furthermore, NtRac5 bears a C terminus CSIL, a motif predicted to be recognized by protein-geranylgeranyltransferases (Maurer-Stroh and Eisenhaber, 2005), and thus presumably depends on the MEP pathway in plants. For this reason, the modified protein might be a good candidate to indirectly control the biosynthesis of capsidiol production in N. tabacum. Inhibitors of the MEP pathway, such as fosmidomycin, block the production of the plastidial GGPP units used as substrates by the protein prenyltransferase catalyzing the transfer onto the protein necessary for the signaling cascade (Fig. 9). The absence of prenylation of this putative protein has a direct impact on its correct subcellular localization and thus the whole signaling cascade would be interrupted. Consequently, transcriptional activation of HMGR would be down-regulated (Fig. 3) and that of EAS would not be initiated; thus, capsidiol production should be prevented. By contrast, inhibitors such as mevinolin or alendronate directly block the biosynthetic pathway toward capsidiol and thus obstruct its biosynthesis. Finally, inhibition of protein prenyltransferase activity by S-carvone would lead to the same effect as that caused by inhibitors affecting the MEP pathway, thereby avoiding geranylgeranylation of the protein involved in the elicitor-induced signaling pathway (Fig. 9).

Interestingly, the metabolic origin of the prenyl group used to modify the protein (MEP pathway) is different from that of the generated secondary metabolite (MVA pathway). It appears therefore that an MEP-derived geranylgeranylated protein is mandatory for the biosynthesis of an MVA-derived secondary metabolite. A similar mode of regulation has already been described in Catharanthus roseus for the biosynthesis of indole.
alkaloid monoterpenes (Hedhili et al., 2007). The inhibition of HMGR and protein prenylation prevents the production of these MEP pathway-derived indole alkaloid monoterpenes (Imbault et al., 1996; Courdavault et al., 2005), suggesting that an MVA-derived isoprenylated protein is required in this case. Conceivably, such biosynthetic regulations may also occur in other plants. The production of the sesquiterpenoid artemisinin in Artemisia annua was shown to be MVA derived (Schramek et al., 2010), but its biosynthesis is inhibited by treatments with mevinolin or fosmidomycin (Towler and Weathers, 2007). In a similar manner, the biosynthesis of the diterpene taxadiene in Taxus species is dependent upon the biosynthesis of MEP (Eisenreich et al., 1996), but compactin (an analog of mevinolin) and fosmidomycin both block the accumulation of this product (Soliman et al., 2011). Altogether, these observations imply that the two compartmentalized MVA and MEP pathways somehow conspire at different levels. Of course, they exchange metabolic precursors for the biosynthesis of isoprenoid compounds (for review, see Henmerlin et al., 2012), but it also appears that metabolites derived from one pathway control the biosynthesis of metabolites coming from the other one.

Monoterpenes as Biologically Active Compounds

We showed that oxygenated monoterpenes, such as carvone or perillyl alcohol, inhibit the production of MVA-derived phytoalexin capsidiol in N. tabacum. As a whole, monoterpenes emitted by plants into the atmosphere constitute a considerable part of volatile organic compounds. It has been proposed that they have specific functions in plant defense (Gershenzon and Dudareva, 2007) and at high concentrations, many monoterpenes act as strong growth inhibitors in allelopathic interactions with other plants. It has been shown that monoterpenes inhibit plant growth, development, and cell division (Singh et al., 2006; Yoshimura et al., 2011; Sakai and Yoshimura, 2012). However, such physiological properties are still a matter of debate. One main reason is that the amounts of product needed to see any biological activity are rather high. Yet real concentrations that likely reach the target are fundamentally unknown. Several studies propose to use essential oils as herbicide compounds. Indeed, like other monoterpenes, S-carvone was found to inhibit seed germination of angiosperms (Vaughn and Spencer, 1993), a peculiarity that was employed to reduce the premature sprouting of potatoes occurring during long storage periods (Hartmans and Diepenhorst, 1994). Volatile monoterpenes inhibit also stomatal opening in some species (Rai et al., 2003), a process otherwise mediated by abscisic acid. Interestingly, Arabidopsis knock-out mutants defective in protein prenyltransferase activities (erl1, plp, or ggb mutants) exhibit an abscisic acid enhanced phenotype inducing closure of stomata (Pei et al., 1998; Running et al., 2004; Johnson et al., 2005). These results are consistent with the negative effects of monoterpenes on the activity of PPTases as well as on stomata opening. Protein prenylation is a widespread posttranslational modification, and such proteins are involved in regulating biological processes such as cell division, hormone signaling, and so forth (Crowell and Huizinga, 2009; Sorek et al., 2009). Therefore, the negative effect of S-carvone on potato sprout meristem development could conveniently be explained by inhibiting the prenylation of a specific, but still unknown, protein.

A further concern, but interesting observation, is that a monoterpane might act as a negative regulator on the production of a phytoalexin sesquiterpene. Some studies illustrate this potential function to some extent. For example, defense responses are primed in lima bean (Phaseolus lunatus) exposed to (E)-β-octimene emitted by engineered N. tabacum plants overexpressing the corresponding synthase (Muroi et al., 2011). S-carvone hinders the biosynthesis of a defense-related compound and therefore makes the whole system vulnerable. At this stage, if and how the plant relies on such a mechanism can only be speculated. Nevertheless, it became increasingly evident through our study that the biosynthesis of a primary metabolite, with important cellular functions, is somehow targeted by S-carvone. It appeared that treatments with S-carvone constrain the biosynthesis of other MVA-derived compounds. Indeed, besides sesquiterpenes, the biosynthesis of other MVA-derived lipids such as phytosterols is also arrested (Threlfall and Whitehead, 1988; Vogeli and Chappell, 1988). This was also observed in intact animal cells treated with perillyl alcohol (Ren and Gould, 1994). In N. tabacum and Arabidopsis, inhibition of sterol biosynthesis leads to a positive feedback regulation of HMGR activity (Wentzinger et al., 2002; Nieto et al., 2009), which would explain to some extent the activation of HMGR in our study. However, monoterpenes also interfere with protein prenylation (Crowell et al., 1991; Gelb et al., 1995; Hardcastle et al., 1999). Some other monoterpenes are described as inhibitors of plant protein isoprenylation. Perillyl alcohol belongs to this category, whereas limonene is inefficient (Morehead et al., 1995; Courdavault et al., 2005). In agreement with this statement, we were not able to block capsidiol production with limonene, but enantiomeric perillyl alcohols were as efficient as carvone. In contrast, the modification and the levels of the isoprenylated rat sarcoma protein are affected by limonene and S-perillyl alcohol treatments in human cell lines (Hohl and Lewis, 1995), and treatments with those monoterpenes blocked protein prenylation in NIH3T3 mouse cells and human mammary epithelial cells (Crowell et al., 1991). However, contrary to perillyl alcohol, in vitro enzyme assays using protein extracts isolated from rat brain or purified mammalian and yeast enzymes showed that limonene is only a weak inhibitor of protein prenyltransferases (Gelb et al., 1995; Hardcastle et al., 1999). Limonene must therefore be metabolized to become a biologically active compound. In humans, the oxidation of limonene into perillyl alcohol is catalyzed by CYP2C19
(Miyazawa et al., 2002). The absence of such an enzyme in plants, especially in N. tabacum, might explain why limonene is only effective in animal tissues. Furthermore, it was previously proposed that monoterpene biosynthetic activity requires an oxygen function (Vaughn and Spencer, 1993).

In conclusion, this study provides convincing evidence for another facet of how the compartmentalized isoprenoid biosynthesis pathways might interact in regulatory processes in plants.

MATERIALS AND METHODS

Chemical Materials

1-[3H]Farnesyl diposphate (205 Ci/mmol) used for 5-epi-aristolochene synthase assays was purchased from NEN Radiochemicals. 1-[3H]Farnesyl diposphate (60 Ci/mmol) or 1-[3H]geranylgeranyl diposphate (60 Ci/mmol) used for protein prenyltransferase assays was purchased from American Radiolabeled Chemicals, Inc. 1-[3C]Oxaloacetate was enzymatically synthesized as described by Hemmerlin et al. (2006) and (R,S)-2-[13C]mevalonolactone was purchased from Amersham. (1-13C)-Glc (99% isotope abundance) was supplied by Sigma-Aldrich.

Capsidiol Production, Extraction, and Quantification by GC

Leaf discs (3-cm diameter) were punched from mature Nicotiana tabacum var Xanthi plants and used in a floating assay for capsidiol production. The discs were positioned adaxial side upward into petri dishes (one disc per dish) containing water supplemented either with 0.5% (w/v) cellulase RS (Yakult Pharmaceutical Industry) or mevinolin (20 mM), or in the presence or absence of 5-cm diameter prenyltransferases protein substrates His6-GFP-BD-CVIL, His6-GFP-BD-CVIM, and His6-GFP-BD-SVIL were produced in Escherichia coli as recombinant proteins, purified using Ni2+ spin columns (Qiagen) and used at a final concentration of 20 μM. Activity measured with a total protein extract (20 μg) was assayed for 1 h. After denaturation, proteins were separated on a SDS-PAGE that was treated with Amplify (Amersham) before being exposed for 6 weeks at ~80°C to a preflashed x-ray film (Kodak).

Transgenic N. tabacum BY-2 cell suspensions expressing prenylable GFP fusion protein GFP-BD-CVIL were used for evaluation of protein isoprenylation in vivo as previously described in detail (Gerber et al., 2009). Protein prenylation, characterized by a localization of the GFP within the plasma membrane, was visualized by confocal microscopy, based on the procedure described by Gerber et al. (2009).

The N. tabacum BY-2 cell suspension (200 μL) was incubated with 1 μCi [2-14C]MVA or 1 μCi [1-14C]-[2-14C]mevalonolactone was converted to its open acid form before being exposed for 6 weeks at ~80°C to a preflashed x-ray film (Kodak).

Protein Prenyltransferase Enzyme Assays

N. tabacum protein prenyltransferases were produced as described in the Supplemental Materials and Methods S1 and their activities were determined using a continuous fluorometric enzyme assay previously described (Cassidy et al., 1995) with some modifications. The peptide synthesis facility at the Institut Génétique Biologie Moléculaire Cellulaire synthesized the dansyl-pentapeptide and dansyl-pentapeptide, His6-GFP-BD-CVIL, His6-GFP-BD-CVIM, and His6-GFP-BD-SVIL were produced in Escherichia coli by recombinant proteins, purified using Ni2+ spin columns (Qiagen) and used at a final concentration of 20 μM. Activity measured with a total protein extract (20 μg) was assayed for 1 h. After denaturation, proteins were separated on a SDS-PAGE that was treated with Amplify (Amersham) before being exposed for 6 weeks at ~80°C to a preflashed x-ray film (Kodak).


Capsidiol was characterized by GC-MS, ultra-high performance liquid chromatography–mass spectrometry, and 1H-NMR and 13C-NMR. For each mass spectrometric technique, the retention time and the mass spectrum of the product isolated from N. tabacum were compared with those of an authentic reference. Details of the procedures are described in the Supplemental Materials and Methods S1.

Evaluation of Protein Isoprenylation in N. tabacum Protein Crude Extracts and In Vivo

A wild-type N. tabacum BY-2 cell suspension was used to evaluate protein prenyltransferase activities in crude protein extracts. Cells in stationary phase were diluted 5 times in modified Murashige and Skoog medium as previously described (Hemmerlin and Bach, 1998) and cultured for 24 h at 27°C. Chemical treatment of the cells was performed as described in the figure legends. Filtered cells were frozen in liquid nitrogen, powdered in a mortar, and homogenized in PPTase buffer (50 mM HEPEs pH 7.5, 20 mM MgCl2, 5 mM dithioerythritol [DTE], and 100 μM phenylmethylsulfonyl fluoride). The homogenate was centrifuged for 10 min at 8000 rpm and at 4°C. The supernatant was used as an enzyme source and a Bradford protocol (Bio-Rad) was used to quantify proteins in each sample. Protein prenyltransferase activity was evaluated using a radiometric assay based on the protocol described by Gerber et al. (2009) with some modifications. Instead of a 1-μCi concentration, samples were incubated with a lower concentration (0.25 μCi) of [3H]JFP or [3H]GFP. The prenyltransferase protein substrates His6-GFP-BD-CVIL, His6-GFP-BD-CVIM, and His6-GFP-BD-SVIL were produced in Escherichia coli by recombinant proteins, purified using Ni2+ spin columns (Qiagen) and used at a final concentration of 20 μM. Activity measured with a total protein extract (20 μg) was assayed for 1 h. After denaturation, proteins were separated on a SDS-PAGE that was treated with Amplify (Amersham) before being exposed for 6 weeks at ~80°C to a preflashed x-ray film (Kodak).

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Supplemental Table S3. $^{13}$C-Isotope abundance of isoprene units of α-cembratrieniol after incorporation of [1-13C]Glc.

Supplemental Table S4. Effect of carvone and known inhibitors of protein isoprenylation on capsidil production in cellulase-elicited N. tabacum leaf discs.

Supplemental Table S5. Oligonucleotides used in this study.

Supplemental Materials and Methods S1. Identification of capsidil by 1H- and 13C-NMR, by GC-MS, and by UHPLC-ESI-MS/MS as well as cloning and heterologous expression of N. tabacum PPTases and determination of inhibition of cell growth, cell death, toxicity, and AOS production induced by chemical treatment.

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LITERATURE CITED


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