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Research paper

Farnesol-mediated shift in the metabolic origin of prenyl groups used for protein prenylation in plants

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ABSTRACT

Little is known about how plant cells regulate the exchange of prenyl diphosphates between the two compartmentalized isoprenoid biosynthesis pathways. Prenylation of proteins is a suitable model to study such interactions between the plastidial methylerythritol phosphate (MEP) and the cytosolic mevalonate (MVA) pathways because prenyl moieties used to modify proteins rely on both origins. Tobacco cells expressing a prenylatable GFP were treated with specific MEP and/or MVA pathways inhibitors to block the formation of prenyl diphosphates and therefore the possibility to modify the proteins. Chemical complementation assays using prenyl alcohol precursors restore the prenylation. Indeed, geranylgeraniol (C_{20} prenyl alcohol) and to a lesser but significant level C_{15}-farnesol restored the prenylation of a protein bearing a geranylgeranylation CaaX motif, which under standard conditions is modified by a MEP-derived prenyl group. However, the restoration takes place in different ways. While geranylgeraniol operates directly as a metabolic precursor, the C_{15}-prenyl alcohol functions indirectly as a signal that leads to shift the metabolic origin of prenyl groups in modified proteins, here from the plastidial MEP pathway in favor of the cytosolic MVA pathway. Furthermore, farnesol interferes negatively with the MEP pathway in an engineered *Escherichia coli* strain synthesizing isoprenoids either starting from MVA or from MEP. Following the cellular uptake of a fluorescent analog of farnesol, we showed its close interaction with tobacco plastids and modification of plastid homeostasis. As a consequence, in tobacco farnesol supposedly inhibits the plastidial MEP pathway and activates the cytosolic MVA pathway, leading to the shift in the metabolic origin and thereby acts as a potential regulator of crosstalk between the two pathways. Together, those results suggest a new role for farnesol (or a metabolite thereof) as a central molecule for the regulation of isoprenoid biosynthesis in plants.

1. Introduction

In eukaryotes, proteins with C-terminal CaaX motifs are modified by a zinc cation-mediated sulfur alkylation catalyzed by specific protein prenyl transferases (PPTs), referred to as type-I protein prenylation [1]. Similar to other isoprenoid molecules, protein prenylation depends on the biosynthesis of cellular isopentenyl diphosphate (IPP), the common biosynthetic precursor of this family of compounds. The PPT enzymes use one of two prenyl diphosphate substrates: farnesyl diphosphate (C_{15}, FPP) or geranylgeranyl diphosphate (C_{20}, GGPP) and a protein-CaaX substrate. CaaX motifs share a common C-terminal pattern described as follows: Cys-aliphatic amino acid-aliphatic amino acid, and X as an amino acid providing prenylation specificity [1]. Along with geranyl

Abbreviations: DX, 1-deoxy-D-xylulose; FOS, fosmidomycin; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; IPP, isopentenyl diphosphate; MEcPP, 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MV, mevinolin; MVA, mevalonic acid; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PPTs, protein prenyltransferases.

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diphosphate (C_{10}, GPP), FPP and GGPP are the major isoprenoid precursors required for the biosynthesis of sterols, side chains of ubiquinones, of carotenoids, and a large variety of secondary isoprenoid metabolites [2,3]. In plants the organization of isoprenoid biosynthesis is considerably more complex than in other eukaryotes. One of the reasons of this increased intricacy is the huge number of molecules plants synthesize simultaneously. As a consequence, isozymes with dedicated functions operate in parallel. In addition, IPP is synthesized in the cytosol from mevalonic acid (MVA) by the same pathway found in animals and fungi, but also in the plastidial compartment via the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway commonly found in bacteria [3]. Recently, we demonstrated that a C_{20}-geranylgeranyl moiety of plastidic origin and formed via the MEP pathway, was used for geranylgeranylation of a chimeric GFP-CVIL protein substrate [4]. The limited use of a MEP pathway-derived prenyl group to modify the protein is characterized by an inhibition of protein prenylation when cells are treated with inhibitors of PPTs but also more particularly of MEP pathway specific enzymes (Fig. 1). Conversely, blocking the MVA pathway does not apparently affect the geranylgeranylation of the protein.

Accordingly, we proved that plastids are involved in the regulatory mechanism for supplying isoprenyl diphosphate used for protein prenylation in the cytoplasm of higher plants. Inhibition of protein geranylgeranylation with inhibitors of the MEP pathway can be chemically complemented by isoprenols such as geranylgeraniol, but also by farnesol, which is five carbons shorter than geranylgeraniol [4] (Fig. 1). The goal of this new study was to understand why farnesol showed some capacity to reestablish prenylation of a protein bearing a geranylgeranyl motif.

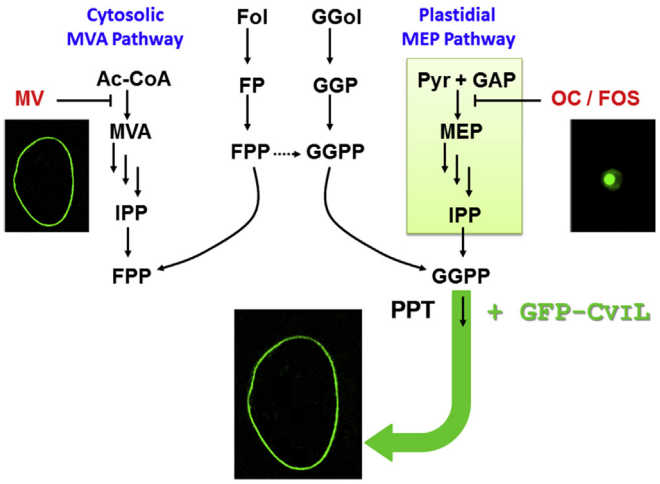

Fig. 1. Protein geranylgeranylation in tobacco BY-2 (TBY-2) cells involves the plastidial MEP pathway. The geranylgeranyl moiety used by the protein prenyltransferase (PPT) to modify a GFP-CVIL chimeric protein bearing a specific C-terminal geranylgeranylation CaaX motif (CVIL) is generated via the plastidial MEP pathway [4] and is characterized by a plasma membrane localized fluorescence of the GFP. In the presence of specific inhibitors of MEP pathway enzymes (OC: oxoclomazone; FOS: fosmidomycin) this plasma membrane protein is not further modified by a hydrophobic prenyl group and delocalizes into the nucleus. The absence of an isoprenoid pool synthesized from the MVA pathway (MV: mevinolin) has no effect on the prenylation of this protein. Complementation by exogenous prenyl alcohols such as farnesol (Fol) or geranylgeraniol (GGol) that are phosphorylated by endogenous kinases into the corresponding prenyl diphosphates (farnesyl diphosphate/FPP and geranylgeranyl diphosphate/GGPP) is indicated. They can virtually be utilized as prenyl diphosphates substrates by protein prenyltransferases. Ac-CoA: acetyl coenzyme A; IPP: isopentenyl diphosphate; Pyr: pyruvate; GAP: glyceraldehyde 3-phosphate.

2. Materials and methods

2.1. Chemicals

All-*trans*-farnesol, *trans*-geraniol, all-*trans*-geranylgeraniol, farnesyl acetate, dexamethasone, fosmidomycin and mevalonolactone were purchased from Sigma-Aldrich (Saint Quentin Fallavier). Mevinolin was a kind gift from Drs. M. Greenspan and A. W. Alberts (Merck Sharp and Dohme). Before use, lactone forms of mevinolin and mevalonolactone were converted into their free salt forms as described by Gerber et al. [4]. 1-deoxy-D-xylulose (DX) was obtained from AlsaChim (Illkirch Graffenstaden, France). The fluorescent analog to farnesol, NBD-geraniol (geranyl-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) alcohol) was obtained from Prof. Herbert Waldmann (MPI, Dortmund) and utilized as described by Hemmerlin et al. [5].

2.2. Biological materials and growth conditions

The tobacco (*Nicotiana tabacum*) Bright Yellow-2 (TBY-2) cell line was cultured in MS-medium as described previously [4]. The TBY-2 cells expressing the chimeric GFP-CVIL prenylation substrate (TBY2::GFP-CVIL) [4] were cultured under identical conditions. For protein prenylation inhibition analyses, 7-day-old cells were diluted 5-fold into fresh MS-medium, treated for 3 h with chemicals, before protein expression was induced with dexamethasone (15 μM) and cells incubated for an additional 15 h. Working stock solutions of isoprenols were prepared in ethanol and used at a final 1:1000 (v:v) dilution in the culture medium. Controls were treated with the solvent alone. For transit expression of PT5-RFP, TBY-2 cells were transformed by tungsten particle shooting with an inflow gun as described [6], then incubated for 5 h before being recovered in liquid MS medium and cultured in the presence of farnesol (1–6 h) as indicated.

The engineered *dxs::KAN* disrupted *Escherichia coli* mutant strain expressing the *Lactobacillus plantarum* MVA metabolizing operon was obtained as described previously [7]. This strain grows at 28 °C on LB medium supplemented with kanamycin (15 μg/mL), ampicillin (100 μg/mL) and requires a source of isoprenoid precursors either in the form of MVA or DX (750 μM). The nature of the precursor used to grow defines the pathway that is activated and used to synthesize isoprenoids in this strain, either through the *Lactobacillus plantarum* MVA pathway when MVA is used, or the endogenous MEP pathway when DX is used to initiate cell growth.

2.3. Microscopy methods

As previously described in detail, the subcellular localization of a GFP fusion protein with a C-terminal prenylatable CaaX sequence was used to evaluate protein prenylation *in vivo* [4]. This technique has the added benefit of providing information about the metabolic origin of the prenyl groups used to modify a chimeric GFP-fusion protein bearing a geranylgeranylation motif (GFP-CVIL) by inhibiting specific enzymes within the isoprenoid biosynthesis pathways [8]. Confocal laser scanning microscopy (CLSM) was used to monitor the subcellular location of the modified GFP, which, if prenylated, integrates into the plasma membrane, otherwise dislocates to the nucleus. Protein prenylation was visualized with a Zeiss Confocal LSM700 microscope. Co-localization experiments were performed in multitrack mode. Excitation/emission wavelengths were set as follows: 488 nm/500–600 nm for GFP and 555 nm/585–615 nm for RFP. Images were acquired using Carl Zeiss ZEN software and processed using Adobe Photoshop.

2.4. Statistical analysis

Cells (>300) were analyzed in two independent measurements and classified by visual selection into the following categories: plasma membrane-localized, nucleus-localized, or both. The efficiency of cellular-treatments with inhibitors was evaluated using chi-squared tests against the null hypothesis being true. Significant differences were assigned to p-values <0.01 (**).

2.5. Biochemical methods

Deoxyxylulose kinase assays using purified *Escherichia coli* recombinant xylulose kinase were performed as described previously [6]. Chlorophyll and carotenoid contents of leaf disks (diameter 1.7 cm) were quantified by UV–VIS spectroscopy according to the protocol described by Lichenthaler and Buschmann [9].

3. Results

3.1. Farnesol interferes with MEP-derived protein geranylgeranylation

To test if a precise isoprenol chain length is mandatory for protein prenylation complementation, we examined the capacity of prenyl alcohols with decreasing lengths (C_{20} to C_{10}) to restore the plasma membrane localization of the GFP-CVIL fusion protein in presence of various inhibitors blocking either enzymes in the MEP pathway (fosmidomycin: FOS), the MVA pathway (mevinolin: MV) or both (MV/FOS) (Fig. 2). This pharmacological approach allows the selection of the active isoprenoid biosynthesis pathway and an evaluation of its implication in providing the IPP pool used to modify proteins with CaaX motifs. Applying the microscope-based methodology we developed previously, results were consistent with our previous observations: a functional MEP pathway is mandatory for modifying GFP-CVIL, reflecting the exclusive use of the MEP pathway-derived geranylgeranyl group [4]. Geranylgeraniol complements deficiencies, regardless of the MVA pathway remaining active or not (Fig. 2). In contrast, farnesol restores partially prenylation only if the MVA pathway remains active. Moreover, we noticed several cell clusters being unable to modify the protein when the MVA pathway is not functional (Fig. 2), suggesting that farnesol behaves under certain circumstances, as inhibitor of the MEP pathway. Overall, we showed that C_{15} and C_{20} isoprenyl alcohols are not equivalent in their ability to restore protein geranylgeranylation. If we consider protein geranylgeranyltransferase-I as being responsive for the modification of GFP-CVIL using GGPP *in vivo*, it could be expected that farnesol being primarily converted into FPP, is less efficient than geranylgeraniol. More intriguing was the incapacity of farnesol to restore prenylation when both isoprenoid pathways are non-operational. For this reason, both molecules cannot be considered as equivalent substrates in chemical complementation assays. Finally, treatment with C_{10}-geraniol could not promote prenylation of GFP-CVIL, i.e. treated cells looked like controls, suggesting that only C_{15} and C_{20} isoprenyl alcohols are substrates or regulators of protein prenylation in tobacco cells.

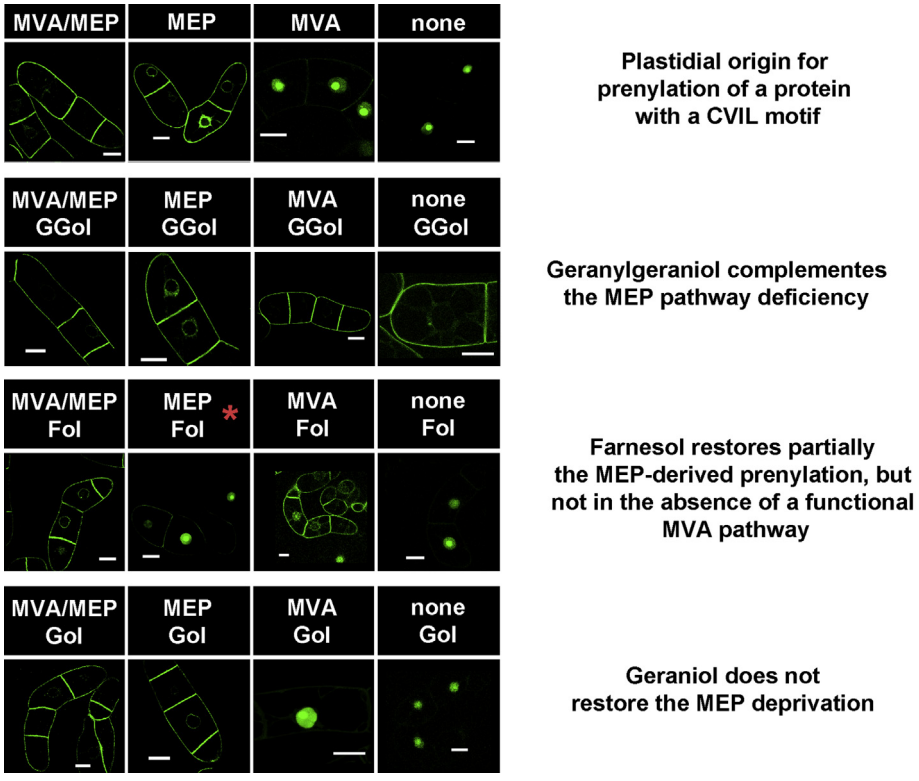

Fig. 2. Chemical complementation assays of protein prenylation inhibition with prenyl alcohols in TBY-2 cells expressing GFP-CVIL. Pictures were recorded by CLSM (bars = 20 μm). The nucleus labeling of tobacco cells indicates that prenyl diphosphates cannot be utilized by protein prenyltransferases to modify the GFP-CVIL. The active pathway(s) that can be involved in prenyl diphosphate biosynthesis, is(are) indicated at the top of each picture as well as the prenyl alcohol used for complementation assays. If chemicals can be converted to prenyl diphosphates and be used as substrates by protein prenyltransferases A pharmacological approach has been used to inactivate either the MVA pathway (mevinolin), the MEP pathway (fosmidomycin) or both (mevinolin + fosmidomycin). Five-fold diluted TBY-2 cells were treated as indicated in the materials and methods section at following concentrations: fosmidomycin (50 μM, FOS), mevinolin (5 μM, MV), geraniol (20 μM, Gol), farnesol (20 μM, Fol) and geranylgeraniol (20 μM, GGol). The red * indicates a subcellular localization we could observed in cells treated with MV (active MEP pathway) and Fol, but not when cells were treated with MV and GGol. However, a large amounts of cells showed a membrane localization (see Fig. 3).

To gain a better insight into these unexpected results, we conducted statistical analyses for a selected number of conditions to evaluate the reproducibility of the phenomena (Fig. 3). We choose to exclude geraniol because this molecule was, as expected, unable to reverse any inhibition. Instead, we considered farnesyl-acetate to evaluate the specificity of the C_{15} chain length. As already suggested in Fig. 2, farnesol as such impacts protein prenylation. This effect is intensified when the MVA pathway is blocked. The plasma membrane was labeled in 55% of the cells treated with farnesol when the MEP pathway is blocked with fosmidomycin, but not when the MEP and MVA pathways are simultaneously blocked with fosmidomycin and mevinolin (Fig. 3). Farnesyl-acetate is considered as not being metabolized *in vivo* unless some esterases cleave the acetate ester and has been identified as an inhibitor of DNA replication in animal cells [10]. When cells were treated with farnesyl acetate, reversion was less effective than with farnesol alone. However, the compound behaves like the C_{15}-prenyl alcohol: unlike geranylgeraniol, it cannot overcome the inhibition of the MEP and the MVA pathway. Together, it appears now questionable of whether the partial reversion of the MEP-pathway depletion is caused by the chemical complementation or if an alternative interpretation should be verified.

The data set shows that farnesol does not substitute for geranylgeraniol as a substrate for protein prenylation. A 30% mislocalization (totally or partially in the nucleus) when cells were treated both with farnesol and mevinolin has been estimated. In tobacco BY-2 cells, farnesol has been characterized as efficiently stimulating HMGR activity through transcriptional activation of the stress form *HMGR2* [11,12]. As a consequence, under those conditions, the MVA pathway should be strongly stimulated. Accordingly, the inhibition by mevinolin suggests that the potentially activated MVA pathway by farnesol might provide the isoprenoid pool capable of modifying GFP-CVIL. In the absence of mevinolin, farnesol is less efficient in permitting prenylation. This result further exemplifies that farnesol might be responsible in mediating a shift from the MEP pathway to the activated MVA pathway for synthesis of the substrates used for protein prenylation. Since mevinolin inhibits prenylation only in the presence of farnesol, the switch to use the MVA pathway appears to be associated with some down-regulation of the MEP pathway.

3.2. Farnesol prevents growth of bacteria using the MEP pathway

An engineered *Escherichia coli* strain producing isoprenoids either through the MEP pathway or the MVA pathway was used to test if farnesol might deactivate the MEP pathway [7]. Growth was examined on media supplemented with MVA or DX in the presence of farnesol (Fig. 4). The colonies were similar when grown on plates supplemented with 750 μM concentrations of either compound. However, the size of the colonies after 3 days at 28 °C was considerably smaller when supplemented with DX plus farnesol (Fig. 4A). In addition, overnight cultures in liquid medium supplemented with DX and farnesol showed a significant decrease of turbidity, indicative of lower bacterial cell division (Fig. 4B). In contrast, farnesol did not noticeably alter growth when the cells were grown on MVA. We considered the possibility that farnesol could directly interfere with 1-deoxy-D-xylulose (DX) phosphorylation needed for the bacterial growth. To rule out this possibility, we tested the activity of recombinant *Escherichia coli* xylulose kinase (EcXK) responsible of DXP formation *in vivo* [13] (Fig. 5). EcXK was assayed using 3.5 mM DX as a sugar substrate and 1 mM farnesol that represented the upper limit for farnesol solubility in the reaction mixture. Over 15 min, farnesol does not inhibit DX kinase activity, which is needed to convert DX into DXP (Fig. 5). From this it can be concluded that farnesol retards growth when the bacteria rely on the MEP pathway for isoprenoid biosynthesis, but not when they utilize the optional MVA pathway. As a consequence, it looks like if farnesol (or a metabolite thereof) down-regulates one or more of the enzymes of the MEP pathway after DXP synthase, the first enzyme in the pathway.

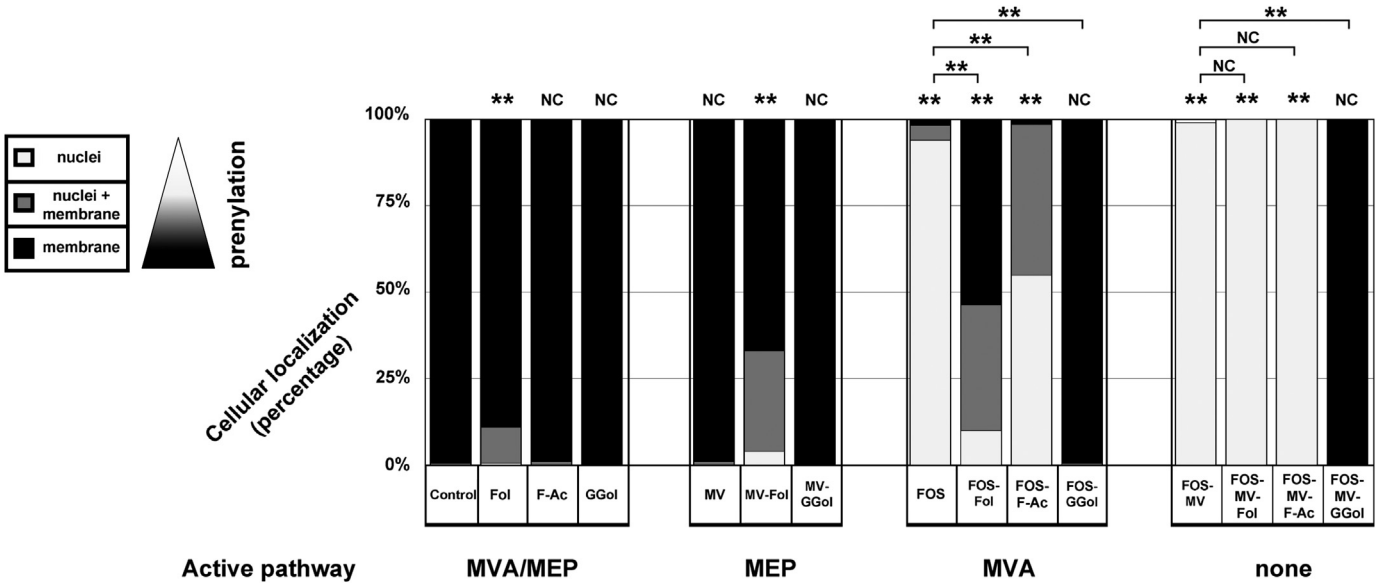

Fig. 3. Quantification of protein prenylation capacity *in vivo* following chemical complementation of isoprenoid pathway inhibitors-induced delocalization in *Nicotiana tabacum* BY-2 (TBY-2) cells expressing GFP- CVIL. Five-fold diluted cells were treated as indicated in the materials and methods section at following concentrations: fosmidomycin (50 μM, FOS), mevinolin (5 μM, MV), farnesol (20 μM, Fol), farnesol-acetate (20 μM) and geranylgeraniol (20 μM, GGol). Prenylation was analyzed by classifying the subcellular localization of GFP into three categories: plasma membrane-localized, nucleus-localized, or both. Increasing dark colors indicate increasing prenylation capability *in vivo*. Categorized values were compared by chi-squared tests against the null hypothesis (control) and were considered to be consistent (significant) when $P > 0.01$ (**) and non-consistent (NC) if not. To show the inconsistency between the complementation of a MEP deficiency or a MVA/MEP deficiency, chi-squared tests were performed as indicated against results obtained when cells were treated with fosmidomycin or fosmidomycin and mevinolin.

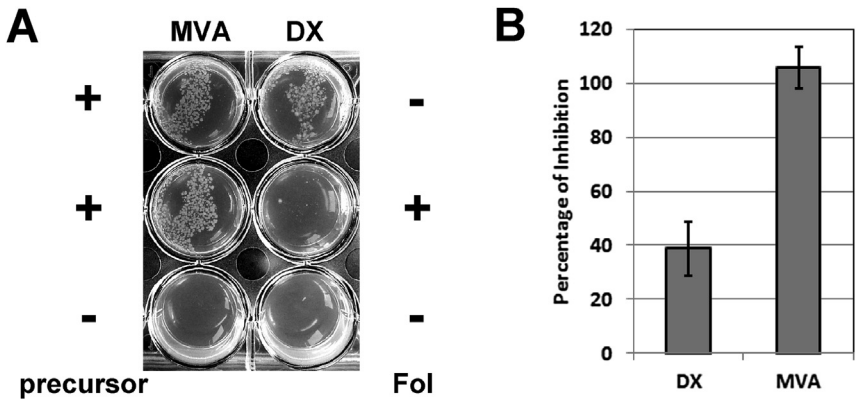

Fig. 4. Farnesol blocks bacterial growth if IPP is synthesized via the MEP pathway but not via the MVA pathway. (A) *Escherichia coli dxs* deletion mutants expressing the *Lactobacillus plantarum* MVA operon were grown in selective liquid medium to the stationary phase, then diluted 50,000 fold into LB medium and 10 μL were plated onto a plate-well (Ø = 2 cm). Isoprenoid precursors (MVA or DX, 750 μM) or farnesol (4 mM) were supplemented to the solid LB medium, as indicated. Development of colonies was allowed at 30 °C and phenotypic analysis was performed. (B) The engineered *Escherichia coli* strain was grown to the stationary phase, then diluted 200,000 fold into LB medium and 25 μL were transferred into 10 mL LB medium containing either LB medium, LB medium supplemented DMSO (1%) and with an isoprenoid precursor (DX or MVA at 250 μM) in the presence of absence of farnesol (4 mM). After 24 h, optical density was measured at 600 nm and percentage of inhibition was calculated. Three independent replicates have been realized and result is considered to be consistent (significant) when $P > 0.01$ (***).

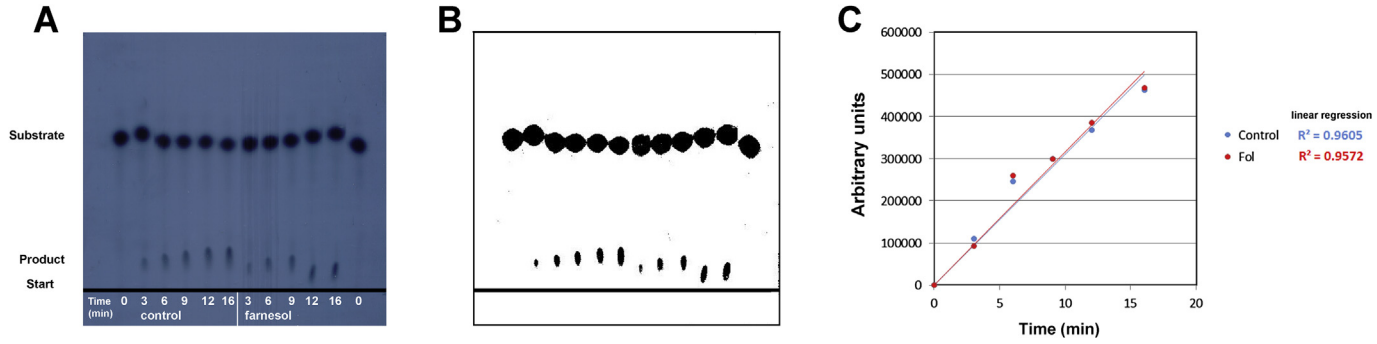

Fig. 5. Farnesol does not interfere with xylulose kinase activity necessary for phosphorylation of DX into DXP in *E. coli*. (A) Enzyme assays were carried out as described by Hemmerlin et al. [6] in the presence or absence of 1 mM farnesol. To stop the reaction, aliquots (2 μL) were spotted on a silica plate at 3, 6, 9, 12 and 16 min that was thereafter developed in (6:1:3, v/v/v) n-propyl alcohol:ethyl acetate:water before being exposed to a X-ray film. (B) The autoradiogram described in A. was analyzed using the open software Image J. To that purpose, the raw image was converted into a binary image and general threshold was adjusted before spots intensities were analyzed. (C) Raw intensities values (arbitrary units) were represented as a function of time (min). Blue dots are corresponding to control assays, while red dots represent assays containing farnesol (1 mM).

3.3. Interaction of farnesol with plastids

Should farnesol negatively interfere with the plastidial MEP pathway, the molecule (or a metabolite derived thereof) would need to translocate into this subcellular compartment. We used a fluorescent analog of farnesol [7] to visualize this possibility. Transiently transformed TBY-2 cells expressing an RFP (PT5-RFP) that specifically labels the stroma of plastids [6] were co-cultured with the analog for 3 h before red (plastids) and green (farnesol) fluorescence was recorded to look for co-localization of the signals, which would indicate translocation of the molecule into plastids (Fig. 6A). For better visibility, a magnified picture is depicted in Fig. 6B. The merged image indicates a strong labeling surrounding the red signal observed in the stroma and a much weaker signal within the plastids (Fig. 6B). These observations indicate that the fluorescent analog might be incorporated into the plastid envelope and to some extent appears internalized inside these organelles. More conclusive data were obtained from time-dependent treatments with farnesol. The number or the size of labeled plastids appears to increase over time (Fig. 6C). To test this hypothesis we evaluated in approximatively 10 cells the number of plastids per cell volume (Fig. 6D). It appears that the number is not significantly modified in treated cells. The effect on plastids was further exemplified in a merged illustration that was realized from a flattened reconstruction from z-stack series passing through the cell (Fig. 6E). The possibility of farnesol to decrease MEP derived isoprenoid accumulations (pigment content) was tested on tobacco leaf disks treated with increasing concentrations of farnesol (Fig. 6F). Only a slight decrease in pigment content could be observed at 100 μM of farnesol. Taken together, these results confirmed our hypothesis that farnesol interacts with plant plastids, thereby possibly also with the plant MEP pathway, although a significant decrease of pigment content has not been observed when tobacco leaves have been treated with farnesol over 72 h.

4. Discussion

There remain many open questions as to the regulation of isoprenoid biosynthesis in plants. In response to various external stimuli, plants reorganize their biosynthetic capacities as a way to satisfy needs for the synthesis of specific compounds. This is especially true for isoprenoids where inducible biosynthesis of metabolites is required under certain conditions [14,15]. Metabolic flexibility is enhanced in plants by the synthesis of prenyl diphosphate precursors via the two distinct IPP biosynthesis pathways [3]. It is particularly poorly understood how dysregulation of metabolic

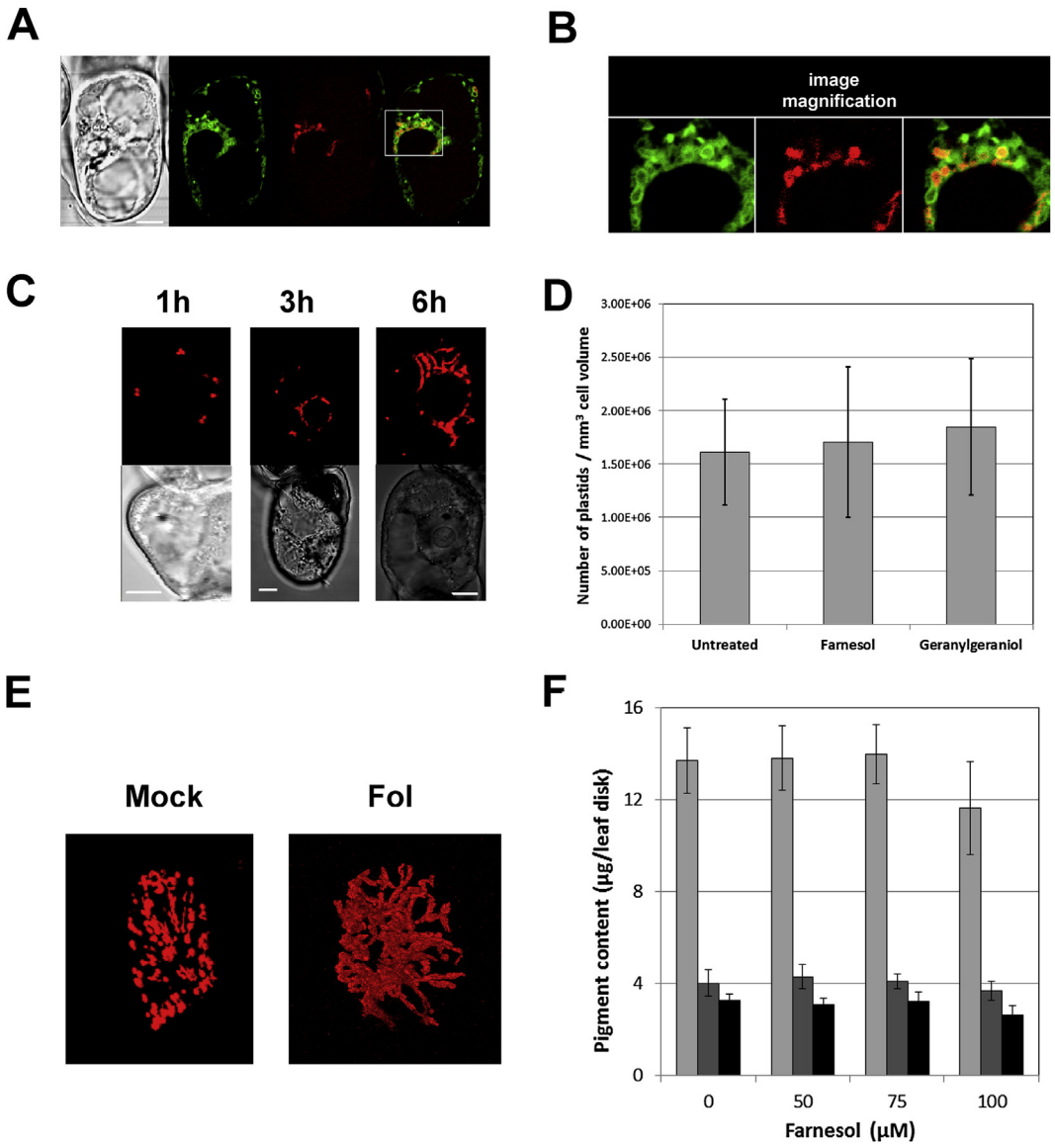

Fig. 6. Effects of farnesol on plastid homeostasis. (A) A fluorescent analog of farnesol-interacts with plastid in tobacco BY-2 cells. Cells were transiently transformed to express PT5-RFP that labels plastids. The fluorescent analog of farnesol (30 μM) was added for 3 h and images were acquired by CLSM. From right to left: phase contrast, green emission corresponding to farnesol emission, red emission corresponding to plastids, and an overlay between green and red. (B) An image magnification of the white scare depicted in (A) is represented. (C) Cells expressing PT5-RFP were treated for 1, 3 or 6 h with farnesol (30 μM) and a single-track image was recorded. Bars = 10 μm. (D) Numbering plastids contained in an equivalent of a virtually 1 mm^3 cell volume. Approximatively 10 measurements were realized. Cells were treated with 20 μM isoprenol or were untreated. (E) Flattened reconstruction from 30 z-stack acquisitions with 0.77 μm slices of a cell expressing PT5-RFP treated for 6 h with farnesol (Fol) as compared to an untreated control (mock). (F) Quantification of MEP-derived metabolites (chlorophylls and carotenoids) in tobacco leaves disks (diameter 1.7 cm) treated for 72 h with increasing concentrations of farnesol.

intermediates availability is inducing a rebalancing between both pathways. We discovered a link between farnesol and the two pathways providing the precursors for prenylation of proteins in plants. Farnesol is used as an activator of an alternative biosynthetic route that will provide the metabolic pool necessary to meet the needs for protein prenylation under restrictive conditions.

Overall, farnesol appears to be a central regulatory component in plants, which reinforces the idea of a strong contribution of this molecule in the regulation of physiological functions. This property is underlined by two observations: First, a parallelism between the action of farnesol and the function of the phytohormone abscisic acid (ABA) in stomatal opening [16] and flower development [17]. Further, a FPP-salvage machinery explicitly allows for recycling of the farnesyl moiety from degradation of farnesylated proteins into FPP, which then can be used for other biosynthetic purposes [18]. Thus, under some conditions, farnesol can be phosphorylated to yield FPP. The conversion of farnesol to FPP is required for its incorporation into prenylated proteins, ubiquinone and cholesterol in rat C6 glial cells and in African green monkey kidney cell lines [19]. In plants, farnesol is the substrate for specific kinases [20,21] that synthesize FPP for the synthesis of isoprenoid metabolites [20,22]. Farnesol is a major component in many essential oils from various plant species [23–25]. This suggests that non-metabolized FPP can be dephosphorylated. Activity for phosphatases capable of hydrolyzing FPP to farnesol have been described in cell-free protein extracts [26]. For instance, in kiwi flowers, farnesol that accumulates at concentrations that are toxic for other cell types [5,11] appears to compete for the available pool of FPP utilized for (*E*)-nerolidol sesquiterpene biosynthesis [23]. This observation suggests that at elevated concentrations, farnesol might also be

involved in regulating the production of secondary metabolites.

The results of this study suggest a central and regulatory role for farnesol in protein prenylation. Our data indicate that farnesol is not recycled to FPP that would subsequently be utilized as a substrate for protein prenylation. Rather, the molecule acts through shifting the metabolic origin of the prenylation substrates from the MEP to the MVA pathway. These conclusions are consistent with other observations we made when *S*-carvone was used to inhibit PPTs, where *S*-carvone was more effective when cells were treated simultaneously with *S*-carvone plus farnesol [27]. The action of farnesol on the MEP and MVA pathways is also consistent with studies showing a relatively low incorporation rate of [^{3}H]farnesol, as compared to [^{3}H]geranylgeraniol, into proteins of tobacco cells pretreated with mevinolin [20]. A regulatory process in which a metabolic intermediate leads to use an alternative biosynthetic pathway instead of serving as a source by itself, is certainly a matter of debate. To address this concern, we would need to answer the simple question of how important it is to modify a dedicated protein either with a geranylgeranyl or a farnesyl group? Plants adopted two pathways, two enzymes and two prenyl diphosphates to modify prenylatable proteins. This organization allows large degrees of flexibility to modify proteins even when a specific metabolic pool becomes limiting.

To explain how farnesol might down-regulate the MEP pathway, several possibilities can be considered. First, the alcohol could be important in retrograde signaling, as seen for 2-*C*-methyl-D-erythritol-2,4-cyclodiphosphate (MEcPP), an intermediate of the MEP pathway [28]. Alternatively, farnesol might interfere with an enzyme in the MEP pathway and alter its catalytic efficiency. We found that in an *Escherichia. coli* strain engineered to express a functional MVA pathway in parallel to the native MEP pathway, farnesol interferes with cell growth only if cells are utilizing the MEP pathway. This work indicates that farnesol specifically interferes with isoprenoid biosynthesis by the MEP pathway. The precise mode of action for farnesol at a molecular level awaits its elucidation, but it is likely that the compound or one of its metabolites negatively regulates one of the plastidial MEP enzymes, as was suggested for the bacterial 2*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase [29]. In this scenario, farnesol, or a related metabolite such as FPP, must be synthesized in or imported into the plastidial compartment. Export of FPP from plastids to the cytosol has been reported during sesquiterpene biosynthesis [30]. However, except for (*Z*,*Z*)-FPP-derived sesquiterpenoids [31], there are no convincing data clearly demonstrating that plants synthesize C_{15}-isoprenoid precursors in plastids. A more likely scenario involves synthesis of (*E*,*E*)-FPP from MVA in the cytosol followed by a subsequent accumulation of farnesol in plastids. Several studies, based on incorporation of ^{13}C labeled precursors, followed by analysis of metabolites by NMR, provided evidence for incorporation of an MVA-derived FPP unit into plastidial diterpenoids in some plants [32,33]. Our data are consistent with an import from the cytosol.

In conclusion, the function of farnesol as a factor that shifts the metabolic origin of isoprenoid compounds from the MEP to the MVA pathway opens new avenues. Additional studies should provide more insight into its mechanism of action for regulation of protein prenylation and, more generally, isoprenoid metabolism.

Authors' contributions

AnH, designed research; AlH, MSB, EG, DT and AnH performed research; AlH, MSB, EG, DT, TJB and AnH analyzed and interpreted data; AnH wrote the paper. All authors contributed in drafting the manuscript, approved the final version of the manuscript, and are in agreement to have the article published.

Conflict of interest

The authors declare no conflict of interest to this work.

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