CHAPTER THIRTEEN

Tomato Fruits—A Platform for Metabolic Engineering of Terpenes

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Abstract

Terpenoids are a large and diverse class of plant metabolites including mono-, sesqui-, and diterpenes. They have numerous functions in basic physiological processes as well as the interaction of plants with their biotic and abiotic environment. Due to the tight regulation of biosynthetic pathways and the resulting limited natural availability of terpenes, there is a strong interest in increasing their production in plants by metabolic engineering for agricultural, pharmaceutical, and industrial applications. The tomato fruit system was developed as a platform for metabolic engineering of terpenes to overcome detrimental effects on overall plant growth and photosynthesis traits, which are affected when terpenoid engineering is performed in vegetative tissues. Here we describe how the use of fruit-specific promoters for transgene expression can avoid

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these unwanted effects. In addition, targeting the expression of the introduced terpene biosynthetic gene to fruit tissue can take advantage of the large precursor pool provided by the methylerythritol-phosphate (MEP) pathway, which is highly active during tomato fruit ripening to facilitate the accumulation of carotenoids. We also discuss how the production of high levels of target terpene compounds can be achieved in fruits by the expression of individual or a combination of (i) the MEP or mevalonic acid pathway enzymes, (ii) prenyltransferases, and/or (iii) terpene synthases. Finally, we provide a brief outline of how the emitted as well as internal pools of terpenes can be analyzed in transgenic tomato fruits.

1. INTRODUCTION

Terpenoids are a large and diverse class of plant metabolites that are involved in numerous physiological and ecological processes. These compounds are vital for basic plant processes like photosynthesis and respiration (the phytyl side chain of chlorophylls and quinones), regulation of growth and development (the hormones gibberellins, abscisic acid, strigolactones, and brassinosteroids), modulation of membrane fluidity (sterols), and photoprotection and energy transfer (carotenoids). In addition, this metabolite class includes monoterpenes (C_{10}), sesquiterpenes (C_{15}), and diterpenes (C_{20}), which play important roles in the interactions of plants with their biotic and abiotic environment. Due to their volatile nature, many mono-, sesqui-, and some diterpenes contribute to plant reproduction by attracting pollinators and seed dispersers, as well as to the direct and indirect plant defense against pests and pathogens (Gershenzon & Dudareva, 2007; Pichersky & Gershenzon, 2002; Unsicker, Kunert, & Gershenzon, 2009).

All terpenoids originate from the universal five-carbon building blocks, isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP), both of which are produced by two independent, compartmentally separated pathways in plants (Ashour, Wink, & Gershenzon, 2010; Hemmerlin, Harwood, & Bach, 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 exclusively in plastids (Fig. 1). Although these two pathways are located in different subcellular compartments, there is ample evidence for metabolic cross talk between them, with IPP- and to a lesser extent DMAPP-exchange occurring in both directions (Dudareva et al., 2005; Furumoto et al., 2011; Hemmerlin et al., 2003; Kasahara

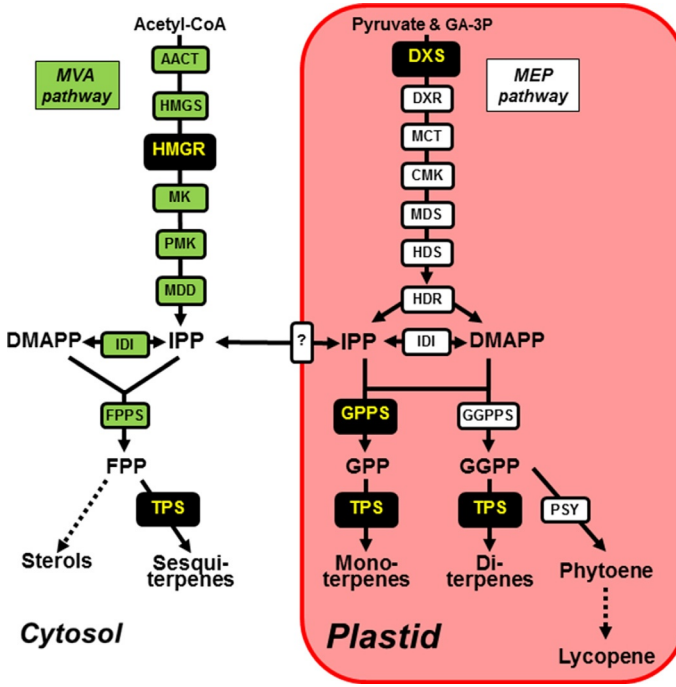

Fig. 1 Metabolic pathways involved in terpenoid biosynthesis and enzymatic steps introduced for terpene metabolic engineering in tomato fruits. Enzymes of the MEP pathway and lycopene biosynthesis are localized in plastids and are highlighted in *white*. Enzymes of the MVA and sterol biosynthesis are primarily localized in the cytosol and are highlighted in *green/gray*. Enzymes engineered into transgenic tomato fruits are highlighted in *black*. The enzymatic steps are indicated by *arrows* and *dashed lines* indicate multiple enzymatic steps. Individual enzymes are depicted as *boxes* with the abbreviation of their names. Abbreviations: *AACT*, acetoacetyl-CoA thiolase; *CMK*, 4-(cytidine 5′-diphospho)-2-*C*-methyl-D-erythritol kinase; *DMAPP*, dimethylallyl diphosphate; *DXR*, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; *DXS*, 1-deoxy-D-xylulose 5-phosphate synthase; *FPP*, farnesyl diphosphate; *FPPS*, farnesyl diphosphate synthase; *GA-3P*, D-glyceraldehyde 3-phosphate; *GGPP*, geranylgeranyl diphosphate; *GGPPS*, geranylgeranyl diphosphate synthase; *GPP*, geranyl diphosphate; *GPPS*, geranyl diphosphate synthase; *HDR*, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate reductase; *HDS*, (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase; *HMG-CoA*, 3-hydroxy-3-methylglutaryl-CoA; *HMGR*, 3-hydroxy-3-methylglutaryl-CoA reductase; *HMGS*, 3-hydroxy-3-methylglutaryl-CoA synthase; *IDI*, isopentenyl diphosphate isomerase; *IPP*, isopentenyl diphosphate; *MCT*, 2-*C*-methyl-D-erythritol 4-phosphate cytidylyltransferase; *MDS*, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate synthase; *MEP*, 2-*C*-methyl-D-erythritol 4-phosphate; *MK*, mevalonate kinase; *MVA*, mevalonate; *MDD*, mevalonate diphosphate decarboxylase; *PMK*, phosphomevalonate kinase; *PSY*, phytoene synthase; *TPS*, terpene synthases (including mono-, sesqui-, and diterpene synthases).

et al., 2002; Laule et al., 2003; Nagata, Suzuki, Yoshida, & Muranaka, 2002; Schuhr et al., 2003) via a yet unidentified transporter (Bick & Lange, 2003; Flügge & Gao, 2005; Soler, Clastre, Bantignies, Marigo, & Ambid, 1993). IPP and DMAPP are subsequently used in multiple compartments as substrates by short-chain *trans*-prenyltransferases to form larger prenyl diphosphate intermediates, including geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15), and geranylgeranyl diphosphate (GGPP, C20), that ultimately serve as substrates for terpene synthases (TPSs) (Fig. 1). GPP, the precursor of all monoterpenes, is formed in the *trans* (*E*) configuration by the head-to-tail condensation of one DMAPP molecule with one IPP molecule in a reaction catalyzed by GPP synthase (GPPS), which is localized exclusively in plastids. FPP is the result of the condensation of one DMAPP with two IPP molecules and FPP synthase (FPPS) is responsible for its formation. FPPSs are localized in the cytosol and mitochondria, thus providing FPP for sesquiterpene, homoterpene, triterpene, sterol, brassinosteroid, and polyprenol biosynthesis (Fig. 1). GGPP synthase (GGPPS) catalyzes the condensation of one DMAPP with three IPP molecules to form GGPP. GGPPSs reside in plastids, mitochondria, and the endoplasmic reticulum, producing precursors for diterpenes, gibberellins, homoterpenes, carotenoids, phytyl side chains for chlorophyll/tocopherols/quinones, polyprenols, oligoprenols, abscisic acid, and strigolactones. Recently, in addition to *trans*-prenyltransferases, *cis*-prenyltransferases that synthesize *cis*-FPP and neryl diphosphate (NPP), the *cis*-isomer of GPP, from IPP and DMAPP, have been isolated from trichomes of wild and cultivated tomato along with TPSs, which utilize these cisoid substrates for sesquiterpene and monoterpene formation, respectively (Akhtar et al., 2013; Gonzales-Vigil, Hufnagel, Kim, Last, & Barry, 2012; Sallaud et al., 2009; Schilmiller et al., 2009).

Due to their involvement in specialized biological processes the biosynthesis of monoterpenes, sesquiterpenes, and diterpenes in plants is highly regulated. Their formation is often: (i) restricted to specific tissues or cell types such as flowers (Aros et al., 2012; Dudareva, Cseke, Blanc, & Pichersky, 1996; Dudareva et al., 2003; Lee & Chappell, 2008; Nieuwenhuizen et al., 2009) or glandular trichomes found at the surface of plant organs (Iijima et al., 2004; Schilmiller, Last, & Pichersky, 2008), (ii) limited to distinct developmental stages and/or times of the day (eg, formation of terpenes in flowers takes place after anthesis and is frequently diurnally or nocturnally regulated) (Dudareva et al., 1996, 2003; Guterman et al., 2002), and (iii) induced by an environmental stimulus such as a

herbivore attack (Arimura et al., 2008; Fäldt, Arimura, Gershenzon, Takabayashi, & Bohlmann, 2003; Martin, Gershenzon, & Bohlmann, 2003; Schnee, Köllner, Gershenzon, & Degenhardt, 2002; Unsicker et al., 2009) and pathogen infection (Huang et al., 2003; Leitner et al., 2008). Due to this tight regulation of terpene formation in plants, natural sources often do not produce sufficient amounts of compounds required for agricultural or industrial uses. Moreover, terpene production in many crop plants has been severely compromised over the last decades by conventional breeding efforts that have focused on other attributes such as overall yield, shelf life, or resistance to environmental stresses, thus often resulting in the concomitant loss of unselected traits such as aroma and flavor. In addition to terpenoid production being a valuable agronomic trait, plant-derived terpenes are routinely used as flavors, fragrances, cosmetics, preservatives, insect repellents, and pharmaceutical products. However, many of these high-value terpene compounds are not produced in crop or model plants and found only in wild plants, which, as a consequence, are at risk of being overexploited and even going extinct.

There are two potential approaches to overcome limitations in terpene production in plants for agricultural, pharmaceutical, and industrial applications. When traits contributing to sufficient and/or required formation of target terpene compounds are available in the germplasm of a selected plant species, breeding and back-crossing can be used to introduce, combine, or improve the desired traits. While this approach is potentially useful and has been utilized in some plants, including crops (eg, corn [Degenhardt et al., 2009; Schnee et al., 2006]) and medicinal plants (eg, *Artemisia annua* [Graham et al., 2010]), it is a laborious and lengthy process. Furthermore, a breeding approach is not a viable option for many wild plant species, which are rich in terpenes but lack genetic resources, or in crop plants that do not produce a target terpene compound. In these cases, metabolic engineering represents an alternative and often highly efficient approach to either increase or modify terpene formation in a naturally producing plant species amenable to genetic modification, or to introduce a terpene biosynthetic pathway into a crop or model plant naturally devoid of the target compound. Numerous attempts have been made over the last decade to engineer the formation of selected terpene products in different plant species (summarized in Lange & Ahkami, 2013; Vickers, Bongers, Liu, Delatte, & Bouwmeester, 2014). While some of these attempts indeed achieved the formation of significant amounts of the terpene compound(s) of interest, others were less successful or resulted in unexpected outcomes including

undesired product modifications or detrimental effects on plant growth and performance. The expression of a prenyl transferase or of several TPSs in vegetative tissues of *Arabidopsis thaliana*, potato, or tobacco led to severe growth retardation and in some cases to chlorotic phenotypes (Aharoni et al., 2003, 2006; Besumbes et al., 2004; Orlova et al., 2009). Further analyses of these transgenic potato and tobacco plants demonstrated that chlorophyll, carotenoid, and/or gibberellin levels were reduced, which could be due to (i) depletion of the isoprenoid precursor pool by the introduced biosynthetic pathway (Aharoni et al., 2003), (ii) potential toxic effects of the newly introduced terpene products in plant cells (Aharoni et al., 2003), or (iii) inhibitory effects of the introduced products on endogenous terpenoid biosynthetic pathways (Gutensohn et al., 2014). To overcome these detrimental effects on overall plant growth and photosynthesis, which ultimately result in reduced biomass and productivity, subsequent engineering strategies were designed and successfully carried out to restrict terpene production to specific plant tissues or organs such as fruits or glandular trichomes (Bleeker et al., 2012; Zhang et al., 2015).

2. TERPENOID FORMATION IN TOMATO FRUITS

The fruits of cultivated tomato (*Solanum lycopersicum*) contain various distinct terpenoid compounds that accumulate at different stages of fruit development and ripening. The early stages of fruit development are highly active in the production of sterols, which are essential for rapid cell division and subsequent cell expansion (Gillaspy, Ben-David, & Gruissem, 1993). At these early stages of tomato fruit development hydroxymethylglutaryl-CoA reductase (HMGR1), a key regulatory enzyme of the MVA pathway, and FPPS are highly expressed, but not during later stages of fruit ripening (Gaffe et al., 2000; Jelesko, Jenkins, Rodríguez-Concepción, & Gruissem, 1999; Narita & Gruissem, 1989).

The genome of cultivated tomato contains 44 TPS genes with 29 being functional or potentially functional (Falara et al., 2011). Expression of only six TPS genes was detected in red fruits (Falara et al., 2011). Sixteen TPS genes are expressed in green immature fruits, which possess glandular trichomes (Li et al., 2004) that dry up and fall off during fruit maturation. A large number of TPS genes are expressed in glandular trichomes of young leaves and stem (14 and 16 genes, respectively). These trichomes contain high quantities of terpenes, which likely act as defense compounds (Kang et al., 2010; Schilmiller et al., 2009; van Schie, Haring, & Schuurink, 2007).

The ripening process of tomato fruits is characterized by the massive accumulation of carotenoids, primarily lycopene, which are all tetraterpene pigments (Fraser, Truesdale, Bird, Schuch, & Bramley, 1994). Accumulation of lycopene begins at the "breaker" stage when the first flush of color becomes visible, which occurs only after the tomato fruit has reached its final size at the "mature green" stage and cell expansion ceases. The first step of the plastid localized lycopene biosynthetic pathway (Cunningham & Gantt, 1998), the head-to-head condensation of two GGPP molecules, is catalyzed by phytoene synthase (PSY). Subsequently two enzymes, phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), introduce double bonds to ultimately convert phytoene to lycopene. Transcript levels of the *Psy* and *Pds* genes were shown to increase significantly in tomato fruits at the "breaker" stage and to remain high until fruits are fully ripe (Giuliano, Bartley, & Scolnik, 1993; Pecker, Chamovitz, Linden, Sandmann, & Hirschberg, 1992; Ronen, Cohen, Zamir, & Hirschberg, 1999). In line with this, the expression of two genes encoding key enzymes of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and hydroxymethylbutenyl diphosphate reductase (HDR), is strongly upregulated during ripening of tomato fruits to provide GGPP substrate for PSY (Botella-Pavia et al., 2004; Lois, Rodríguez-Concepción, Gallego, Campos, & Boronat, 2000; Paetzold et al., 2010). Despite the fact that the MVA and MEP pathways are highly active during fruit growth and ripening, respectively, tomato fruits produce only very small amounts of monoterpenes and no detectable sesquiterpenes (Baldwin, Scott, Shewmaker, & Schuch, 2000; Buttery, Teranishi, Ling, & Turnbaugh, 1990; Davidovich-Rikanati et al., 2007, 2008; Lewinsohn et al., 2001). Such limited terpene production is consistent with the fact that only a small number of endogenous monoterpene synthases and no sesquiterpene synthases are expressed in tomato fruits (Bleeker et al., 2011; Falara et al., 2011). Moreover, the few terpenes found in ripe tomato fruits are primarily formed by degradation of carotenoids (Lewinsohn, Sitrit, Bar, Azulay, Ibdah, et al., 2005; Lewinsohn, Sitrit, Bar, Azulay, Meir, et al., 2005) rather than by de novo synthesis from prenyl diphosphate intermediates.

Ripening tomato fruits thus represent an ideal platform for the metabolic engineering of terpene production because: (i) the production of terpenes can be restricted to the ripening fruits through the use of fruit-specific promoters so that the growth, development, and performance of the rest of the tomato plant will not be affected; (ii) the MEP pathway, which provides precursors for newly introduced terpene compounds, is highly active during

fruit ripening; (iii) many fruits can be obtained simultaneously; (iv) changes in the carbon flux toward terpene production often can be visually assessed based on the level of carotenoids; and (v) low levels of endogenous terpenes in ripening fruits make the metabolic profiling of transgenic plants easy. In this chapter we outline the potential approaches that can be utilized to achieve high-level terpene production in tomato fruits and summarize the outcome of respective metabolic engineering studies performed in our and other labs over the recent years.

3. TRANSGENE EXPRESSION IN RIPENING TOMATO FRUITS

3.1 Stable Transformation Under Fruit Ripening-Specific Promoters

In general, the first step in metabolic engineering is the introduction of transgene(s) encoding the biosynthetic enzymes involved in the formation of the target compound into a respective host plant. Tomato plants, like many other *Solanaceae* plants, are highly amenable for the integration of transgenes, and protocols for *Agrobacterium tumefaciens*-mediated stable transformation are well established. A critical aspect of terpene metabolic engineering in tomato fruits is the selection of suitable promoters that regulate the introduced transgene and restrict its expression to ripening fruits (Fig. 2A), thereby avoiding potential negative effects on vegetative tissues and plant development, and synchronizing the engineered terpene biosynthesis with high MEP pathway activity and precursor availability. To date three different promoters have been successfully used to express terpene metabolic genes in ripening tomato fruits.

The first fruit-specific promoter selected for terpene metabolic engineering in tomato was the promoter region of the ethylene-responsive tomato *E8* gene (Deikman & Fischer, 1988). Although the function of the *E8* gene is not completely understood, its promoter activity and expression have been extensively characterized. The *E8* promoter is activated at the onset of tomato fruit ripening (Deikman & Fischer, 1988; Deikman, Kline, & Fischer, 1992; Lincoln & Fischer, 1988) and gives rise to uniform transgene expression throughout the fruit pericarp (Kneissl & Deikman, 1996). The *E8* promoter has already been successfully used for genetic engineering in tomato fruits (Giovannoni, DellaPenna, Bennett, & Fischer, 1989; Good et al., 1994; Sandhu et al., 2000) including production of folates, carotenoids, and aromatic amino acids (Díaz de la Garza, Gregory, & Hanson,

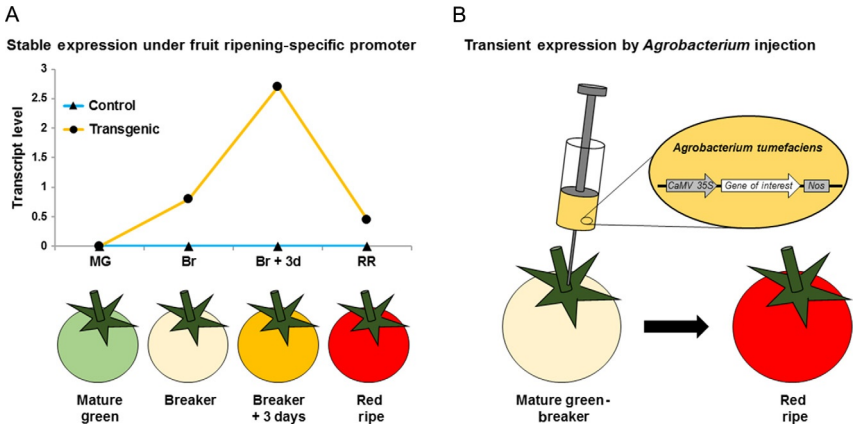

Fig. 2 Two alternative strategies for the expression of terpene biosynthetic transgenes in ripening tomato fruits. (A) Expression of transgene in stable transgenic tomato plants under control of a fruit ripening-specific promoter that restricts expression to the ripening period between the breaker and red ripe stages when carotenoids accumulate. (B) Transient expression of transgene in ripening tomato fruits upon *Agrobacterium* injection of fruits prior to or at the breaker stage.

2007; Sun et al., 2012; Tzin et al., 2013). When the *E8* promoter was used for terpene metabolic engineering, it has been shown that fruits of transgenic tomato plants carrying a linalool synthase gene (*LIS*), responsible for formation of the monoterpene linalool, contained no monoterpene products at the "mature green" stage, while monoterpenes began to accumulate at the "breaker" stages reaching maximum in fully ripe fruits (Lewinsohn et al., 2001).

A second promoter utilized for metabolic engineering in tomato fruits originated from the pepper *fibrillin* (*fib*) gene, which encodes a major chromoplast protein involved in the deposition of carotenoids into lipoprotein fibrils in ripening pepper fruits (Deruère, Bouvier, et al., 1994; Deruère, Römer, et al., 1994). When this promoter is used to drive transgene expression in tomato fruits, practically no expression is observed at early stages of fruit development. However, transgene expression levels increase between the "mature green" and "breaker" stages, and remain high throughout ripening of tomato fruits (Kuntz et al., 1998; Simkin et al., 2007). When the key MEP pathway enzyme DXS was expressed in transgenic tomato plants under the control of the *fibrillin* promoter, DXS activity was significantly increased in ripening tomato fruits 4–6 days after the "breaker" stage (Enfissi et al., 2005).

The tomato *polygalacturonase* (*PG*) promoter is the most frequently used promoter for metabolic engineering of terpenes in ripening tomato fruits. Polygalacturonase is responsible for the degradation of polyuronides in the fruit cell wall, thus contributing to the fruit softening. The expression of the *PG* gene is tightly regulated with no expression in vegetative tissues including roots, stems, and leaves (Biggs & Handa, 1989). Furthermore, no transcripts are detected at the earlier stages of fruit development up to the "mature green" stage. *PG* mRNA begins to accumulate at the "breaker" stage, reaches the highest level a few days after the "breaker" stage, and then drops once the fruit becomes fully ripe (Biggs & Handa, 1989; DellaPenna, Lincoln, Fischer, & Bennett, 1989). In contrast, the amounts of polygalacturonase protein and enzyme activity continue to increase consistently after their first appearance at the "breaker" stage and reach the highest levels in red ripe fruits (Biggs & Handa, 1989). Essentially identical fruit ripening-specific expression patterns were observed when two reporter genes were expressed in transgenic tomato plants under the control of either a short (1.4 kb) or a long (4.8 kb) version of the *PG* promoter (Bird et al., 1988; Fernandez et al., 2009; Montgomery, Pollard, Deikman, & Fischer, 1993; Nicholass, Smith, Schuch, Bird, & Grierson, 1995). While both promoter fragments were capable of driving transgene expression in the pericarp of ripening fruits, higher expression levels were achieved with the longer one. This longer version of the *PG* promoter has been successfully used to express two prenyltransferases as well as various TPSs in transgenic tomato plants (Davidovich-Rikanati et al., 2008, 2007; Gutensohn et al., 2014, 2013; Kovacs et al., 2007). While no transcripts of the respective transgenes were detected in fruits at the "mature green" stage, they began to accumulate at the "breaker" stage as expected, reached the highest level 3 days after the "breaker" stage, and declined in ripe fruits (Davidovich-Rikanati et al., 2008; Gutensohn et al., 2014, 2013).

3.2 Transient Transformation via *Agrobacterium* Injection into Fruits

Although stable transformation of tomato plants is now routine, it is still a time-consuming procedure involving tissue culture and plant regeneration, and it takes several months before fruit-bearing plants are available. Considering the often uncertain outcome of new metabolic engineering strategies, one would prefer to have a rapid and efficient way of testing gene expression constructs and the effect of a given transgene on host plant metabolism prior to the more labor-intensive generation of stable transgenic lines. Transient

transformation via *Agrobacterium* infiltration into the intercellular spaces of leaves was successfully established in various plant species including *Nicotiana benthamiana*, *A. thaliana*, and tomato and is now commonly used for the assessment of transgene function. A comparable strategy for the transient transformation of fleshy fruits was first reported by Spolaore, Trainotti, and Casadoro (2001), in which an *Agrobacterium* suspension was injected into fruits using a syringe with hypodermic needle. This agroinjection procedure was subsequently further optimized for the transient expression and analysis of transgenes in ripening tomato fruits (Orzaez, Mirabel, Wieland, & Granell, 2006). Injection of *Agrobacterium* carrying β-glucuronidase and yellow fluorescent protein reporter gene constructs under control of the *Cauliflower Mosaic Virus* (*CaMV*) *35S* promoter into tomato fruits at the "mature green" stage through the fruit stylar apex resulted in high levels of reporter gene expression around the placenta tissue and moderate levels in the fruit pericarp. When this agroinjection technique was subsequently combined with the *Tobacco Rattle Virus*-based "virus induced gene silencing" (VIGS) to downregulate the carotenoid biosynthetic gene PDS in tomato fruits, these fruits accumulated lower levels of lycopene and lycopene-derived volatiles (Orzaez et al., 2009, 2006). Transient expression also offers the advantage of being able to test gene stacks through introduction of additional constructs in stably transformed plants. Indeed, we have recently used the agroinjection protocol as described by Orzaez et al. (2006) to transiently overexpress MEP and MVA pathway genes under the *CaMV 35S* promoter in the background of stable transgenic tomato lines expressing TPSs under the fruit ripening-specific *PG* promoter (Gutensohn & Dudareva, unpublished data).

4. OVEREXPRESSION OF TERPENE BIOSYNTHETIC GENES IN TOMATO FRUITS

In the past decade numerous attempts have been made to modify the terpenoid profile of tomato fruits. In general, three types of genes encoding enzymes involved in the biosynthesis of terpenes were used to achieve formation of the desired product. They include: (i) MVA and MEP pathway genes to increase the pool of available IPP and DMAPP precursors, (ii) prenyltransferase genes to increase the pool of a required prenyl diphosphate intermediate, and (iii) TPS genes to direct the metabolic flux toward the formation of a target compound. Below we outline examples of terpene metabolic engineering in ripening tomato fruits that have utilized genes

from one of these classes or combinations of genes from several classes and discuss the respective outcomes.

4.1 MEP and MVA Pathway Genes

Despite the fact that the plastidic MEP pathway is highly active in ripening tomato fruits to provide precursors for carotenoid biosynthesis, attempts were made to even further increase flux through this pathway. Expression of *Escherichia coli* DXS targeted to plastids under the control of the *fibrillin* promoter resulted in twofold increases in both DXS enzyme activity and 1-deoxy-D-xylulose 5-phosphate internal pool in ripening transgenic tomato fruits relative to controls (Enfissi et al., 2005). In addition, the carotenoid levels were increased 1.6-fold, indicating that *DXS* overexpression had indeed increased the metabolic flux through the plastidic MEP pathway toward the biosynthesis of downstream carotenoids.

In contrast to the MEP pathway, the cytosolic MVA pathway is highly active only during early stages of fruit development and not during later stages of fruit ripening. Thus, the key MVA pathway enzyme HMGR from *Arabidopsis* was overexpressed in transgenic tomato plants under the control of the constitutive *CaMV 35S* promoter (Enfissi et al., 2005). Despite the fact that no significant increases in HMGR enzyme activity were detected, the phytosterol content in ripe fruits of these transgenic lines was increased up to 2.4-fold compared to controls. This indicates that overexpression of *HMGR* increases metabolic flux through the cytosolic MVA pathway toward the biosynthesis of downstream phytosterols, similar to the effect of *DXS* overexpression on carotenoid biosynthesis. Despite these encouraging results up to date the stable overexpression of *DXS* or *HMGR* has not been further utilized for the metabolic engineering of terpene formation in tomato fruits.

4.2 Prenyltransferases

Besides the importance of having high metabolic flux through the upstream MEP and MVA pathways, the availability of sufficient pools of the required prenyl diphosphate intermediates is critical for efficient engineering of terpene formation. Of the two GGPP synthases present in tomato, *GGPPS2* is highly expressed in ripening tomato fruits (Ament, Van Schie, Bouwmeester, Haring, & Schuurink, 2006) and provides GGPP for the massive accumulation of lycopene and other carotenoids. In contrast, FPPS is highly expressed during tomato fruit development (Gaffe et al., 2000), while the putative small subunit of a heterodimeric GPPS is only weakly

expressed in ripening tomato fruits (Gutensohn et al., 2013; Wang & Dixon, 2009). The *FPPS* and *GPPS* expression profiles suggest that only small pools of the FPP and GPP precursors are likely available in ripening fruits for sesqui- and monoterpene biosynthesis, respectively.

To increase the amount of available GPP and divert the metabolic flux from GGPP and carotenoid formation toward monoterpene biosynthesis we expressed a noncatalytic small subunit of snapdragon (*Antirrhinum majus*) GPPS (*GPPS-SSU*) under control of the *PG* promoter in tomato (Gutensohn et al., 2013). Heterodimeric GPPSs, like that found in snapdragon, contain a catalytically inactive GPPS-SSU that interacts with a *bona fide* GGPPS and changes its product specificity, thus forming a heterodimer, which catalyzes the formation of GPP (Tholl et al., 2004; Wang & Dixon, 2009). Overexpression of snapdragon *GPPS-SSU* significantly increased the total GPPS activity in ripening tomato fruits (Gutensohn et al., 2013). In contrast to control fruits, which contain only minute amounts of monoterpenes due to expression of limited number of endogenous TPSs (Bleeker et al., 2011; Falara et al., 2011), transgenic *GPPS-SSU* tomato fruits produced an array of monoterpenes including geraniol, the dephosphorylated form of GPP, geranial, neral, citronellol, citronellal, and geranic acid. The amounts of monoterpenes positively correlated with the *GPPS-SSU* transcript and protein levels in various transgenic lines. However, the monoterpenes produced were not a result of the action of endogenous monoterpene synthases, but of endogenous phosphatases (Ganjewala & Luthra, 2009; Izumi, Ashida, Yamamitsu, & Hirata, 1996; Perez, Taucher, & Cori, 1980), reductase(s) and alcohol dehydrogenase(s) (Bicsak, Kann, Reiter, & Chase, 1982; Davidovich-Rikanati et al., 2007), activity of which were previously shown to be present in tomato fruits (Davidovich-Rikanati et al., 2007). While *GPPS-SSU* expression had no deleterious effects on vegetative tissues and development of transgenic tomato plants, the lycopene levels of ripe fruits were strongly reduced. This severe reduction of lycopene could not be exclusively attributed to a redirection of metabolic flux from GGPP to GPP formation, but was the result of a posttranscriptional control likely via feed-forward regulation of lycopene biosynthetic enzymes by one (or several) product(s) formed in transgenic lines (Gutensohn et al., 2013). Thus, the observed monoterpene formation and reduction of lycopene levels in the transgenic fruits indicate that the expression of *GPPS-SSU* indeed leads to the redirection of metabolic flux and an increase in the GPP pool, which can be utilized for further metabolic engineering of monoterpenes in ripening tomato fruits.

Although most monoterpene synthases use GPP as substrate, it was recently found that some monoterpene synthases accept NPP, the *cis*-isomer of GPP, as substrate (Falara et al., 2011; Gonzales-Vigil et al., 2012; Matsuba et al., 2013; Schilmiller et al., 2009; Zhang, Liu, Li, & Yu, 2013). However, in tomato plants *NDPS1*, encoding the *cis*-prenyltransferase that catalyzes the NPP formation, is primarily expressed in trichomes of leaves and stems but not in fruits (Akhtar et al., 2013; Gutensohn et al., 2014; Schilmiller et al., 2009). Thus, to achieve formation of NPP-derived monoterpenes in tomato fruits, we have expressed the tomato *NDPS1* under the control of the *PG* promoter (Gutensohn et al., 2014). *NDPS1* transgenic fruits produced large amounts of nerol, the dephosphorylated form of NPP, as well as minor amounts of neral, geranial, and geraniol. Similar to the conversion of GPP into an array of monoterpenes in transgenic *GPPS-SSU* fruits, produced NPP was subjected to the action of endogenous phosphatases and alcohol dehydrogenases. As in the case of the *GPPS-SSU* expressing fruits, *NDPS1* expression led to a severe reduction in lycopene levels, which can be only partially explained by the redirection of metabolic flux from carotenoids toward NPP formation. Indeed, subsequent analyses demonstrated that the produced NPP serves as an inhibitor of GGPPS activity in tomato fruits. This finding was further corroborated by the fact that transcript levels of *GGPPS2* were 2.5-fold increased in *NDPS1* fruits (Gutensohn et al., 2014).

In contrast to our successful engineering of GPP and NPP formation in ripening tomato fruits by expression of *GPPS-SSU* and *NDPS1*, respectively (Gutensohn et al., 2014, 2013), to the best of our knowledge no comparable attempt has been made so far to increase the available FPP pool by expression of an FPPS in ripening tomato fruits.

4.3 Terpene Synthases

While the overexpression of MEP and MVA pathway genes increases the metabolic flux through the respective pathways, and overexpression of prenyltransferases, such as GPPS and NPPS, redirects the metabolic flux from carotenoids toward increased formation of corresponding prenyl diphosphate intermediates, only the expression of TPSs ultimately determines which terpene products, eg, mono-, sesqui-, or diterpenes, are formed in transgenic tomato fruits. The expression of *Clarkia breweri S-linalool synthase* (*LIS*) under the control of the *E8* promoter represents a first example of monoterpene engineering in tomato fruits (Lewinsohn et al., 2001). The

resulting transgenic fruits indeed produced high levels of *S*-linalool as well as substantial levels of 8-hydroxylinalool and a number of unknown products. The appearance of 8-hydroxylinalool suggests the presence of an endogenous hydroxylating activity in ripening tomato fruits that accepts linalool as substrate. Despite the accumulation of monoterpenes in the *LIS* transgenic fruits the levels of other terpenoids such as carotenoids and tocopherols were unaltered. Expression of the *Ocimum basilicum geraniol synthase* (*GES*), a second monoterpene synthase used for tomato fruit metabolic engineering, under the control of the *PG* promoter, resulted in the formation of geraniol in transgenic fruits (Davidovich-Rikanati et al., 2007). In addition, these *GES* transgenic fruits accumulated other monoterpenes, including nerol, citronellol, geranial, neral, citronellal, geranic acid, neric acid, and citronellic acid, which all are derived from geraniol through the action of endogenous enzymes such as alcohol dehydrogenases and reductases. The amount of geraniol and its derivatives accumulated in the *GES* fruits (about 3500 ng/g FW) was significantly higher than the amount of linalool and its derivatives detected in the *LIS* fruits (400–800 ng/g FW) (Lewinsohn et al., 2001). In contrast to *LIS* fruits, lycopene levels in *GES* fruits were reduced by 50% indicating a substantial redirection of the metabolic flux from carotenoids toward monoterpenes. Recently, *phellandrene synthase 1* (*PHS1*), which is naturally only expressed in tomato trichomes and accepts both NPP and GPP substrates to form different monoterpenes, respectively (Schilmiller et al., 2009), was expressed in tomato fruits under control of the fruit ripening-specific *PG* promoter (Gutensohn et al., 2014). The resulting transgenic tomato fruits produced small amounts of myrcene and ocimene, the GPP-derived monoterpene products of PHS1 (Schilmiller et al., 2009). The low level of total monoterpene production in *PHS1* fruits (~50 ng/g FW) relative to that in the *LIS* and *GES* fruits (Davidovich-Rikanati et al., 2007; Lewinsohn et al., 2001) is likely due to the low affinity of PHS1 for GPP (Schilmiller et al., 2009). Overexpression of *PHS1* also uncovered the absence of an endogenous NPP pool in the fruits. Taken together, the above-described metabolic engineering efforts with three monoterpene synthases clearly demonstrated that despite the low expression level of a *GPPS* small subunit and the low GPPS activity in ripening tomato fruits (Gutensohn et al., 2013; Wang & Dixon, 2009) a sufficient GPP pool is available for the introduced monoterpene synthases.

The positive outcomes of the described monoterpene engineering approaches in tomato fruits fulfilled expectations because the high activity of the MEP pathway during ripening provides precursors for the

overexpressed monoterpene synthases in plastids. In contrast, it was questionable if sesquiterpene formation could also be successfully achieved in ripening tomato fruits. Sesquiterpene synthases are localized in the cytosol where the MVA pathway genes, like *HMGR1*, as well as *FPPS* are expressed at low levels during fruit ripening (Gaffe et al., 2000; Jelesko et al., 1999; Narita & Gruissem, 1989). However, when *α-zingiberene synthase* (*ZIS*), a sesquiterpene synthase from *Ocimum basilicum*, was expressed in tomato fruits under the control of the *PG* promoter, the resulting transgenic fruits accumulated high levels of α-zingiberene and other sesquiterpenes such as α-bergamotene, 7-*epi*-sesquithujene, β-bisabolene, and β-curcumene (Davidovich-Rikanati et al., 2008). No significant differences between *ZIS* transgenic and control fruits were found in sterol levels, which are also synthesized from MVA pathway-derived precursors. In contrast, a slight decrease in carotenoids was observed in *ZIS* transgenic fruits. The significant accumulation of sesquiterpenes (up to 1000 ng α-zingiberene/g FW) in *ZIS* transgenic fruits indicates that despite the low expression level of *HMGR1* and *FPPS* there is a sufficient supply of FPP in ripening tomato fruits to drive the production of engineered sesquiterpenes.

Besides mono- and sesquiterpenes, the formation of diterpenes has been successfully engineered in tomato fruits as well. Taxans, such as taxadiene, are polycyclic diterpenes that are naturally produced in small amounts in various species of yew trees (*Taxus* spec.) and used as anticancer drugs. The taxadiene synthase from *Taxus baccata* was expressed under the control of both, the constitutive *CaMV 35S* promoter and the fruit ripening-specific *PG* promoter, in the background of the *yellow flesh* tomato mutant (Kovacs et al., 2007). This tomato mutant is deficient in the fruit-specific PSY and thus does not consume GGPP for carotenoid biosynthesis (Fray & Grierson, 1993). Resulting transgenic fruits accumulate taxadiene, with amounts being higher upon fruit-specific promoter-driven expression (400–600 μg/g DW) relative to constitutive promoter-driven expression (140–400 μg/g DW). Independent of the promoter used, seed formation in transgenic fruits was severely affected, which could, however, be fully restored by treatment of flowers with giberellic acid.

4.4 Pyramiding Multiple Terpene Biosynthetic Genes

After the successful engineering of individual metabolic steps in the terpene biosynthetic pathway including MEP/MVA pathway genes, prenyltransferases, and TPSs, the next step to achieve high levels of terpene production in ripening tomato fruits is to combine several transgenes in the same host plant. To obtain transgenic lines coexpressing a prenyltransferase and

a TPS in ripening tomato fruits we have crossed parental lines that express respective transgenes under the control of the *PG* promoter. The resulting F1 plants were initially tested for the presence of both transgenes by PCR on genomic DNA using gene-specific primers. Subsequently, the expression levels of both transgenes were analyzed by quantitative real-time PCR in ripening fruits of these F1 plants and compared to the expression levels in the respective parental lines. Coexpression of the snapdragon *GPPS-SSU* with the *Ocimum basilicum GES* led to a more than threefold increase in the overall formation of monoterpenes in tomato fruits relative to the parental *GES* line (Gutensohn et al., 2013). Remarkably, geraniol emission from *GPPS-SSU* × *GES* fruits was sevenfold higher than from parental *GES* fruits, while no significant differences in internal monoterpene pools were detected. These results suggest that the metabolic flux through the engineered pathway was increased, while the internal monoterpene pools were saturated. A similar increase in monoterpene formation was achieved upon coexpression of snapdragon *GPPS-SSU* and tomato *PHS1* in tomato fruits, with a more than twofold increase in myrcene and ocimene emission, as well as a 3.8- and 8-fold increase in respective internal pools in *GPPS-SSU* × *PHS1* fruits compared to *PHS1* fruits (Gutensohn et al., 2014). In order to attain metabolic engineering of NPP-derived monoterpenes in tomato fruits, we generated transgenic lines coexpressing both tomato *NDPS1* and *PHS1* (Gutensohn et al., 2014). The monoterpenes found in the fruits of *NDPS1* and *PHS1* parental lines, nerol and myrcene/ocimene, respectively, were absent in *NDPS1* × *PHS1* fruits and instead they produced a blend of monoterpenes including β-phellandrene. These results indicate that the NPP pool produced by the overexpressed *NDPS1* is utilized by the introduced PHS1 for monoterpene formation in tomato fruits.

5. ANALYSIS OF TERPENES IN TOMATO FRUITS

For the development of metabolic engineering strategies it is not only important to express genes required for the production of a target compound in the host plant, but also to have sensitive methods for its detection. Here we outline some technical aspects of the characterization of volatile terpene production, which are specific to tomato fruits and different from the common analysis of leaf or flower volatiles. A comprehensive overview of the terpenoids produced in transgenic tomato fruits can be obtained by analyzing the terpenes emitted from fruits as well as the internal terpene pools accumulated in fruits.

5.1 Analysis of Emitted Terpenes

Volatiles emitted from ripe tomato fruits are collected using a closed-loop stripping method (Dudareva et al., 2005) at 21°C under growth chamber conditions. Ripe tomato fruits are freshly harvested, washed with cold tab water, dried with paper towels, and then depending on the size of the fruit vertically cut into six to eight triangular slices. Ten milliliters of a 5% (w/v) sucrose solution is pipetted into the volatile collection chamber. The tomato fruit slices (in total 30–50 g fresh weight) are then inserted into the collection chamber upright in such a way that the side of the fruit that had been attached to the plant has some contact with the sucrose solution, thus allowing the fruit to take up sucrose during the period of the collection. Volatile collections are performed for 24 h using Porapak Q traps and collected volatiles are subsequently eluted from the traps with 200 μL dichloromethane.

5.2 Analysis of Internal Terpene Pools

For the analysis of internal volatile pools approximately 30 g of pericarp from fresh ripe tomato fruits are cut into small pieces, transferred into 250 mL screwcup bottles, and extracted with 100 mL methyl *tert*-butyl ether. After vigorous shaking overnight, the extract is filtered to remove the fruit pieces. Since the resulting extract contains substantial amounts of water from the fruit tissue in addition to the organic solvent, it is subsequently allowed to separate into two phases. The ethereal phase is separated off, additionally dried with anhydrous $CaSO_4$ to remove residual water, and concentrated to 200 μL under a gentle N_2 stream.

The collected emitted as well as the extracted tomato fruit volatiles are supplemented with 3.33 μg of naphthalene as internal standard, and a 2 μL aliquot is subsequently analyzed by gas chromatography–mass spectrometry (GC–MS) (Dudareva et al., 2005). Representative terpene standards are used to determine response factors, which then allow the quantification of analyzed compounds.

6. CONCLUSIONS

Progress in the discovery and characterization of TPSs, prenyltransferases, and the MEP and MVA pathway biosynthetic genes made it possible to metabolically engineer terpenoid formation in plants. However, when genetic manipulations were performed in vegetative tissue they

often resulted in undesired effects on the overall performance of the host plant. Therefore, the tomato fruit system was developed as an alternative platform for the efficient production of high levels of terpenes using fruit-specific promoters. This platform is beneficial not only because it avoids the deleterious effects of engineering on normal plant development, but also because it provides a sufficient pool of precursors for terpene biosynthesis, as the MEP pathway is highly active during the accumulation of carotenoids, which accompanies tomato fruit ripening. Thus, the tomato fruits can be used not only as an experimental system for studying metabolic pathways and engineering steps but also as biofactories for a large-scale production of desired target terpene compounds, since it is easy to grow this horticultural crop and scale up its production.

Another benefit of the tomato fruit system is the availability of transient transgene expression via the developed agroinjection method (Orzaez et al., 2006), which allows rapid testing of the effects of introduced transgenes on production of the target compounds prior to the more labor-intensive and time-consuming creation of stable transgenic lines. This transient expression system also permits assessment of the effectiveness of gene stacks via introduction of additional transgenes in stably transformed plants. Earlier terpene engineering studies have demonstrated that the expression of individual biosynthetic transgenes in host plants often resulted in a lower terpene production than transgenic plants coexpressing multiple terpene biosynthetic enzymes, for example, prenyltransferases and TPSs (Wu et al., 2006). Previously, stable gene stacks have been generated in the tomato fruit system by crossing of individual parental lines containing single transgenes (Gutensohn et al., 2014, 2013). However, this approach is impractical for staking a larger number of transgenes in one plant. Thus, the next step in the metabolic engineering of terpene formation in the tomato fruits will be utilization of newly developed techniques that allow the introduction of multiple transgenes under the control of one promoter via one transformation event. In one such approach, multiple transgenes are fused in a single transcriptional unit such that the coding sequences of the individual proteins are separated by the recognition sequence of the tobacco vein mottling virus (TVMV) NIa proteinase (Dafny-Yelin & Tzfira, 2007). The resulting polyprotein is then processed and cleaved into individual proteins by the coexpressed TVMV NIa proteinase. An alternative approach for multicistronic expression of transgenes in plants is based on the use of either viral internal ribosomal entry sites (Ngoi, Chien, & Lee, 2004) or viral 2A sequences which result in ribosomal skipping (de Felipe et al., 2006; Donnelly et al., 2001), to

separate translation of individual transgenes in such transcriptional units for synchronized production of multiple proteins. The latter two techniques have recently been successfully used to engineer the formation of carotenoids in rice endosperm (Ha et al., 2010).

Metabolic engineering often leads to unpredicted results, highlighting our lack of a comprehensive understanding of plant metabolism including silent metabolism (Lewinsohn & Gijzen, 2009). In general, the production and metabolic fate of a newly synthesized compound depend on the entire biochemical repertoire within a given plant tissue; thus it is difficult to predict how much of the target compound will remain in the desired form. The newly synthesized compound may be affected by enzymes that are endogenously present in the cell and have broad substrate specificity, such as dehydrogenases, phosphatases, and others. A comprehensive understanding of the entire terpenoid metabolic network in plants is required for future rational and precise metabolic engineering, as was recently exemplified by the unexpected discovery of isopentenyl phosphate kinase and its role in terpenoid biosynthesis in plants (Henry, Gutensohn, Thomas, Noel, & Dudareva, 2015). Undoubtedly the application of omics approaches, such as transcriptomics and metabolomics, in tomato fruits will improve our understanding of the entire metabolic network in this platform, including silent metabolism. The identification and subsequent manipulation of enzymatic steps, which convert the desired terpene target compound into undesired downstream products, will result in more predictable outcomes of terpene metabolic engineering in the tomato fruit system.

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