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Metabolic engineering of monoterpene biosynthesis in tomato fruits via introduction of the non-canonical substrate neryl diphosphate

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ABSTRACT

Recently it was shown that monoterpenes in tomato trichomes (*Solanum lycopersicum*) are synthesized by phellandrene synthase 1 (PHS1) from the non-canonical substrate neryl diphosphate (NPP), the *cis*-isomer of geranyl diphosphate (GPP). As PHS1 accepts both NPP and GPP substrates forming different monoterpenes, it was overexpressed in tomato fruits to test if NPP is also available in a tissue highly active in carotenoid production. However, transgenic fruits overexpressing *PHS1* produced only small amounts of GPP-derived PHS1 monoterpene products, indicating the absence of endogenous NPP. Therefore, NPP formation was achieved by diverting the metabolic flux from carotenoids via expression of tomato *neryl diphosphate synthase 1* (*NDPS1*). *NDPS1* transgenic fruits produced NPP-derived monoterpenes, including nerol, neral and geranial, while displaying reduced lycopene content. *NDPS1* co-expression with *PHS1* resulted in a monoterpene blend, including β-phellandrene, similar to that produced from NPP by PHS1 *in vitro* and in trichomes. Unexpectedly, *PHS1* × *NDPS1* fruits showed recovery of lycopene levels compared to *NDPS1* fruits, suggesting that redirection of metabolic flux is only partially responsible for the reduction in carotenoids. *in vitro* assays demonstrated that NPP serves as an inhibitor of geranylgeranyl diphosphate synthase, thus its consumption by PHS1 leads to recovery of lycopene levels. Monoterpenes produced in *PHS1* × *NDPS1* fruits contributed to direct plant defense negatively affecting feeding behavior of the herbivore *Helicoverpa zea* and displaying antifungal activity against *Botrytis cinerea*. These results show that NPP-derived terpenoids can be produced in plant tissues; however, NPP has to be consumed to avoid negative impacts on plant metabolism.

1. Introduction

Terpenoids, compounds found in all branches of life, represent one of the largest and diverse classes of metabolites within the plant kingdom and are involved in various physiological and ecological processes. Such compounds including carotenoids, chlorophylls, sterols, and the hormones gibberellins, abscisic acid, and brassinosteroids are essential for basic processes like photosynthesis, growth, and development. In addition, mono- (C10), sesqui- (C15) and di- (C20) terpenes (referred to as "terpenes") play important roles in mediating interactions between plants and their biotic environment. Due to their volatile nature, many of these terpenes contribute to reproduction by attracting pollinators and seed dispersers, as well as to direct and indirect plant defense against herbivores and pathogens (Gershenzon and Dudareva, 2007; Pichersky and Gershenzon, 2002; Unsicker et al., 2009). The biosynthesis of terpenes in plants is often restricted to specific tissues or cell types like flower organs (Aros et al., 2012; Dudareva et al., 1996, 2003; Lee and Chappell, 2008; Nieuwenhuizen et al., 2009) or glandular trichomes, the hair-like protuberances found at the surface of leaves, stems and fruits (Iijima et al., 2004; Schilmiller et al., 2008).

All terpenoids originate from the C5 building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which in plants are synthesized by two alternative pathways (Ashour et al., 2010; Hemmerlin et al., 2012 and references within). The mevalonic acid (MVA) pathway is localized in the cytosol and partially in peroxisomes, while the methylerythritol-phosphate (MEP) pathway operates in plastids. IPP and DMAPP are subsequently utilized by prenyltransferases to form larger prenyl diphosphate intermediates that ultimately serve as substrates for terpene synthases (TPSs) generating mono-, sesqui- and diterpenes. Upon the head-to-tail

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condensation of one molecule DMAPP with one or several IPP molecules, the isoprene units can be joined in the *trans* (*E*) or *cis* (*Z*) configuration leading to the formation of different prenyl diphosphate isomers. It has long been known that the isoprene units of some long-chain plant terpenoids, like rubber and dolichols, are linked in the *cis* (*Z*) conformation (Asawatreratanakul et al., 2003; Cunillera et al., 2000) as they are synthesized from *Z*-prenyl diphosphates formed by *cis*-prenyltransferases (Akhtar et al., 2013; Asawatreratanakul et al., 2003; Oh et al., 2000). In contrast, it has generally been observed that geranyl diphosphate (GPP), in which one DMAPP and one IPP molecule are joined in the *trans* (*E*) configuration, is the substrate for monoterpene biosynthesis by TPSs. Although GPP undergoes isomerization to a neryl intermediate during biosynthesis of cyclic monoterpenes, neryl diphosphate (NPP), the *cis*-isomer of GPP, has not been found to be a substrate for many monoterpene synthases (Croteau, 1987). Likewise all-*trans*-farnesyl diphosphate (*E*,*E*-FPP) was considered for a long time as the exclusive precursor for sesquiterpene formation by sesquiterpene synthases (Sallaud et al., 2009).

Recently a *cis*-prenyltransferase has been isolated from trichomes of the wild tomato *Solanum habrochaites* that synthesizes *cis*-FPP (*Z*,*Z*-FPP) from IPP and DMAPP along with two TPSs, santalene/bergamotene synthase (ShSBS) and 7-epizingiberene synthase (ShZIS) that utilize this *cisoid* substrate for sesquiterpene formation (Gonzales-Vigil et al., 2012; Sallaud et al., 2009). At the same time, another *cis*-prenyltransferase, neryl diphosphate synthase 1 (NDPS1), catalyzing the formation of NPP from IPP and DMAPP in plastids (Akhtar et al., 2013), was isolated from glandular trichomes of cultivated tomato (*Solanum lycopersicum*) (Schilmiller et al., 2009). In parallel, the TPS phellandrene synthase 1 (PHS1) was identified that uses NPP as substrate to produce a mixture of monoterpenes, identical to that found in tomato glandular trichomes with β-phellandrene being the main product (Schilmiller et al., 2009). Remarkably, when incubated *in vitro* with GPP, PHS1 catalyzed the formation of two acyclic monoterpenes, myrcene and ocimene, although its K_m for GPP was >300-fold higher than for NPP (Schilmiller et al., 2009). In the meanwhile, several additional TPSs that use NPP as substrate for the synthesis of various monoterpenes have been identified in *S. lycopersicum* (SlTPS19) (Matsuba et al., 2013), *S. habrochaites* (ShLMS and ShPIS) (Gonzales-Vigil et al., 2012) and *Solanum pennellii* (SpeTPS19) (Falara et al., 2011; Matsuba et al., 2013), all of which are expressed predominantly in trichomes.

These recent findings raise the question whether NPP is available and can serve as substrate for monoterpene formation by TPSs in tissues other than trichomes. The presence of NPP or its formation by an enzyme responsible for the synthesis of this non-canonical *cis*-prenyl diphosphate, could provide the opportunity to engineer new terpenes in plant tissues when TPSs able to utilize such substrate are also expressed. To address this issue, a metabolic engineering approach was taken to express *S. lycopersicum PHS1* in tomato fruit pericarp to test the availability of the GPP and NPP substrates. In addition, *PHS1* was co-expressed with the small subunit of heterodimeric snapdragon GPP synthase (*GPPS-SSU*) as well as tomato *NDPS1* to elucidate the effect of increased pools of each of the two prenyl diphosphate substrates on monoterpene formation. Tomato fruit is a good system to test this approach, since the plastidic MEP pathway is highly active in ripening tomato fruits, providing precursors for the accumulation of lycopene and other carotenoids (Botella-Pavia et al., 2004; Lawrence et al., 1997; Lois et al., 2000; Paetzold et al., 2010). All transgenes were introduced into tomato plants under control of the polygalacturonase (*PG*) promoter to limit their expression to ripening fruits and thus avoid potential negative effects on vegetative tissues and plant development (Orlova et al., 2009).

Here we show that in tomato fruit pericarp only an endogenous GPP, but no NPP, pool is available, and was utilized by PHS1 for myrcene and ocimene formation. Overexpression of *NDPS1* in tomato fruits led to the formation of a large pool of NPP, which PHS1 was able to utilize for the formation of β-phellandrene and several other monoterpenes. However, overexpression of *NDPS1* alone resulted in a drastic reduction in the carotenoid level due to NPP being a potent inhibitor of geranylgeranyl diphosphate synthase (GGPPS), a key enzyme in the carotenoid biosynthetic pathway.

2. Materials and methods

2.1. Growth conditions and generation of PHS1 and NDPS1 transgenic plants

Tomato plants (*S. lycopersicum*, line MP1) (Barg et al., 1997) were grown under a 14-h photoperiod in standard greenhouse conditions. For plant transformation the coding regions of tomato *PHS1* and *NDPS1* (Schilmiller et al., 2009) were cloned into the binary vector pBIN19 carrying the tomato *PG* promoter and terminator, and the kanamycin-resistance marker *NPTII* driven by the *CaMV 35S* promoter (Nicholass et al., 1995). Transgenic tomato plants were generated via *Agrobacterium tumefaciens* (strain c58C1/pMP90) (Koncz and Schell, 1986) mediated transformation as described (McCormick et al., 1986). Transgenic plants rooted on kanamycin selection (100 mg L^{-1}) were transferred to soil, adapted to greenhouse conditions and subsequently propagated by re-rooting of cuttings.

2.2. RNA isolation and qRT-PCR analysis

Total RNA was isolated from tomato fruits as described (Eggermont et al., 1996). For qRT-PCR analysis, RNA was pretreated with RNase-free DNase (Promega) and cDNA was synthesized using Reverse Transcriptase (Superscript II, Invitrogene). Gene specific primers were designed using the PrimerExpress software (Applied Biosystems) and showed more than 90% efficiency at final concentrations of 300 or 500 nM (Table S4). qRT-PCR reactions were performed and transcript levels were determined as previously described (Gutensohn et al., 2013; Orlova et al., 2009) using the StepOne Real-Time PCR system (Applied Biosystems). For the absolute quantification of *PHS1*, *NDPS1* and *GPPS-SSU* transcript levels by qRT-PCR, respective cDNA fragments were purified, diluted from 2 ng/ml to 3.2 pg/ml, and used as templates to obtain standard curves in qRT-PCR with gene-specific primers (Table S4). Based on standard curves, absolute quantities of individual transcripts were calculated and expressed as a percentage of total mRNA. Alternatively, transcript levels of *PHS1*, *NDPS1* and *GPPS-SSU*, as well as transcript levels of tomato genes *Sl-DXS* (AF143812), *Sl-DXR* (AF331705), *Sl-GGPPS-2* (DQ267903), *Sl-PSY-1* (EF534739), *Sl-PSY-2* (EF534738), *Sl-PDS* (NM_001247166) and of *Botrytis cinerea ActinA* were analyzed by qRT-PCR using gene specific primers (Table S4), normalized to the tomato reference gene *Actin-Tom52* and expressed as relative expression. Each data point represents an average of at least three independent biological samples with three technical replicates for each sample.

2.3. Measurement of carotenoid levels in tomato fruits

The lycopene contents of ripe (Br+10 stage) MP1, *NDPS1*, and *PHS1* × *NDPS1* fruits were determined as described (Nagata and Yamashita, 1992). Fruit tissue was homogenized in liquid N_2, carotenoids were extracted with an acetone–hexane (4:6) mixture and absorbance of the organic phase at 663, 645, 505 and 453 nm measured in a spectrophotometer. The lycopene contents were calculated using the equation: lycopene (mg/100 ml extract) = –

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0.0458 A_{663}+0.204 A_{645}+0.372 A_{505}−0.0806 A_{453} and then converted to nmol per gram fresh weight.

2.4. Collection, extraction and analysis of monoterpenes from tomato fruits

Volatiles emitted from ripe tomato fruits (Br+10) were collected for 24 h using a closed-loop stripping method as described previously (Donath and Boland, 1995; Dudareva et al., 2005; Gutensohn et al., 2013). For analysis of internal pools of volatiles pericarp from ripe tomato fruits (Br+10) was extracted with methyl *tert*-butyl ether (MTBE) as described previously (Davidovich-Rikanati et al., 2007; Gutensohn et al., 2013). Emitted and MTBE extracted volatiles were analyzed by GC–MS as described (Dudareva et al., 2005) using naphthalene as internal standard. Representative monoterpene standards were used to determine an average response factor, which was applied for the quantification of analyzed compounds. For analysis of NPP hydrolysis, 250 µM NPP in prenyltransferase assay buffer (Orlova et al., 2009), overlaid with hexane, was hydrolysed by addition of HCl and incubated for 30 min at 30 °C. Hydrolysis products were extracted into the hexane phase and analyzed by GC–MS.

2.5. GGPPS enzyme assays

Crude protein extracts were prepared from MP1 tomato fruits (Br+3 stage) in extraction buffer as described (Nagel et al., 2012) followed by desalting on 2-mL Zeba Spin columns (7K MWCO, Thermo Scientific, Rockford, IL, USA). GGPPS prenyltransferase activity was detected as described (Orlova et al., 2009) in the presence of 20 mM of the IPP isomerase inhibitor, iodoacetamide (Sigma-Aldrich), using [1-^{14}C]–IPP (55 mCi/mmol) and DMAPP. For inhibition experiments different amounts of NPP (0–750 µM final concentration in the reaction) were added to the assays. Each reaction containing 20 µg of protein was overlaid with 1 mL of hexane and incubated for 30 min at 30 °C. Assays were stopped by adding 3N HCl and additionally incubated for 20 min at 30 °C. Hydrolysis products were extracted into the hexane phase and aliquots were analyzed by thin layer chromatography (TLC) as described (Gutensohn et al., 2013). Formation of GGPP was analyzed and quantified using a phosphor imager (Molecular Dynamics Typhoon).

2.6. Bioassays with Helicoverpa zea larvae and fungal pathogen B. cinerea

Herbivory experiments were performed with larvae of the tomato fruitworm *H. zea*. Eggs (Benzon Research Inc., Carlisle, PA, USA) were hatched and larvae reared on the artificial diet provided. No-choice feeding bioassays were performed by placing 1 week old *H. zea* larvae on ripe MP1 and *PHS1* × *NDPS1* B10 tomato fruits. Larval body mass was determined before and after 5 days of feeding on fruits. The culture of *B. cinerea* (strain *BO5-10*) and preparation of conidial spores were described previously (Mengiste et al., 2003) and spore suspension for disease assays was provided by T. Mengiste (Department of Botany/Plant Pathology, Purdue University). Ripe MP1 and *PHS1NDPS1* B10 tomato fruits were harvested, washed and punctured (3 mm depth, 1 mm wide) at 6 sites. 5 sites were inoculated with 10 µL of spore suspension, while the sixth site received 10 µL of sterile water. All fruits were incubated at room temperature and in high humidity. For quantification of fungal growth 4 days after inoculation, infection sites were excised from fruits, RNA extracted and *B. cinerea ActinA* transcript levels determined by qRT-PCR.

3. Results

3.1. Is there a natural pool of NPP in tomato fruit pericarp?

To evaluate whether an internal NPP pool exists in tissues other than trichomes, we overexpressed *PHS1* in tomato fruit pericarp under control of the ripening specific *PG* promoter (Fig. S1A) (Nicholass et al., 1995) and searched for β-phellandrene production. Three independent lines, E3, G5 and E1, with various *PHS1* expression levels, determined by quantitative real-time PCR (qRT-PCR) (Fig. 1A), were chosen for analysis. To examine the effect of *PHS1* expression on monoterpene formation, volatiles were collected from ripe tomato fruits (Br+10) and analyzed by gas chromatography–mass spectrometry (GC–MS). The only difference in the profile of emitted monoterpenes between transgenics and control was the formation of myrcene and ocimene in *PHS1* expressing fruits, which were absent in MP1 controls (Fig. 1B and C, Table S1). Similarly, quantitative analysis of internal monoterpene pools, analyzed upon extraction from fruits, revealed accumulation of myrcene and ocimene in ripe *PHS1* transgenic fruits but not in controls (Table S1). The amounts of both emitted and extracted acyclic monoterpenes displayed a positive correlation with the *PHS1* expression levels in different transgenic lines (Fig. 1A and C, Table S1). These results are consistent with previous *in vitro* analysis of recombinant PHS1 protein, which showed that PHS1 is capable of using GPP as substrate albeit with low catalytic efficiency, producing myrcene and ocimene as the major products (Schilmiller et al., 2009).

To test if an increased GPP pool can positively affect monoterpene formation by PHS1 in tomato fruits, we have crossed line E3 showing the highest *PHS1* expression level (Fig. 1A) with a tomato line expressing the snapdragon small subunit of heterodimeric GPP synthase (*GPPS-SSU*) under control of the *PG* promoter, shown to enlarge the GPP pool available for monoterpene formation in fruits (Gutensohn et al., 2013). In the resulting F1 (*PHS1* × *GPPS-SSU*) plants the *PHS1* and *GPPS-SSU* transcript levels in fruits were comparable with those of parental lines (Fig. S1B). Quantitative analysis of volatiles produced by ripe *PHS1* × *GPPS-SSU* fruits (Fig. 1C, Table S1) indeed revealed a 2.7- and 2.3-fold increase in emitted myrcene and ocimene, respectively, in addition to a 3.8- and 8.0-fold increase in their respective internal pools, compared to the *PHS1* parental line. Besides these PHS1-derived monoterpenes, *PHS1* × *GPPS-SSU* fruits produced the same set of monoterpenes, including citronellal, citronellol, neral, geraniol and geranial (Table S1), which were previously observed in *GPPS-SSU* fruits (Gutensohn et al., 2013). All of those monoterpenes were absent in *PHS1* tomatoes, except a small amount of geranial that was also present in MP1 controls (Fig. 1C, Table S1), indicative of the low level of GPP present in these fruits.

3.2. Formation of NPP in tomato fruits reduces lycopene levels

Since the above results indicate that the preferred NPP substrate of PHS1 is not present in tomato fruit pericarp and *NDPS1*, in contrast to glandular trichomes, is naturally not expressed in fruits (Fig. S2A), *NDPS1* was overexpressed under control of the *PG* promoter (Fig. S2B) (Nicholass et al., 1995) to divert the metabolic flux from carotenoid formation, which is highly active in ripening tomato fruits, to NPP. Five independent lines with different *NDPS1* expression levels, as determined by qRT-PCR (Fig. 2A), were selected for further characterization. *NDPS1* overexpression led to reduced carotenoid accumulation (Fig. S3A), with levels of lycopene, the most abundant carotenoid in tomato fruits, being reduced by 13.9–88.7% in transgenic relative to control fruits (Fig. 2B). In addition, *NDPS1* transgenic fruits produced nerol (Fig. S4), the product of NPP dephosphorylation (Fig. S5; Schilmiller

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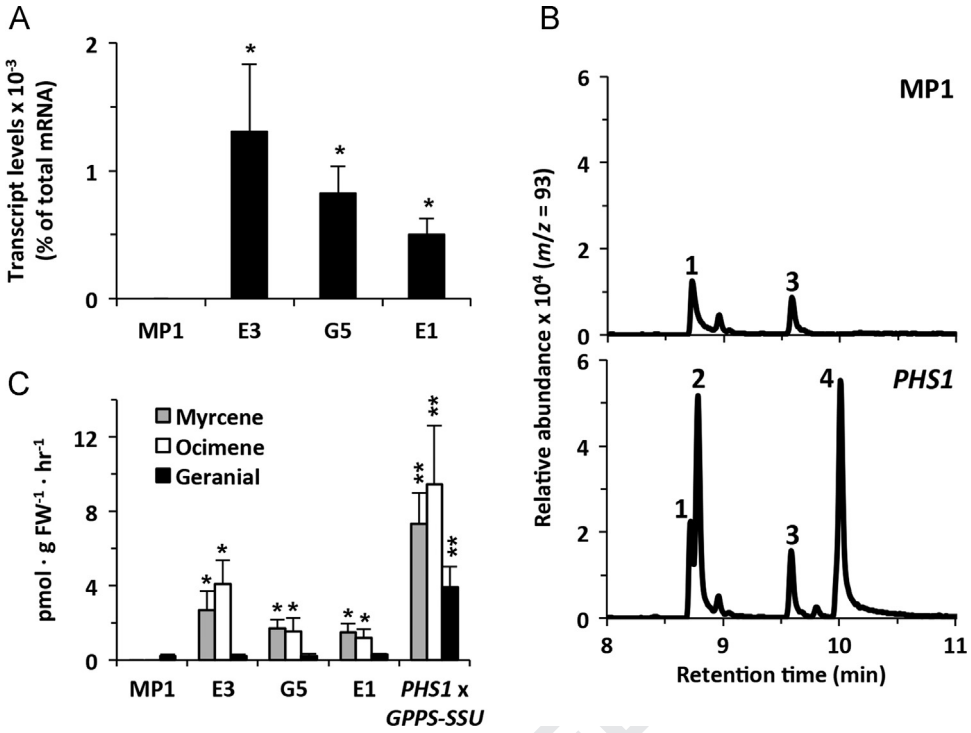

Fig. 1. Profiling of transgenic tomato lines overexpressing *PHS1* under control of the *PG* promoter. A, Transcript levels of *PHS1* in Br+3 stage fruits of MP1 (control) and *PHS1* lines E3, G5 and E1 determined by qRT-PCR (means ± SEM, $n=3$ biological replicates). B, Profiling of monoterpenes collected as emitted volatiles from ripe tomato fruits (MP1 control and *PHS1* line E3) and analyzed by GC–MS. Numbered peaks are: 1, 6-methyl-5-heptene-2-one; 2, myrcene; 3, limonene; 4, ocimene. C, Quantitative changes in monoterpenes collected as emitted volatiles from fruits of MP1, *PHS1* and *PHS1* × *GPPS-SSU* lines. Data are means ± SEM ($n \geq 3$). $^{*}P < 0.01$ *PHS1* lines relative to MP1 and $^{**}P < 0.03$ *PHS1* × *GPPS-SSU* line relative to *PHS1* line E3 by Student's *t*-test. FW, fresh weight.

et al., 2009), which accumulated up to 20 nmol g FW^{-1} and was absent in control fruits (Table S2). Transgenic fruits also formed minor amounts of neral, geranial and geraniol as well as linalool, α-terpineol, terpinen-4-ol and limonene, while all of these volatiles were absent in control fruits except for small amounts of geranial and limonene. Remarkably, *Z,Z*-farnesol, the dephosphorylated form of the *cis,cis* FPP isomer (*Z,Z*-FPP), was found in the internal pools of *NDPS1* fruits as well, but absent in controls (Table S2). The amounts of monoterpenes, in particular nerol, as well as *Z,Z*-farnesol, collected as emitted volatiles and/or extracted from fruits with exception of geranial and geraniol, positively correlated with the *NDPS1* expression level in different transgenic lines (Fig. 2A and C, Table S2).

3.3. NPP produced in tomato fruits inhibits geranylgeranyl diphosphate synthase

The reduction in carotenoids in *NDPS1* expressing lines could be due to a redirection of metabolic flux from carotenoids toward NPP formation in combination with either transcriptional regulation of genes involved in carotenoid biosynthesis and/or post-translational regulation of carotenoid biosynthetic enzymes by one (or several) products formed in transgenic fruits. Analysis of expression of the MEP pathway genes, *1-deoxy-D-xylulose 5-phosphate synthase* (*DXS*) and *1-deoxy-D-xylulose 5-phosphate reductoisomerase* (*DXR*), as well as the carotenoid biosynthetic genes, *phytoene synthase* (*PSY-1*, *PSY-2*) and *phytoene desaturase* (*PDS*), revealed no changes in their transcript levels in *NDPS1* transgenic fruits relative to controls (Fig. 3A). In contrast, the transcript level of *geranylgeranyl diphosphate synthase 2* (*GGPPS-2*), the only *GGPPS* gene expressed in tomato fruits (Ament et al., 2006), was increased almost 2.5-fold in *NDPS1* fruits (Fig. 3A). Since geranylgeranyl diphosphate synthase (GGPPS) catalyzes the key branch-point towards carotenoid biosynthesis, the increase in its transcript level with simultaneous reduction in lycopene levels suggested the involvement of posttranscriptional regulatory mechanisms. Thus, GGPPS activity was analyzed *in vitro* using crude extracts from control tomato fruits in the presence of increasing concentrations of NPP, the prenyl diphosphate produced in *NDPS1* transgenic lines. Indeed, GGPPS activity was inhibited by NPP with an I_{50} value of ~25 μM (Fig. 3B), while no inhibition was observed with nerol. Further analysis revealed that inhibition of GGPPS activity by NPP was competitive with respect to DMAPP (Fig. 3C) and non-competitive with respect to IPP (Fig. 3D). These results indicate that inhibition of GGPPS by NPP likely contributes to lycopene reduction in transgenic *NDPS1* fruits in addition to flux redirection.

To test this hypothesis, we examined whether the lycopene level could be at least partially recovered in the *NDPS1* expressing tomato fruits when the endogenous NPP pool is reduced via its consumption by the monoterpene synthase PHS1. Moreover, less recovery would be expected in fruits with higher *NDPS1* expression, and subsequently higher NPP levels, *versus* fruits with lower *NDPS1* expression when similar amounts of NPP are consumed by PHS1. To achieve co-expression of *NDPS1* and *PHS1*, two *NPDS1* lines with the lowest and highest *NDPS1* expression levels (Fig. 2A), B1 and B10, respectively, were crossed with *PHS1* expressing line E3. The *PHS* transcript levels in both F1 plants (*PHS1* × *NDPS1* B1 and *PHS1* × *NDPS1* B10) were similar with that of the *PHS1* parental line (Fig. 4A), however, the *NDPS1* transcript levels were comparable only between the *NDPS1* B1 parental line and the *PHS1* × *NDPS1* B1 cross, but were reduced by ~50% in the *PHS1* × *NDPS1* B10 cross compared to *NDPS1* line B10 (Fig. 4A). In both crosses, the *PHS1* expression in the *NDPS1* background rescued the lycopene level. While in the cross with B1, the line with the lowest *NDPS1* expression, lycopene was recovered to wild type level, that in the B10 cross was only partially recovered (Figs. 4B and S3B). It is possible that 50% reduction in the *NDPS1*

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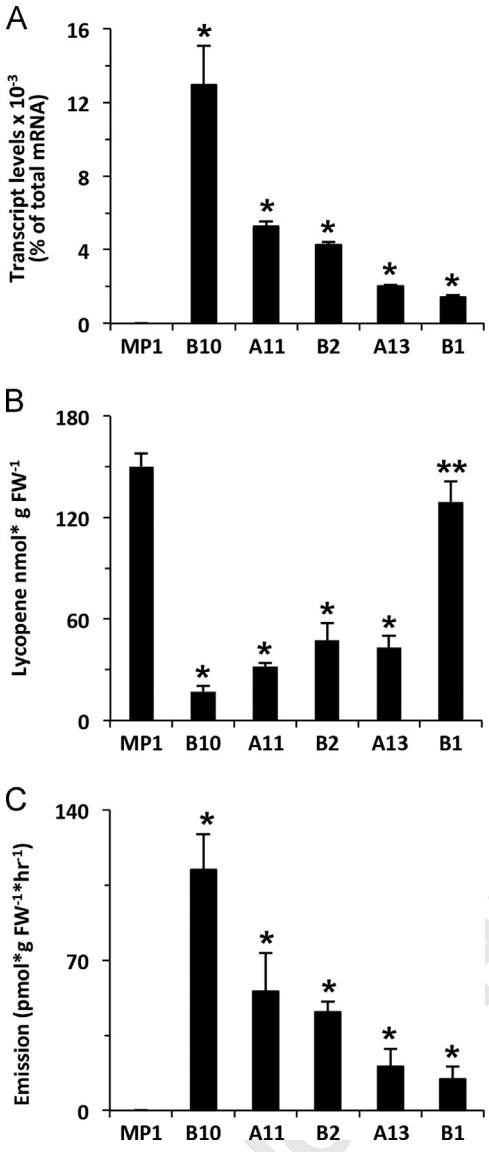

Fig. 2. Profiling of transgenic tomato lines overexpressing *NDPS1* under control of the *PG* promoter. A, Transcript levels of *NDPS1* in Br+3 stage fruits of MP1 (control) and *NDPS1* lines B10, A11, B2, A13 and B1 determined by qRT-PCR. B, Lycopene levels (in nmol per g fresh weight) in Br+10 fruits of MP1 and *NDPS1* transgenic tomato lines. C, Quantitative changes in amounts of nerol collected as emitted volatile from fruits of MP1 and *NDPS1* lines. All data are means ± SEM ($n \geq 3$). $^{*}P < 0.01$ and $^{**}P < 0.04$ *NDPS1* lines relative to MP1 by Student's *t*-test. FW, fresh weight.

transcript level in the *PHS1* × *NDPS1* B10 cross relative to the parental *NDPS1* B10 line (Fig. 4A) could by itself lead to a higher lycopene level in the cross. However, *NDPS1* transcript level in the *PHS1* × *NDPS1* B10 cross was very similar to that in the *NDPS1* A11 line (50% and 38%, respectively, both relative to *NDPS1* expression in B10 line; Figs. 2A and 4A). In contrast, the lycopene level in the *NDPS1* A11 line was more than 2-fold lower than that in the *PHS1* × *NDPS1* B10 line (31.92 ± 2.07 nmol g FW^{-1} and 76.29 ± 12.61 nmol g FW^{-1}, respectively; Figs. 2B and 4B), suggesting that lycopene recovery in the *PHS1* × *NDPS1* B10 cross was mainly due to consumption of NPP by PHS1. This statement was further supported by the fact that the *PHS1* × *NDPS1* B10 and *NDPS1* A11 lines with similar *NDPS1* transcript levels contained considerably different internal nerol pools with *NDPS1* A11 line having more than 13-fold excess (15,653 *versus* 1151 pmol g FW^{-1}, respectively). Transcriptional upregulation of carotenoid biosynthetic genes did not contribute to the recovery in lycopene levels in *PHS1* × *NDPS1* lines, since no changes in *DXS*, *DXR*, *PSY-1*, *PSY-2* and *PDS* expression levels were observed relative to *NDPS1* lines (Fig. 3A). However, *GGPPS-2* transcript levels, which were significantly increased in the *NDPS1* parental line (Fig. 3A), reverted back to the MP1 control levels in *PHS1* × *NDPS1* fruits (Fig. 3A).

The metabolic profiling of emitted and extracted metabolites further confirmed that the produced NPP was consumed by PHS1, as the level of nerol, the product of NPP dephosphorylation, was drastically reduced by 89–97% in the *PHS1* × *NDPS1* fruits relative to parental *NDPS1* lines (Table S3). Moreover, *PHS1* × *NDPS1* fruits produced a blend of monoterpenes including ß-phellandrene and 2-carene, as major compounds, with smaller amounts of pinene, α-phellandrene, α-terpinene, limonene and 2-carene-10-al (Fig. 4C), all of which, with exception of limonene, were absent in the *NDPS1* and *PHS1* parental lines (Table S3). Consistent with the different levels of *NDPS1* expression, the total amounts of emitted monoterpenes were 2.9-fold higher in *PHS1* × *NDPS1* B10 than in *PHS1* × *NDPS1* B1 fruits (Fig. 4D, Table S3).

3.4. Monoterpenes produced in *PHS1* × *NDPS1* fruits contribute to direct plant defense

To examine whether monoterpenes naturally produced in the glandular trichomes of tomato leaves and stems can also protect fruits against herbivores, no-choice feeding assays with larvae of the moth *H. zea*, a major agricultural pest also known as tomato fruitworm, corn earworm and cotton bollworm were performed. After 5 days of feeding on ripe *PHS1* × *NDPS1* B10 tomato fruits *H. zea* larvae showed a 45% reduced gain in body mass as compared to larvae feeding on ripe MP1 fruits (Fig. 5A), thus indicating that monoterpenes produced by PHS1 from NPP negatively influence feeding behavior and/or growth of herbivores like *H. zea*. To analyze whether these monoterpenes also have an inhibitory activity against growth of fungal pathogens, the susceptibility of *PHS1* × *NDPS1* tomato fruits to the necrotrophic fungus *B. cinerea*, the causal agent of grey mold disease responsible for considerable damage in commercial tomato production, was tested. When *PHS1* × *NDPS1* B10 and MP1 tomato fruits were inoculated with *B. cinerea* spores, significantly less fungal growth was observed after 4 days with transgenic fruits (Fig. S4C). Hyphal growth, as quantified by determining *B. cinerea ActinA* transcript level, was reduced by almost 55% in *PHS1* × *NDPS1* B10 fruits compared to MP1 controls (Fig. 5B), indeed confirming antifungal activity of the monoterpenes produced in these metabolically engineered fruits.

4. Discussion

4.1. Tomato fruit pericarp is naturally devoid of NPP

It is well known that some long-chain terpenoids found in plants, like rubber or dolichols, are synthesized from *cis* prenyl diphosphates, formed by respective *cis*-prenyltransferases linking IPP and DMAPP in the *cis* conformation (Akhtar et al., 2013; Asawatreratanakul et al., 2003; Cunillera et al., 2000; Oh et al., 2000). However, the recent identification of the *cis*-prenyltransferase NDPS1 and the terpene synthase PHS1, using the NDPS1 *cis* prenyl diphosphate product, in trichomes of cultivated tomato (Schilmiller et al., 2009) revealed that the short-chain *cis* prenyl diphosphate NPP can serve as substrate for the biosynthesis of monoterpenes like β-phellandrene. In the meanwhile several other monoterpene synthases, capable of using NPP as substrate, have been identified (Falara et al., 2011; Gonzales-Vigil et al., 2012; Matsuba et al., 2013; Zhang et al., 2013), which all, except of one, are highly expressed in leaf and stem trichomes. Moreover, the existence of small NPP pools in tobacco leaves (Zhang et al., 2013)

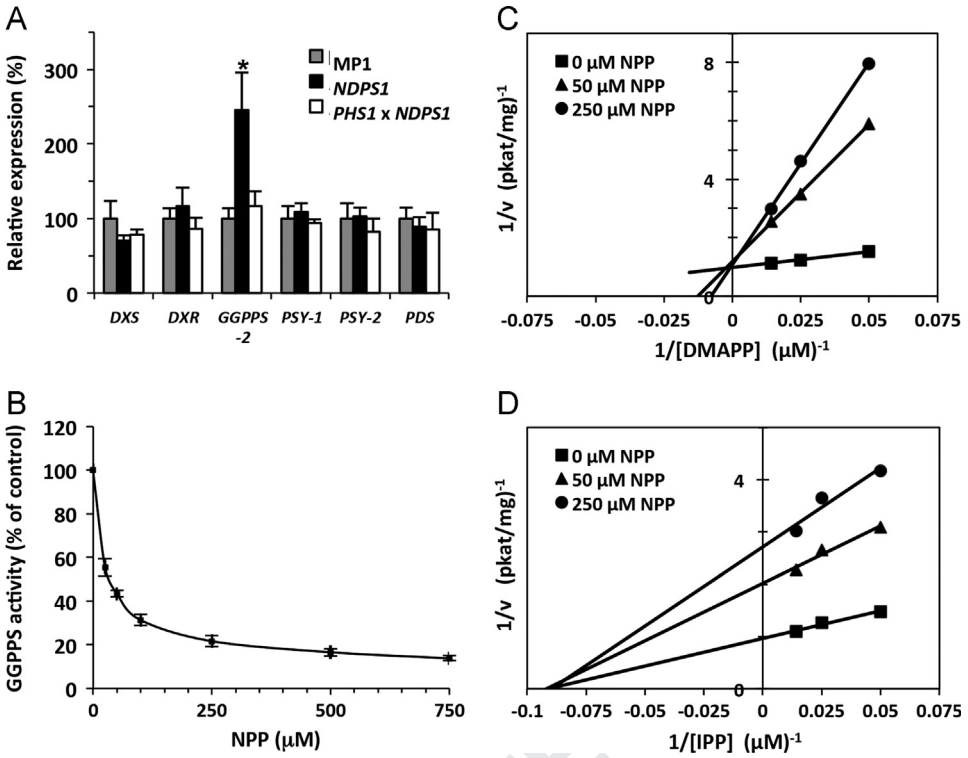

Fig. 3. Effect of NPP formation on the expression of MEP pathway and carotenoid biosynthetic genes, and on GGPPS activity in tomato fruits. A, Transcript levels of the MEP pathway genes *1-deoxy-D-xylulose 5-phosphate synthase* (*DXS*) and *1-deoxy-D-xylulose 5-phosphate reductoisomerase* (*DXR*), the fruit specific geranylgeranyl diphosphate synthase (*GGPPS-2*), and the carotenoid biosynthetic genes *phytoene synthase* (*PSY-1*, *PSY-2*) and *phytoene desaturase* (*PDS*) determined by qRT-PCR in Br+3 stage fruits of MP1 (control), *NDPS1* line B10, and the *PHS1* × *NDPS1* B10 cross. B, GGPPS prenyltransferase activity in crude extracts from MP1 control tomato fruits (measured with IPP and DMAPP substrate) in the presence of increasing concentrations of NPP. All data are means ± SEM ($n=3$). $^*P < 0.04$ *NDPS1* relative to MP1 by Student's *t*-test. C and D, Kinetic analysis of the inhibition of GGPPS activity by NPP. Shown are double-reciprocal plots of initial velocities in the presence of different NPP concentrations, with varied DMAPP and fixed IPP concentrations (C), or varied IPP and fixed DMAPP concentrations (D).

and non-trichome cells of tomato leaves (Akhtar et al., 2013) was recently demonstrated using a genetic approach. These findings not only challenged the long standing assumption that the *trans* prenyl diphosphate GPP represents the exclusive substrate for monoterpene synthases, but in addition raised the question whether NPP is available ubiquitously in many plant tissues or only in leaves and leaf/stem trichomes.

Here, we overexpressed *PHS1* in transgenic tomato plants under control of the *PG* promoter restricting its expression to the pericarp of ripening fruits to investigate the existence of internal NPP pools in tissues other than trichomes (Nicholass et al., 1995). In general, ripening tomato fruits produce very small amounts of monoterpenes due to the expression of a limited number of terpene synthases (Bleeker et al., 2011; Falara et al., 2011), despite the fact that the MEP pathway is highly active to support the accumulation of carotenoids, in particular lycopene (Botella-Pavia et al., 2004; Lawrence et al., 1997; Lois et al., 2000; Paetzold et al., 2010). *PHS1* overexpression in tomato fruits led to production of two new compounds, ocimene and myrcene (Fig. 1B), which are the major products formed by PHS1 from GPP *in vitro* (Schilmiller et al., 2009), indicating that PHS1 uses a small endogenous GPP pool available in tomato fruits (Fig. S6). Even higher ocimene and myrcene levels were achieved (Fig. 1C) by increasing the plastidic GPP pool via co-expression of snapdragon *GPPS-SSU* and *PHS1* in transgenic tomato fruits (Figs. S1B and S6). Our data are consistent with previous results showing the existence of a small GPP pool in ripening tomato fruits when the GPP-dependent basil monoterpene synthase, geraniol synthase (GES) was overexpressed (Davidovich-Rikanati et al., 2007). Moreover, when *GES* was co-expressed with *GPPS-SSU* the resulting enlarged GPP pool led to a several-fold increase in the formation of GES derived monoterpenes (Gutensohn et al., 2013). The lack of any β-phellandrene formation in the *PHS1* transgenic fruits (Table S3) at the same time provides evidence for the absence of a natural NPP pool in tomato fruit pericarp, in particular when considering the significantly higher affinity of PHS1 for NPP than for GPP (Schilmiller et al., 2009).

4.2. NPP-dependent monoterpene formation can be achieved in transgenic tomato fruits

In the absence of an endogenous NPP pool, we redirected the metabolic flux of MEP pathway-derived IPP and DMAPP towards NPP formation by overexpressing *NDPS1* in tomato fruits (Figs. 2A, S2B and S6). *NDPS1* overexpression resulted in the production of several monoterpenes, in particular nerol, both as emitted volatiles and internal pools (Figs. 2 and S4, Table S2), the levels of which positively correlated with *NDPS1* transcript levels (Fig. 2A), thus providing evidence for NPP formation *in planta*. The observed monoterpenes in *NDPS1* fruits could not be completely attributed to the action of endogenous monoterpene synthases since only few of the 44 TPSs recently identified in the tomato genome are expressed in fruits (Bleeker et al., 2011; Falara et al., 2011). Among those, only one, TPS5/MTS1, represents a monoterpene synthase and catalyzes the formation of linalool detected in *NDPS1* fruits, however it is formed from GPP substrate (van Schie et al., 2007). The produced nerol is likely the result of enzymatic hydrolysis of NPP by endogenous phosphatases (Fig. S6), which were previously shown to be active in tomato fruits (Davidovich-Rikanati et al., 2007). Nerol seems to be further oxidized by an alcohol dehydrogenase(s) (Fig. S6), present in several plant species (Bicsak et al., 1982; Sekiwa-Iijima et al., 2001; Singh Sangwan et al., 1993), to the

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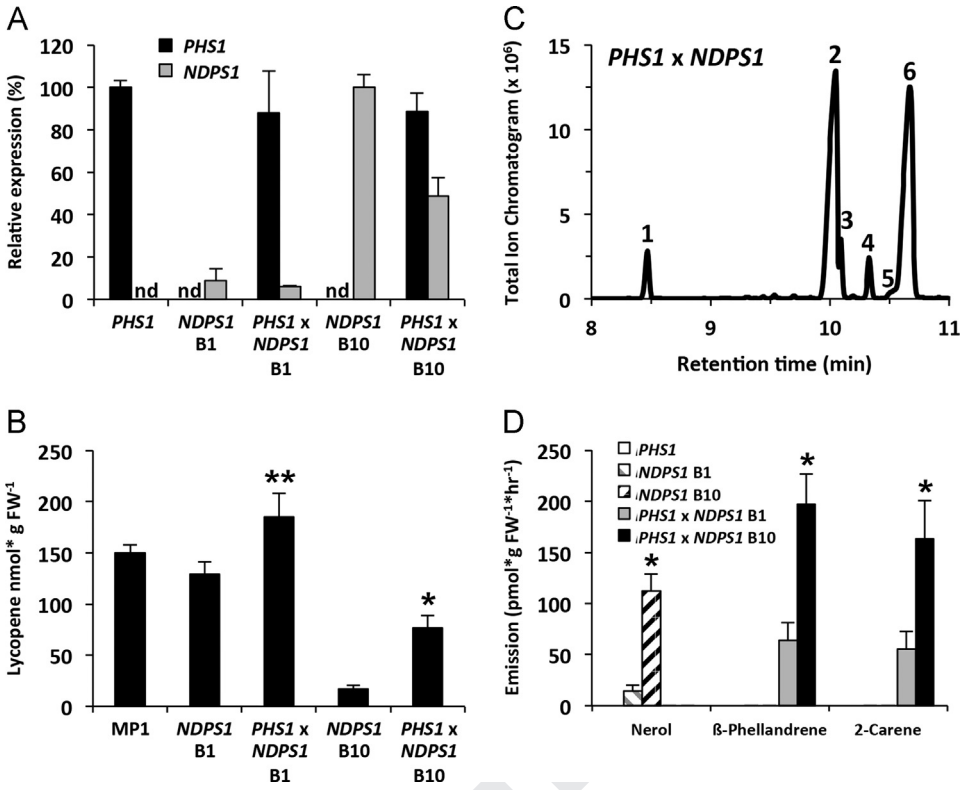

Fig. 4. Characterization of tomato lines co-expressing *PHS1* and *NDPS1* under control of the *PG* promoter. A, *PHS1* and *NDPS1* transcript levels in Br+3 stage fruits of *PHS1* E3, *NDPS1* B1, *NDPS1* B10, *PHS1* × *NDPS1* B1 and *PHS1* × *NDPS1* B10 lines determined by qRT-PCR (means ± SEM, $n=3$). Expression in *PHS1* × *NDPS1* lines is presented as percentage of the corresponding value in the parental lines *PHS1* E3 and *NDPS1* B10. B, Carotenoids were extracted from Br+10 fruits and lycopene levels were quantified. Values are means ± SEM ($n=3$) in nmol per g fresh weight. $^{*}P<0.01$ and $^{**}P<0.05$ *PHS1* × *NDPS1* fruits relative to the respective parental *NDPS1* line by Student's *t*-test. C, Volatiles collected from ripe fruits of the *PHS1* × *NDPS1* B10 cross were analyzed by GC–MS and the total ion current is shown. Compounds were identified based on their mass spectra and retention time: 1, pinene; 2, δ-2-carene; 3, α-phellandrene; 4, α-terpinene; 5, limonene; 6, β-phellandrene. D, Quantitative changes in amounts of nerol, β-phellandrene and δ-2-carene collected as emitted volatile from fruits of *PHS1* E3, *NDPS1* B1, *NDPS1* B10, *PHS1* × *NDPS1* B1 and *PHS1* × *NDPS1* B10 lines. Data are means ± SEM ($n\geq 3$). FW, fresh weight. $^{*}P<0.01$ *PHS1* × *NDPS1* B10 fruits relative to *PHS1* × *NDPS1* B1 fruits by Student's *t*-test.

monoterpene aldehyde neral, which then non-enzymatically tautomerizes to geranial.

Similarly, endogenous phosphatase activity on GPP and alcohol dehydrogenase activity on geraniol (Fig. S6) were recently reported in *GPPS-SSU* expressing tomato fruits which produce a spectrum of monoterpenes comprised of geraniol, linalool, geranial and neral (Gutensohn et al., 2013). Products formed by endogenous enzymes including alcohol dehydrogenases and reductases were also observed in tomato fruits overexpressing *GES* (Davidovich-Rikanati et al., 2007). Interestingly, similar to the previous efforts to metabolically engineer terpenoid production in tomato fruits [*e.g.* by overexpressing *GES* (Davidovich-Rikanati et al., 2007) or linalool synthase (Lewinsohn et al., 2001)], *NDPS1* fruits produced other monoterpenes, such as linalool, limonene, terpinen-4-ol and α-terpineol (Table S2), which cannot be attributed to the action of introduced enzymes. Thus, their origin and the enzymes involved in their biosynthesis require further investigation.

The fact that observed monoterpenes in *NDPS1* transgenic fruits are not products of TPSs derived from NPP suggests that there are no endogenous TPSs in tomato fruit pericarp, which are capable to use the available NPP pool for monoterpene biosynthesis. However, in transgenic but not in control fruits (Table S2) the sesquiterpene *Z,Z*-farnesol was found, which likely originates from the enzymatic hydrolysis of *Z,Z*-FPP by phosphatases. It has been recently shown that *Z,Z*-FPP can be formed from NPP by the action of SlCPT6 (Fig. S6), a member of the *cis*-prenyltransferase (CPT) gene family in tomato, which was found to be expressed in roots and fruits (Akhtar et al., 2013).

The absence of endogenous terpene synthases in tomato fruit pericarp utilizing NPP along with the presence of NPP metabolizing enzymes, like phosphatases and *cis*-prenyltransferase SlCPT6, raised the question whether the *cis* prenyl diphosphate synthesized in *NDPS1* fruits is available for an introduced NPP-dependent monoterpene synthase. Overexpression of *PHS1* in the *NDPS1* background revealed the production of a blend of monoterpenes including ß-phellandrene, 2-carene, pinene, α-phellandrene, α-terpinene, limonene and 2-carene-10-al (Figs. 4C and S6, Table S3), which was similar to that produced by PHS1 *in vitro* with NPP and found in tomato trichomes (Schilmiller et al., 2009) and different from monoterpenes formed by PHS1 from GPP *in vitro* and *in planta* (Fig. 1B and C). These results suggest that the NPP pool produced by overexpressed *NDPS1* in plastids (Schilmiller et al., 2009; Akhtar et al., 2013) was available to the introduced PHS1 for monoterpene formation in fruit pericarp and that the availability of prenyl diphosphate substrate determines the type of product formed. The absence of *Z,Z*-farnesol and a strong reduction in nerol in *PHS1* × *NDPS1* relative to *NDPS1* fruits (Table S3) further indicate that the NPP pool was largely consumed by PHS1.

The amount of emitted and internal monoterpenes produced by PHS1 in *PHS1* × *NDPS1* B1 and *PHS1* × *NDPS1* B10 transgenic lines positively correlated with the *NDPS1* expression levels (Fig. 4, Table S3), indicating that the internal NPP pool size determined the amount of formed product. Moreover, higher levels of emitted and internal monoterpenes were observed in *PHS1* × *NDPS1* than in *NDPS1* lines suggesting that utilization of the produced NPP by the introduced TPS led to an increase in flux through the pathway. A similar increase in the metabolic flux towards monoterpenes

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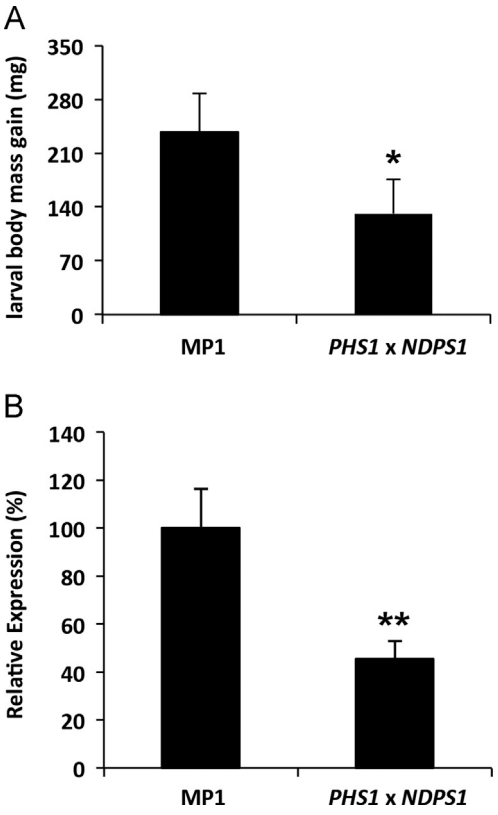

Fig. 5. Effect of *PHS1* and *NDPS1* co-expression on *Helicoverpa zea* larval feeding and *Botrytis cinerea* hyphal growth. A, No-choice bioassays were performed by placing *H. zea* larvae on ripe MP1 and *PHS1* × *NDPS1* B10 tomato fruits. Data shown are means ± SEM ($n=14$) of gained larval body mass after 5 days of feeding on fruits. B, Ripe MP1 and *PHS1* × *NDPS1* B10 tomato fruits were spot inoculated with a *B. cinerea* spore suspension. Fungal growth 4 days after inoculation was quantified by determining *B. cinerea ActinA* transcript levels by qRT-PCR (means ± SEM, $n=3$ biological replicates). $^{*}P<0.01$ and $^{**}P<0.05$ *PHS1* × *NDPS1* fruits relative to MP1 by Student's *t*-test.

was previously observed upon co-expression of GPP synthases and monoterpene synthases (Gutensohn et al., 2013; Wu et al., 2006; see also Fig. 1C). Interestingly, the internal pools of NPP-derived monoterpenes were 5- and 26-fold higher in *PHS1* × *NDPS1* B1 and *PHS1* × *NDPS1* B10 transgenic lines, respectively, when compared to the internal pools of GPP-derived monoterpenes in the *PHS1* × *GPPS-SSU* line (Tables S1 and S3). These results suggest that the MEP pathway can sustain a high production of monoterpenes in tomato fruits. The total amount of formed monoterpene products depends on the amount of GPPS and NDPS1 proteins (since the same *PHS1* parental line was used in both crosses), affinity of GPPS and NDPS1 towards IPP and DMAPP, internal pools of GPP and NPP in tomato fruits, and affinity of PHS1 towards GPP and NPP substrates. The observed lower production of GPP-derived monoterpenes could be explained by a poor affinity of PHS1 towards GPP (Schilmiller et al., 2009) and its competition for GPP substrate with GGPPS, which is highly active in ripening tomato fruits, and likely other TPSs.

4.3. NPP serves as an inhibitor of GGPPS causing reduction in lycopene accumulation

An unexpected outcome of the *NDPS1* overexpression in transgenic tomato fruits was the severe reduction in lycopene levels, the most abundant carotenoid in tomato fruits, the level of which was negatively correlated with the *NDPS1* transcript levels and the amounts of nerol formed (Fig. 2). Considering the total amounts of emitted monoterpenes (between 19 and 145 pmol per g FW and hour) and internal monoterpenes (between 5 and 23 nmol per g FW) found with *NDPS1* transgenic fruits (Table S2), it is obvious that the respective reduction in lycopene in these fruits (roughly between 20 and 130 nmol per g FW) can only be partially attributed to the redirection of metabolic flux from carotenoids towards NPP formation. Moreover, the transcript levels of MEP pathway and carotenoid biosynthetic genes were unchanged, while that of *GGPPS-2* was 2.5-fold higher in *NDPS1* fruits compared to controls (Fig. 3A). A similar up-regulation of *GGPPS-2* expression was previously observed in transgenic *GPPS-SSU* tomato fruits that also displayed a severely reduced carotenoid content (Gutensohn et al., 2013). The unchanged or even increased transcript levels of genes involved in carotenoid biosynthesis suggested the involvement of a post-transcriptional mechanism leading to the reduction of lycopene levels in *NDPS1* fruits. Indeed, GGPPS activity in crude extracts of tomato fruits was inhibited by NPP (Fig. 3B–D), suggesting that inhibition of GGPPS by NPP produced in *NDPS1* overexpressing tomato fruits significantly contributes to the observed overall reduction of lycopene levels. Partial or complete recovery of lycopene levels in *PHS1* × *NDPS1* B10 and *PHS1* × *NDPS1* B1 fruits, respectively (Figs. 4B and S3B), provide further evidence for the inhibitory effect of NPP on GGPPS activity, as consumption of NPP by the introduced PHS1 led to an increased carotenoid formation. The inhibition of GGPPS by NPP might provide an explanation as to why *NDPS1* is predominantly expressed in glandular trichomes of tomato leaves and stems (Akhtar et al., 2013; Schilmiller et al., 2009), thereby protecting photosynthetic leaf tissue, where GGPPS activity is required for chlorophyll and carotenoid biosynthesis, from NPP accumulation. Our results show that NPP can be produced in plant tissues highly active in formation of GGPPS-derived terpenoid compounds, however it has to be immediately utilized to avoid negative effects on plant metabolism.

4.4. Monoterpenes formed in PHS1 × NDPS1 fruits elevate resistance to herbivores and pathogens

Emission of volatile organic compounds, such as monoterpenes, is induced by wounding and herbivory, and can play a role in the indirect plant defense against herbivores by attracting predators and parasitoids. Produced monoterpenes can also directly defend plants by repelling or intoxicating attacking herbivores leading to a reduction in their feeding and growth rates (Duffey and Stout, 1996; Seybold et al., 2006; Unsicker et al., 2009; Zou and Cates, 1997). The latter scenario also seems to be true for the monoterpenes produced in the glandular trichomes of cultivated tomato as demonstrated in the *odorless-2* (*od-2*) mutant (Kang et al., 2010). Trichomes from *od-2* leaves contain only trace amounts of the monoterpenes, sesquiterpenes and flavonoids found in the wild type. At the same time, *od-2* plants were found to be highly susceptible to attack by herbivores like Colorado potato beetle (*Leptinotarsa decemlineata*) and tobacco hornworm (*Manduca sexta*) as indicated by significantly increased feeding and larval body mass. Here we show that metabolic engineering of a monoterpene blend, naturally found in tomato glandular trichomes (Schilmiller et al., 2009), in the pericarp of tomato fruits by co-expression of *PHS1* and *NDPS1* had a negative effect on the feeding behavior of a herbivore. In no-choice feeding assays the larvae of the moth *H. zea* fed on ripe *PHS* × *NDPS* B10 tomato fruits showed a 45% reduced gain in body mass as compared to larvae fed on ripe control fruits (Fig. 5A). These results provide direct evidence that feeding behavior and/or growth of a herbivore is negatively influenced by monoterpenes produced by PHS1 from NPP. Similarly, the co-expression in the trichomes of cultivated tomato of *S. habrochaites Z,Z*-FPP synthase with *ShZIS* sesquiterpene synthase that utilizes the *cisoid Z,Z*-FPP substrate for

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sesquiterpene formation (Gonzales-Vigil et al., 2012; Sallaud et al., 2009) resulted in the formation of 7-epizingiberene and improved resistance of transgenic plants against several herbivores (Bleeker et al., 2012).

The antifungal activities of monoterpenes produced in various plant species against pathogenic fungi have been previously demonstrated *in vitro* (*e.g.* Cakir et al., 2004; Krauze-Baranowska et al., 2002; Soylu et al., 2006; Viuda-Martos et al., 2008). In addition, an inhibitory activity of the volatiles emitted from tomato leaves, predominantly mono- and sesquiterpenes against spore germination and hyphal growth of two fungal pathogens has recently been shown *in vitro* (Zhang et al., 2008). Here, we have demonstrated *in planta* that the monoterpene blend produced in tomato fruits co-expressing *PHS1* and *NDPS1* significantly reduces the hyphal growth of the necrotrophic fungus *B. cinerea* (Fig. 5B). In summary our results suggest that NPP-derived monoterpenes formed by PHS1, naturally present in leaf and stem trichomes, contribute to the direct defense of tomato plants against herbivores and fungal pathogens, and that this property can be engineered into other tissues of tomato and eventually also other plant species.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2014.05.008.

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