

Running title: **Triterpene Metabolism in Plants**

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**Engineering Triterpene and Methylated Triterpene Production in Plants Provides
Biochemical and Physiological Insights into Terpene Metabolism¹**

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One sentence summary: Targeting non-native triterpene biosynthesis to different cellular
locations sheds light on the intracellular distribution of cofactors and substrates and
uncovers a new homeostatic mechanism.

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ABSTRACT (250 words)

Linear, branch-chained triterpenes including squalene (C30), botryococcene (C30) and their methylated derivatives (C31-C37) generated by the green algae *Botryococcus braunii* race B, which have received significant attention because of their utility as chemical and biofuel feedstocks. However, the slow growth habit of *B. braunii* makes it impractical as a production system. In this study, we evaluated the potential of generating high levels of botryococcene (C30) in tobacco plants by diverting carbon flux from the cytosolic MVA pathway or the plastidic MEP pathway by the targeted overexpression of an avian farnesyl diphosphate synthase along with two versions of botryococcene synthases. Up to 544 µg/g fresh weight of botryococcene was achieved when this metabolism was directed to the chloroplasts, which is ~90-times greater than that accumulating in the plants engineered for cytosolic production. To test if methylated triterpenes could be produced in tobacco, we also engineered triterpene methyltransferases (TMTs) from *B. braunii* into wild type plants and transgenic lines selected for high-level triterpene accumulation. Up to 91% of the total triterpene contents could be converted to methylated forms (C31, C32) by co-targeting the TMTs and triterpene biosynthesis to the chloroplasts, whereas only 4-14% of total triterpenes were methylated when this metabolism was directed to the cytoplasm. When the TMTs were over-expressed in the cytoplasm of wild type plants, up to 72% of the total squalene was methylated and total triterpenes (C30+C31+C32) content was elevated 7-fold. Altogether, these results point to innate mechanisms controlling metabolite fluxes, including a homeostatic role for squalene.

76 **INTRODUCTION**

77 Terpenes and terpenoids represent a distinct class of natural products (Buckingham, 2003) that
78 are derived from two universal 5-carbon precursors: isopentenyl diphosphate (IPP) and
79 dimethylallyl diphosphate (DMAPP). In eukaryotic fungi and animals, IPP and DMAPP are
80 synthesized via the mevalonate (MVA) pathway whereas in prokaryotes they are synthesized
81 via the methylerythritol phosphate (MEP) pathway. In higher plants, the pathways are present in
82 separate compartments and are believed to operate independently. The MVA pathway in the
83 cytoplasm is predominately responsible for sesquiterpene (C₁₅), triterpene (C₃₀), and
84 polyprenol (>45) biosynthesis and associated with the endoplasmic reticulum (ER) system. The
85 MEP pathway resides in plastids and is dedicated to monoterpenes (C₁₀), diterpenes (C₂₀),
86 carotenoids (C₄₀) and long-chain phytol biosynthesis. All these compounds are usually
87 produced by plants for a variety of physiological (i.e. hormones, aliphatic membrane anchors,
88 maintaining membrane structure) and ecological roles (i.e. defense compounds, insect/animal
89 attractants) (Kempinski et al., 2015). Terpenes are also important for various industrial
90 applications ranging from flavors and fragrances (Schwab et al., 2008) to medicines (Dewick,
91 2009; Niehaus et al., 2011; Shelar, 2011).

92 The utility of terpenes as chemical and biofuel feedstocks has also received considerable
93 attention recently. Isoprenoid-derived biofuels include farnesane (Renninger et al., 2008; Rude
94 et al., 2009), bisabolene (Peralta-Yahya et al., 2011), pinene dimers (Harvey et al., 2010),
95 isopentenal (Withers et al., 2007), and botryococcene (Glikson et al., 1989; Hillen et al., 1982;
96 Mastalerz et al., 1996; Moldowan et al., 1980). The richness of branches within these
97 hydrocarbon scaffolds correlate with their high-energy content, which enables them to serve as
98 suitable alternatives to crude petroleum (Peralta-Yahya et al., 2010). Indeed, some of them are
99 already major contributors to current day petroleum-based fuels. One of the best examples of
100 this is the triterpene oil accumulating in the green algae *Botryococcus braunii* race B, which is
101 considered a major progenitor to oil and coal shale deposits (Moldowan et al., 1980). This alga
102 has been well studied and the major constituents of its prodigious hydrocarbon oil are a group of
103 triterpenes including squalene (C₃₀), and organism-specific botryococcene (C₃₀), methylated
104 squalene (C₃₁-C₃₄) and methylated botryococcene (C₃₁-C₃₇) (Huang et al., 1989; Metzger et
105 al., 1988; Okada et al., 1995), which can be readily converted into all classes of combustible
106 fuels under hydrocracking conditions (Hillen et al., 1982).

The unique biosynthetic mechanism for the triterpenes in *Botryococcus* was recently described by Niehaus et al. (2011), in which a series of novel squalene synthase-like genes were identified (Figure 1). In short, squalene synthase-like enzyme, SSL-1, performs a head-to-head condensation of two farnesyl diphosphate (FPP) molecules into presqualene diphosphate (PSPP), followed by a reductive rearrangement to yield squalene (C30) by the enzyme SSL-2, or converted by SSL-3 to form botryococcene through a different reductive rearrangement (Niehaus et al., 2011). Methylated derivatives are the dominant triterpene species generated by *Botryococcus braunii* race B (Metzger et al., 1988; Metzger et al., 1985) and these derivatives are known to yield higher quality fuels due to their high energy content and the hydrocracking products derived by virtue of having more hydrocarbon branches. Triterpene methyltransferases (TMTs) that can methylate squalene and botryococcene have been successfully characterized by Niehaus et al. (2012). Triterpene methyltransferase 1 (TMT-1) and triterpene methyltransferase 2 (TMT-2) prefer squalene C30 as their substrate for production of mono- (C31) or di-methylated (C32) squalene, while TMT-3 prefers botryococcene as its substrate for the biosynthesis of mono- (C31) or dimethylated (C32) botryococcene (Figure 1). These TMTs are believed to be insoluble enzymes; they exhibit large hydrophobic areas and their activities were only observed *in vitro* using yeast microsomal preparations (no activity was observed when expressed in bacteria) (Niehaus et al., 2012).

Like the majority of identified methyltransferases, these TMTs utilize the methyl donor S-adenosylmethionine (SAM) found ubiquitous in prokaryotes and eukaryotes (Liscombe et al., 2012; Scheer et al., 2011). In plants, SAM is one of the most abundant co-factors (Fontecave et al., 2004; Sauter et al., 2013), and is exclusively synthesized in the cytosol (Wallsgrrove et al., 1983; Ravanel et al., 1998; Ravanel et al., 2004; Bouvier et al., 2006). While it is used predominately as a methyl donor in methylation reaction (Ravanel et al., 2004), it also serves as the primary precursor for the biosynthesis of the ethylene (Wang et al., 2002b), polyamines (Kusano et al., 2008), and nicotianamine (Takahashi et al., 2003), which play a variety of important roles for plant growth and development (Huang et al., 2012; Sauter et al., 2013). The SAM present in organelles, like the chloroplast, appears to be imported from the cytosol by specific SAM/S-adenosylhomocysteine (SAH) exchange transporters that reside on the envelope membranes of plastids (Ravanel et al., 2004; Bouvier et al., 2006). The imported SAM is involved in the biogenesis of aspartate-derived amino acids (Curien et al., 1998; Jander et al., 2009; Sauter et al., 2013) and serves as the methyl donor for the methylation of macromolecules, such as plastid DNA (Ahlert et al., 2009; Nishiyama et al., 2002) and proteins

(Alban et al., 2014; Houtz et al., 1989; Niemi et al., 1990; Trievel et al., 2003; Ying et al., 1999), and small molecule metabolites such as prenylipids (e.g. plastoquinone, tocopherol, chlorophylls and phyloquinone) (Bouvier et al., 2006; Bouvier et al, 2005; DellaPenna, 2005).

Although plants and microbes are the natural sources for useful terpenes, most of them are produced in a very small amount and often as complex mixtures. In contrast, *Botryococcus braunii* produces large quantities of triterpenes, but its slow growth makes it undesirable as a viable production platform (Niehaus et al., 2011). Nevertheless, metabolic engineering and synthetic biology offer many strategies to manipulate terpene metabolism in various biological systems to achieve high-valued terpene production with high yield and high fidelity for particular practical applications (Nielsen et al., 2011). Many successes have been achieved in engineering valuable terpenes in heterotrophic microbes, such as *Escherichia coli* (Nishiyama et al., 2002; Martin et al., 2003; Ajikumar et al., 2010) and *Saccharomyces cerevisiae* (Ro et al., 2006; Takahashi et al., 2007; Westfall et al., 2012; Zhuang and Chappell, 2015). The strategies developed in these efforts usually take advantage of specific microbe strains whose innate biosynthetic machinery are genetically modified to accumulate certain prenyldiphosphate precursors (e.g. IPP or FPP), which can be utilized by other introduced terpene synthase(s) for production of the desired terpene(s). For example, greater than 900 mg/L of bisabolene was produced when bisabolene synthase genes from plants were introduced into FPP-overproducing *E. coli* or *S. cerevisiae* strains (Peralta-Yahya et al., 2011). High levels of farnesane production for diesel fuels was also achieved by reductive hydrogenation of its precursor farnesene, which was generated from a genetically engineered yeast strain using plant farnesene synthases (Renninger, et al., 2008; Ubersax et al., 2010). However, terpene production using microbial platforms is still dependent on exogenous feedstocks (i.e. sugars) and elaborate production facilities, both of which add significantly to their production costs.

Compared to microbial systems, engineering terpene production in plant systems seems like an attractive target as well. This is because plants can take advantage of photosynthesis by using atmosphere CO₂ as their carbon resource instead of relying on exogenous carbon feedstocks. Moreover, crop plants such as tobacco can generate a large amount of green tissues efficiently when grown for biomass production (Schillberg et al., 2003; Andrianov et al., 2010), making them a robust, sustainable and scalable platform for large-scale terpene production. Nonetheless, compared to microbial platforms, there have only been a few examples of elevating terpene production in bioengineered plants. This is due partly to higher plants being complex multicellular organisms, in which terpene metabolism generally utilizes more complex

innate machinery that can be compartmentalized intracellularly and to cell/tissue specificities (Kempinski et al, 2015; Lange and Ahkami, 2013). Significant efforts have been made to overcome these obstacles to improve the production of valuable terpenes in plants, including monoterpenes (Lücker et al., 2004; Ohara et al., 2010; Lange et al., 2011), sesquiterpenes (Aharoni et al., 2003; Kappers et al., 2005; Wu et al., 2006; Davidovich-Rikanati et al., 2008), diterpenes (Besumbes et al., 2004; Anterola et al., 2009), and triterpenes (Inagaki et al., 2011; Wu et al., 2012). Among these, engineering terpene metabolism into a subcellular organelle, where the engineered enzymes/pathway can utilize unlimited/unregulated precursors as substrates, appears most successful. For example, Wu et al. expressed an avian FPP synthase (FPS) with foreign sesquiterpene/triterpene synthases targeted to the plastid, to divert IPP/DMAPP pool from the plastidic MEP pathway to synthesize high levels of the novel sesquiterpenes patchoulol and amorpho-4,11-diene up to 30 µg/g fresh weight, and the triterpene squalene up to 1,000 µg/g fresh weight (Wu et al., 2006; Wu et al., 2012). This strategy appears to be particularly robust because it avoids possible endogenous regulation of sesquiterpene and triterpene biosynthesis which occurs normally in the cytoplasm, as well as relies upon more plastic precursor pools of IPP/DMAPP inherent in the plastid which are primarily derived from the local CO₂ fixation (Wright et al., 2014).

The goal of the current study was to evaluate the prospects for engineering advanced features of triterpene metabolism from *Botryococcus* into tobacco, and thus to probe innate intricacies of isoprenoid metabolism in plants. In order to achieve this, we first introduced the key steps of botryococcene biosynthesis into specific subcellular compartments of tobacco cells under the direction of constitutive or trichome-specific promoters. The transgenic lines expressing the enzymes in the chloroplast were found to accumulate the highest levels of botryococcene. Triterpene methyltransferases were next introduced into the same intracellular compartments of selected high triterpene accumulating lines. A high yield of methylated triterpenes was also achieved in transgenic lines when the TMTs were targeted to the chloroplast. Through careful comparison of the levels of triterpenes and the methylated triterpene products in the various transgenic lines, we have also gained a deeper insight into subcellular distribution of the triterpene products in these transgenic lines, as well as a better understanding of methylation metabolism for specialized metabolites in particular compartments. These findings all contribute to our understanding of regulatory elements that control carbon flux through the innate terpene biosynthetic pathways operating in plants.

RESULTS

Targeting botryococcene biosynthesis to cytoplasm versus chloroplasts

The earlier study demonstrated that plastid-targeted engineering of a foreign squalene synthase and an FPP synthase can successfully divert carbon flux from the MEP pathway to accumulate a high level of squalene in transgenic tobacco (Wu et al., 2012). That study revealed the availability of IPP/DMAPP precursors were adequate and strong regulatory mechanisms were absent in the chloroplast for novel squalene (C30) production to occur. This, in turn, led us to utilize this strategy to engineer botryococcene (C30) biosynthesis into tobacco plants. However, botryococcene biosynthesis requires two squalene synthase-like enzymes, SSL-1 and SSL-3, to catalyze successive reactions to make the botryococcene product. This is in contrast to squalene biosynthesis which requires only a single enzyme, squalene synthase (Figure 1, Niehaus et al., 2011). We chose to over-express two chimeric versions of botryococcene synthase: one is SSL1-3 (Figure 2B), which is a fusion of the SSL-1 and SSL-3 enzymes by a peptide linker, which exhibited a 2-fold greater accumulation of botryococcene when expressed in yeast in comparison to simple co-expression of the two enzymes separately (Niehaus et al., 2011). The second design is referred to as SSL1-3M (Figure 2B) in which the SSL1-3 chimeric enzyme has 71 amino acids of the carboxy-terminus of *Botryococcus* squalene synthase (M) appended to its C-terminal. This construct thus contains a membrane spanning domain that was hypothesized to improve botryococcene productivity in engineered yeast by integrating the enzyme into the ER membrane in order to promote proximity between enzymes for substrates (Niehaus et al., 2011). The overall gene constructs thus consist of either botryococcene synthase SSL1-3 or SSL1-3M directed by cassava mosaic promoter (Pcv) (Verdaguer et al., 1996), and the avian FPS (Tarshis et al., 1994), driven by the 35S-CaMV promoter (Pca) (Benfey et al., 1990). An amino terminal, plastid targeting signal sequence (tp) from the Rubisco small subunit gene of *Arabidopsis* (Lee et al., 2006) was also inserted onto the chimeric SSL1-3 constructs to target these enzymes to the chloroplast compartment, whereas constructs without the signal sequence would target the encoded proteins to the cytoplasmic compartment. The respective gene constructs (Fig. 2B, Table 1) were introduced into *Nicotiana tabacum* accession KY 1068 by standard *Agrobacterium* transformation methodology. Thirty or more T0 independent transgenic lines were generated and the leaf materials from different transgenic plants were extracted and analyzed by GC-MS and GC-FID. When evaluated by GC, a unique molecule was detected in the extraction from some of the transgenic plants (Supplemental Fig. S1E) that was not evident in any of the wild type plants (Supplemental Fig. S1A). This unique

chemical peak had identical retention time and mass spectrum (Supplemental Fig. S2A) as a botryococcene standard (Niehaus et al., 2011), and was confirmed as botryococcene by ¹H-NMR and ¹³C-NMR analyses (Supplemental Figs. S3 and S4).

We observed that transgenic lines engineered with the construct that directed botryococcene synthase (SSL1-3) along with FPS to the chloroplasts (tpSSL1-3+tpFPS) (Fig. 2B) generate a high level of botryococcene (544 µg/g FW, maximum and 269 µg/g FW, average) (Fig. 2C, Table 1), which is about a 70-90 fold increase over the level of botryococcene (6.3 µg/g FW, maximum and 3.5 µg/g FW, average) accumulated in the lines with the same enzymes targeted to the cytoplasm (SSL1-3+FPS) (Fig. 2B). The results indicate that the chimeric SSL1-3 enzyme efficiently uses FPP as a substrate derived from the universal C5 precursors present in the chloroplast compartment supported by the accompanying engineered FPS. In contrast, the failure to enhance botryococcene yield by cytosolic engineering might be due to the cytosolic FPP pool being low and highly regulated, even if the avian FPS is used to over-ride potential regulatory mechanisms in the cytoplasm (Wu et al., 2006; Wu et al., 2012). The overall production of botryococcene by plastid targeting of this metabolism, and its increase over that achieved by cytosolic engineering coincided well with what was found earlier for engineering squalene biosynthesis by Wu et al. (2012).

A similar contrast was also found in comparison of production by engineering the membrane tethered version of botryococcene synthase in the chloroplasts (tpSSL1-3M) to that directing the same metabolism to the cytoplasm (SSL1-3M). A relatively high amount of botryococcene accumulation was achieved by plastidic engineering, with a maximum level of 202 µg/g FW and average level of 131 µg/g FW, which is about a 10 to 20-fold increase over that for cytosolic engineering with a maximum of 16.4 µg/g FW and average of 5.8 µg/g FW (Table 1, Fig. 2). As with the soluble form of SSL1-3, the low production by cytosolic engineering of SSL1-3M suggests a limited flux of carbon and/or restrictive regulation occurring in the cytoplasm, but absent in the chloroplast. The membrane targeting of SSL1-3M to internal membranes like the ER may enhance access to more readily available substrates in the cytoplasm, which might account for why the cytosolic engineered lines of SSL1-3M accumulated a higher level of botryococcene than was achieved by lines engineered for cytosolic, functionally soluble SSL1-3. In contrast, plastid targeted SSL1-3M yielded only half the level of botryococcene as the plastid, soluble SSL1-3 form. Why this differential response of the soluble and membrane forms of the SSL1-3 enzyme in the chloroplast and cytoplasm is unknown, but certainly the stroma

compartment is physically distinct from the cytoplasm, as are the thylakoid membranes versus the ER membranes.

Development dependent and tissue specific accumulation

As expected, botryococcene accumulation demonstrated a significant developmental dependence. The level of botryococcene accumulating in mature leaves was 2 to 4-fold higher than in young leaves (Table 1, Fig. 2). This makes sense biochemically because the engineered botryococcene and FPP synthases were expressed constitutively, and thus had more time to biosynthesize and accumulate botryococcene over the developmental time course of leaf maturation. In addition, there is no known mechanism in plants or any other organisms for the catabolism of botryococcene. Hence, accumulation should primarily reflect biosynthesis.

To determine if the various transgenic plants accumulated triterpenes in other tissues beside leaves, the triterpene chemical profiles across various tissues were determined for select transgenic lines (Fig. 3). The lines selected for this comparison were homozygous for the transgenes (T2 generation) and targeted either squalene or botryococcene biosynthesis to the cytoplasm versus plastid compartments. The squalene accumulating lines were previously described by Wu et al., 2012 and designated as tpSS+tpFPS #5. The botryococcene lines were tpSSL1-3M+tpFPS #31 and tpSSL1-3+tpFPS #10. Triterpene content (either squalene or botryococcene) was found in all the tissues examined, but the levels varied dramatically. Leaf accumulation was greatest and up to 64-fold more than found in roots. Low amounts of triterpenes, never exceeding 25 µg/g, were also observed in other tissues like veins and stems.

Trichome specific expression of botryococcene metabolism

Trichomes are specialized organs located on the surface of the aerial parts of plant species, which can be the site of abundant specialized metabolite biosynthesis, accumulation and secretion. In tobacco, for instance, up to 15% of the leaf dry weight has been attributed to secretion of leaf exudate from trichomes (Wagner et al., 2004). Such a large contribution to leaf biomass relative to the actual volume of the glandular trichomes makes trichome engineering an attractive target for metabolite bioengineering (Ennajdaoui et al., 2010). Hence, in an effort to direct botryococcene biosynthesis to secretory trichomes, the trichome-specific promoters of the cembratrienol synthase and cembratrienol hydroxylase genes (Wang et al., 2002a; Ennajdaoui et al., 2010) were used to direct botryococcene synthase and FPS expression, respectively, to

the secretory trichomes of tobacco. To strengthen the overall trichome specific expression, the 35S double enhancer element was also appended to the 5' prime end of each of the trichome promoters (Wu et al., 2012). Four constructs harboring SSL1-3 or SSL1-3M with FPS, plus/minus chloroplast amino terminal sequences (tp), were thus introduced into the tobacco accession, *Nicotiana tabacum* 1068, an accession documented to have high trichome density (Nielsen et al., 1982). Almost 30 independent transgenic lines were generated for each construct, the resulting transgenic lines propagated in greenhouse facilities, and different sized leaves were analyzed for their botryococcene content (Table 1).

Targeting botryococcene metabolism to the chloroplasts of trichomes resulted in only a modest accumulation of botryococcene in young and maturing leaf tissue (<30 µg/g FW on average), which was 10-20 fold greater than that accumulated in the lines with the enzymes directed to the cytoplasm of trichome cells (Table I). However, more unexpectedly, plants with putative enhanced, trichome-specific expression of SSL1-3 and FPS targeted to the chloroplast showed a strong chlorotic, white, mottling, and dwarf phenotype (Fig. 4A, right and B), which certainly contributed to difficulties in propagating these materials. This phenotype was also much more severe than anything observed with trichome specific expression of squalene biosynthesis (Wu et al., 2012). These adverse phenotypes were surprising because we had hoped to engineer botryococcene metabolism away from tissues important for normal growth and development. Instead, we speculate that the trichome-specific promoters in combination with the double 35S enhancers may not behave in the anticipated manner (that is, not trichome specific) and exhibit ectopic expression (higher than that of the other constitutive promoters used here) in tissues that are more sensitive to this type of metabolism (e.g. meristems) resulting in the deleterious phenotypes described.

Engineering triterpene methyltransferases into particular subcellular compartments of tobacco plants

The success in engineering squalene and botryococcene C30 production in transgenic tobacco led us to take advantage of these high triterpene accumulating lines for possible triterpene methylation. Our working hypothesis was that if we introduced triterpene methyltransferases into these lines, the accumulating triterpene (C30) could be converted to their methylated forms (C31 and C32) if the methyltransferases were targeted to where the triterpenes were synthesized and accumulated, and if the methyl donor substrate S-adenosylmethionine were available in sufficient quantities for the methyltransferase activity. We also wished to evaluate

the substrate specificity of the TMTs for squalene and botryococcenes as was done in yeast by Niehaus et al. (2012). Due to the hydrophobic regions present in the TMTs (which may function as transmembrane domains), it was equally important to evaluate if these enzymes could be expressed and function in the chloroplast and cytoplasm compartments.

To address these questions, all three of the TMTs genes were individually constructed with a strong constitutive promoter (CaMV 35S promoter), plus or minus a plastid targeting signal sequence, and engineered separately into squalene and botryococcenes accumulating lines (Fig. 5, Table II). Many independent transformants for each of the parental lines previously engineered for squalene biosynthesis and accumulation in the plastid compartment (a T2 homozygous line, line #5, tpSQS+tpFPS), or botryococcenes accumulation in the plastid compartment (T1 generation heterozygous for tpSSL1-3+tpFPS, line #10, or T1 generation heterozygous for tpSSL1-3M+tpFPS, line #31) were subsequently transformed with each of the methyltransferases targeted to the cytoplasm (TMT-1, TMT-2 and TMT-3) or plastid compartment (tpTMT-1, tpTMT-2 and tpTMT-3), and the resulting transformants screened for their triterpene chemical content and composition. More specifically, each of the transgenic lines was evaluated for their triterpene (C30), mono- (C31) and di-methylated (C32) triterpene contents by GC-MS (Supplemental Figs. S1 and S2). Because of the large number of transgenic lines generated and evaluated, Table II summarizes the data for all the various lines and Fig. 5 illustrates the design strategies and provides analysis for 3 example lines for each of the TMT constructs.

When the transgenic TMT-1 enzyme was targeted to the chloroplasts of the squalene accumulating line, the maximum level of squalene methylation was 91% with the average across all the lines being 65% (Table II). Of the methylated forms, approximately two-thirds was in the dimethylated form (Table II and Fig. 5). When TMT-2 was plastid targeted in the same parental background line, the maximum level of squalene methylation was 82% of the total, with an average of 51% for the 36 lines accumulating methylated squalenes (Table II). In contrast to the TMT-1 lines, only about one-third of the methylated squalene was in the dimethylated form in the TMT-2 plastid targeted lines (Table II and Fig. 5).

When TMT-1 or TMT-2 were targeted to the cytoplasm rather than the chloroplasts, only 4% of the total squalene, on average, was methylated by either of the methyltransferases. But, 100% of the methylated squalene was in the dimethylated form (Table II and Fig. 5). The small amount of squalene available to the methyltransferases under these conditions could arise from

cytosolic squalene synthesized by the native machinery, or that synthesized by mistargeted engineered squalene synthase not properly transported into the chloroplast.

In contrast, only 5% (average) of the total squalene was methylated when TMT-3 was targeted to the chloroplast of the high squalene producing line, suggesting that TMT-3 exhibited weak catalytic activity towards squalene (Supplemental Table SI), which was also observed when substrate specificity for TMT-3 was investigated in yeast (Niehaus et al., 2012). Targeted expression of TMT-3 to the cytoplasm in the same line did not result in any methylation products, also corroborating the inability of the TMT-3 to utilize the limited amounts of squalene found in this compartment (Supplemental Table SI).

When the TMT-3 enzyme was introduced into the botryococcene (C30) accumulating lines, we observed a large proportion of methylated botryococcene only when TMT-3 was targeted to the chloroplasts (Table II and Fig. 5). Using parental line tpSSL1-3M+tpFPS, 87% of the botryococcene was maximally methylated with 54% of the total botryococcene being methylated on average across all the 18 lines evaluated. In comparison, maximally 66% of the botryococcene was methylated in parental line tpSSL1-3+tpFPS, but more typically 35% (average) was methylated (Table 2). The apparent improved efficiency of botryococcene methylation in the tpSSL1-3M line versus tpSSL1-3 line might, however, be more of a reflection on the total botryococcene levels rather than the efficiency of the methylation reaction itself. The line engineered with tpSSL1-3M accumulates about half as much botryococcene as tpSSL1-3, and assuming similar expression levels of TMT-3 in both parental lines and comparable amounts of SAM availability, the greater percentage of methylated botryococcene in tpSSL1-3M may simply reflect the smaller pool of botryococcene available for secondary modifications. Little to none of the chloroplast synthesized botryococcene was methylated when either TMT-1 or TMT-2 were targeted to the chloroplast (Supplemental Table SI), further demonstrating the striking substrate preference of TMT-3 for botryococcenes, and TMT-1 and TMT-2 for squalene.

By comparison, only a small proportion of methylated botryococcene was formed when TMT-3 was targeted to the cytoplasm. For parental line tpSSL1-3M+tpFPS, 14% maximal and on average only 6% of the total botryococcene was methylated, whereas 10% maximal and on average only 3% of the botryococcene produced in parental line tpSSL1-3+tpFPS was methylated (Table II). Like that suggested above for squalene, the low level of methylated botryococcene produced by cytosolic TMT-3 could arise from either botryococcene (C30)

produced by mistargeted SSL1-3(M) generating triterpene substrate in the cytoplasm, or leakage of botryococcene from the chloroplast synthesized pool.

The observation of methylated squalene in the lines with squalene biosynthesis targeted to the chloroplast, yet the methyltransferases expressed in the cytoplasm, raised a question about what pool of squalene was being methylated. Was it the squalene synthesized in the chloroplast by the engineered squalene synthase, or could it be a reflection of the squalene synthesized by the native biosynthetic machinery operating in association with the endoplasmic reticulum? Because differentiating between pools of native versus engineered squalene in the tpSQS line has proven to be technically difficult, an alternative approach was sought. Gene constructs directing expression of the TMT-1 and TMT-2 enzymes targeted to the chloroplast and cytoplasm were introduced into control, wild type tobacco and the resulting transgenic lines screened for methylated squalene. Interestingly, a significant proportion of methylated squalene (average 41% of total squalene) was observed when TMT-1 was targeted to the chloroplasts (Table III), where there is no evidence for squalene biosynthesis or accumulation (Aharoni et al., 2003). In this case, one plausible explanation is that some mistargeting of the engineered TMT-1 results in methylation of cytoplasmic biosynthesized squalene. In contrast, when TMT-1 and TMT-2 expression were targeted to the cytoplasm of wild type plants, a high proportion of methylated squalene (average 72% and 67% of total squalene respectively) was found (Table III, Fig. 6). But even more surprising, the level of total squalene (C30+C31+C32) in transgenic lines expressing TMT-1 was elevated to a maximum of 55 $\mu\text{g/g}$ FW and an average of 36 $\mu\text{g/g}$ FW (Table III). This was about 4 to 7- fold greater than the level of endogenous squalene (C30) accumulating in wild type plants (Table III and Fig. 6). Equally important, the level of non-methylated squalene remained relatively constant in all these lines at 6 to 10 $\mu\text{g/g}$ FW with all the additional triterpene accumulating as mono- and di-methylated squalene.

Developmental Triterpene Accumulation and Methylation

To explore the possible influence of development processes on the methylation status of squalene and botryococcenes, 3 independent lines for each expression vector combination (squalene biosynthesis targeted to the plastid compartment alone with plastid targeted TMT-1 or TMT-2, and botryococcenes biosynthesis plus TMT-3 directed to the plastid compartment) were grown in the greenhouse for approximately 3 months, then leaves at 4 developmental positions on the plants were profiled for their triterpene levels and methylation status. The data in Fig. 7 hence represents the average determinations for leaves from 3 independent transformants for

each engineered combination. As expected, the total triterpene levels showed a successive increase with leaf maturation. The more mature leaves generally had more total triterpene accumulation (Figure 7, left axis). However, the average ratio of methylated (C31+C32) to total triterpene (C30+C31+C32) at the various leaf positions remained essentially the same from 55% to 75% for all the transgenic lines (Figure 7B, right axis).

Phenotypes of triterpene accumulating plants

Over 75% of the botryococcene accumulating lines directing this metabolism to the chloroplast exhibited moderate to distinguishing phenotypes, including dwarfing, chlorosis and mottling (table I and Figure 8A, B, C). These phenotypes were not observed in any transgenic lines wherein the engineered metabolism was targeted to the cytoplasm (Table I) and was obviously different from what was observed in any of squalene accumulating plants (Fig. 8E middle) (Wu et al., 2012). The visual observations thus indicated that botryococcene accumulation had some undefined effects on chloroplast development, plant morphology and growth while squalene accumulation did not. Moreover, there were not any noticeable differences in phenotypes between triterpene accumulating plants and their respective methylated triterpene counterpart plants (Figure 8D, E), hence methylation of botryococcenes was not able to restore wild type phenotype.

DISCUSSION

The current work successfully transplanted several steps of triterpene metabolism occurring in the algae *Botryococcus braunii* race B into tobacco plants, leading to a high level accumulation of botryococcene and methylated triterpenes. The most robust triterpene accumulation relied upon the strategy of diverting the 5 carbon precursors IPP and DMAPP from the MEP pathway operating in the chloroplast to triterpene (C30) biosynthesis by the co-expression of an FPS plus a triterpene synthase. The accumulating triterpenes (C30) in the transgenic plants could be further modified by targeting yet another enzyme activity, TMT, to the chloroplasts of these transgenic plants. Hence, engineering novel expression of the enzymes FPS, triterpene synthases and TMTs created a new metabolic channel redirecting carbon flux from the MEP

pathway to the biosynthesis and accumulation of unique and unusual triterpenes (Figure 1, 2A and 5A).

The strategy was successful in taking advantage of engineering terpene metabolism in the plant chloroplasts. First, chloroplasts offer an unrestricted abundance of carbon passing through the MEP pathway, and diverting an intermediate and carbon flux from this pathway does not adversely impact the biosynthetic needs in the chloroplasts, for large amounts of carotenoids and chlorophylls. The second, equally important, observation is that the chloroplast provides an ideal environment for heterologous terpene production, perhaps due to the endogenous regulation of the MEP pathway in plastids, allowing for a new carbon sink in the form the introduced triterpenes, as compared to the MVA pathway operating in the cytosol preventing the high IPP/DMAPP flux into these compounds (Kempinski et al., 2015).

This approach has now been demonstrated to be applicable for the metabolic engineering of various types of terpene compounds including monoterpenes, sesquiterpenes, and triterpenes in tobacco plants. However, we also note that accumulation level of each type of terpene differed substantially between the respective terpene targets. Plants engineered for triterpene production accumulated 200 to 1000 $\mu\text{g/g}$ FW of triterpene, whereas sesquiterpene production has not exceeded 30 $\mu\text{g/g}$ FW and monoterpene accumulation is maximally in the range of 1 $\mu\text{g/g}$ FW (Wu et al., 2006; Kempinski et al., 2015). Such stark differences strongly suggest that the limitation in specific terpene class accumulation lies with the engineered terpene synthase or that certain introduced terpene compounds may have differing effects on physiological homeostasis and growth. Consistent with this notion, we found that overexpression and chloroplast targeting of the soluble form of SSL1-3 with FPS yielded similar levels of botryococcene accumulation to that for squalene which was achieved by plastid-targeted engineering of a yeast, soluble SQS along with the avian FPS. However, two times more botryococcene was observed with SSL1-3 than expressing the SSL1-3M enzyme form with FPS targeted to the chloroplast. It suggests that the chimeric enzyme SSL1-3 functions as well as the single yeast SQS enzyme targeted to the chloroplast, and exhibits a higher catalytic capacity than SSL1-3M could.

TMTs are functionally insoluble enzymes, which exhibit an unexpectedly high catalytic activity for the methylation reaction when engineered into both the chloroplast and cytoplasm compartments of the appropriate transgenic plant lines. Up to 91% of the C30 triterpenes accumulating in the high yielding lines was subsequently transformed to mono- or di-methylated

triterpene when one of the three TMT genes targeted methyltransferase activity to the chloroplast. The methylation ratio of 51%-91% by TMTs directed to the plastid compartment versus 3%-14% by TMTs targeted to the cytosol provide additional evidence to show that the distribution of triterpene C30 in the high triterpene accumulating transgenic lines remained in the chloroplast. This was not unexpected because the C30 triterpenes are supposedly synthesized in the chloroplast and methylation in the cytosol would require some mechanism, either active or passive, to export the novel triterpene out of the chloroplasts to the cytoplasm.

Therefore, in order to account for the small but significant methylation of triterpenes occurring in the cytoplasm, at least four possible routes remain plausible: First, the methylated squalene produced by targeting TMT-1 and TMT-2 to the cytoplasm in the wildtype plants proves that natively synthesized squalene can be methylated by TMTs; Second, the small amount of methylated botryococcene generated in plants wherein TMT-3 was directed to the cytoplasm while high botryococcene biosynthesis was directed to the chloroplasts [tpSSL1-3(M)+tpFPS], could arise from a low level of botryococcene (C30) biosynthesized by mistargeted SSL1-3(M) (Table II). This notion implies that TMTs can methylate cytosolic triterpene (C30) produced by mistargeted triterpene synthase as well as by the native triterpene machinery; Third, expressing the tpTMT-1 in wild type plants also resulted in methylated products, which must be derived from cytosolic endogenous squalene catalyzed by mistargeted TMT-1. This evidence, not surprisingly, suggests that our chloroplast targeting strategy is not 100% effective and supports our contention that mistargeted TMTs are also able to methylate cytosolic-localized triterpenes. Fourth, the cytosolic engineered TMTs may have a way to access the plastidic-localized squalene. The recent discovery that plastid envelope localized substrates can be accessed by enzymes targeted to ER membrane through a continuity of ER and chloroplast (Mehrshahi et al., 2013) offers one possible explanation. Of course, the methylation status of triterpenes could come about by some combination of routes, which might also be variable upon plant development and growth habit.

An issue raised during the initial phases of this work was whether there would be sufficient SAM to support formation of the methylated triterpenes. This concern arose because of an appreciation for how important SAM is to methylation of macromolecules as well as very diverse small molecules (Bouvier et al., 2006; Sauter et al., 2013) and its known biosynthesis in the cytoplasm (Ravanel et al., 1998; Ravanel et al., 2004). Fortunately, concern for SAM availability seemed unfounded regardless if the methylation reactions were targeted to the chloroplasts or to the cytoplasm. Although there was reduced TMT efficiency in the cytoplasm, this is most

likely due to the reduced amount of triterpene available for methylation and thus reflects TMT efficiency and not SAM availability.

Equally interesting was the observation that plants engineered for botryococcene accumulation tended to exhibit distinct phenotypic outcomes like dwarfism, chlorosis and mottling, while plants accumulating high levels of squalene did not show any of these adverse effects. Why this might be so is currently unknown. However, if one were able to discern how the plants were able to accumulate high levels of squalene without any negative impact on growth performance, then one might be able to use this information in the engineering of advanced accumulation mechanisms for terpenes like botryococcene. One suggestion worth examining is how botryococcenes versus squalene might differentially interdigitate into membranes and disrupt normal biochemical functions. Hence, engineering alternative means for sequestering these molecules could alleviate physiological consequences and improve overall accumulation (e.g. engineering in lipid droplet forming proteins).

Finally, squalene biosynthesis is known to be a key committed step in sterol biosynthesis, and squalene has been suspected of serving a regulatory role (Wu et al., 2012). The results presented here where introducing cytosolic forms of the TMT enzymes elevated overall squalene and methylated squalene levels 4 to 7 fold higher than normal, directly address this issue. By diverting squalene to its methylated forms, some innate mechanism had to be evoked to allow for additional squalene production to occur. This, we suggest, is providing an important glimpse into the regulatory complexity of squalene biosynthesis, which is crucial for homoeostatic control of sterol biosynthesis in the plants.

MATERIALS AND METHODS

Expression vector construction and plant transformation

Design of gene constructs and assembly for engineering botryococcene biosynthesis were based on the work previously described by Wu et al. (2006) and Wu et al., (2012) using standard molecular methodologies. Gene constructs consisted of a peptide fusion of SSL-1 (GenBank accession: HQ585058.1) and SSL-3 (GenBank accession: HQ585060.1) connected by a triplet repeat peptide linker of GGSG, with or without appending the carboxy-terminal (71 amino acids) of the *Botryococcus* squalene synthase (GenBank AF205791.1) onto the carboxy-

termini of the SSL-3, and the FPS gene (P08836) (Tarshis et al., 1994). The chimeric SSL1-3 genes and FPS genes were inserted downstream of strong constitutive promoters Pcv (Verdaguer et al., 1996) and Pca (Benfey et al., 1990) respectively. For trichome specific expression of triterpene biosynthesis, the trichome-specific promoters Pcbt (Ennajdaoui et al., 2010) or the Pcyp16 (Wang et al., 2002a) were fused to 5' end of botryococcene synthase genes and FPS gene respectively. The duplicated CAMV 35S enhancer elements (Benfey et al., 1990) were fused to the 5' end of each trichome promoter. A chloroplast targeting signal sequence (tp) encoding for the first 58 amino acids of the *Arabidopsis* Rubisco small subunit gene (NM23202)(Lee et al., 2006) was fused in-frame with the 5' end of the respective terpene synthase genes. The gene cassette were assembled together in a helper vector described in Wu et al. (2012) by standard molecular biology methods and the various DNA segments were verified by DNA sequencing. The gene cassettes were then introduced into pBDON, a modified Ti plasmid vector harboring a hygromycin resistance gene by DNA recombination (Wu et al., 2006).

The triterpene methyltransferase genes TMT-1 (JN828962.1), TMT-2 (JN828963.1), and TMT-3 (JN828964.1) were inserted directly into plant transformation vector pKYLx71 (Schardl, et al., 1987) harboring a 35S viral promoter and a kanamycin resistance gene. In order to target TMT genes to the chloroplast, the chloroplast targeting signal sequence (tp) noted above was then inserted in-frame with the 5' termini of the respective TMT genes.

The engineered Ti plasmid vectors were introduced into *Agrobacterium tumefaciens* GV3850 by electroporation, and the resulting *Agrobacterium* lines were used to genetically engineer *Nicotiana tabacum* (tobacco) TI accession 1068 (Nielsen et al. 1982) or transgenic line of tpSQS+tpFPS #5 (T2 homozygous generation) with high level of squalene, as previously described by Wu et al. (2012), or high botryococcene accumulating transgenic lines (tpSSL1-3+tpFPS-10, or tpSSL1-3M+tpFPS-31, T1 heterozygous generation) generated in this study. Leaf explants were transformed with the respective gene constructs and the resulting calli were selected on tissue culture media with hygromycin (50 mg/l) for engineering botryococcene biosynthesis and with both hygromycin (50 mg/L) and kanamycin (250 mg/L) for engineering methylated triterpene biosynthesis. The culture media (1L) contained 4.2 g MS salts (Phytotechnology Laboratories, Overland Park, KS), 0.112 g B5 vitamins (Phytotechnology Laboratories), 30 g sucrose, 9 g agar, 1 mg indole-3-acetic acid and 2.5 mg benzylaminopurine (Sigma). The selected calli were grown under sterile tissue culture conditions to regenerate

584 plantlets. The selected T0 plantlets were then propagated in the greenhouse and assessed for
585 triterpene content by GC–MS or GC-FID analyses.

588 **Plant propagation and segregation selection**

589 All the T0 plantlets after hygromycin or kanamycin selection were grown in common commercial
590 vermiculite/soil blends in a greenhouse and fertilized weekly with water soluble fertilizer (20-20-
591 20 for nitrogen, phosphorus, and potassium). Insect control was performed as needed. The T0
592 plants were allowed to flower in the greenhouse and the T1 seed collected for subsequent
593 cycles of propagation. Segregation of the hygromycin and kanamycin resistance trait in the T1
594 seed lines was also evaluated by germinating sterilized seeds on 50 mg/L hygromycin and 250
595 mg/L kanamycin in T- tissue culture media (4.2 g MS salts, 0.112 g B5 vitamins, 30 g sucrose,
596 and 9 g agar in 1 L medium).

597 **Triterpene (squalene, botryococcene, methylated squalene and botryococcene)** 598 **determinations**

599 Fifty to one hundred and fifty mg of transgenic leaf material were collected from the upper most,
600 fully expanded leaves of tobacco plants grown in a greenhouse. The other plant tissues roots,
601 stem, and veins were collected from plants grown in the tissue culture for chemical analysis.
602 The terpene content for each sample was determined by the methods previously described in
603 Wu et al. (2012). Each plant sample was ground in liquid nitrogen, then extracted with 2-4 ml of
604 a hexane:ethyl acetate mixture (v/v 85:15) containing 200 ng of α -cedrene as an external
605 standard for quantification and calculations of recovery. The extracts were concentrated to 500
606 μ l under a nitrogen stream without drying the sample. The concentrated extracts were then
607 partially purified by passing through a silica column (500 mg, prepared in glass wool plugged
608 glass pipette) and further eluted with 1 ml of the hexane solvent. After concentration of the
609 combined eluate under a stream of nitrogen, aliquots were injected onto a GC–MS equipped
610 with a HP5-MS capillary column (30 m $\times$ 0.32 mm, 0.25 μ m phase thickness) with the following
611 temperature program of 70°C for 1 min, followed by a 4 °C per min gradient to 250 °C. Mass
612 spectra were recorded at 70 eV, scanning from 35 to 500 atomic mass units, and experimental

samples were compared with standards that were previously used in earlier studies (Wu et al., 2012, Niehaus et al., 2011 and 2012) for verification.

The structure of purified botryococcene from tobacco was determined by ^1H -NMR and ^{13}C -NMR spectral analyses, which were also described in an earlier study (Wu et al., 2012). Botryococcene was extracted from leaf material of transgenic line (tpSSL1-3+tpFPS-10) #10 targeting the chimeric botryococcene synthase SSL1-3 and FPS to the plastid compartment under the direction of the constitutive promoters.

One hundred g leaf material was ground in liquid nitrogen, then extracted with 1.2 L of hexane:ethyl acetate (85:15), the extract concentrated to 5 ml, and the extract fractionated on a silica column with 5 ml aliquots of hexane as the eluting solvent. Fractions were monitored by GC-MS for the desired triterpene compound. Enriched fractions were pooled, concentrated under nitrogen, and the entire sample processed by silica HPLC–PDA using hexane as the eluting solvent (Niehaus et al. 2012, Wu et al., 2012). Alternatively, the crude extract was resuspended in hexane and fractionated via silica gel chromatography, a final purification step provided by HPLC. Recovery of 6 mg of purified botryococcene sample with a 50% yield was obtained. ^1H -NMR and ^{13}C -NMR spectra were recorded on a 400-MHz Varian J-NMR spectrometer at 300 K, and chemical shifts were referenced relative to solvent peaks, namely δH 7.24 and δC 77.0 for CDCl_3 .

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Triterpene and methylated triterpene contents were determined in leaf extracts from elite transgenic plants by GC-MS.

Supplemental Figure S2. Mass spectra of C30 botryococcene (A) produced in transgenic lines expressing SSL1-3 or SSL1-3M, C31 monomethylated botryococcene (B) and C32 dimethylated botryococcene (C) produced in lines expressing TMT-3 in a botryococcene accumulating line, C31 monomethylated squalene (D) and C32 dimethylated squalene (E) produced in lines expressing TMT-1, TMT-2 or TMT-3 in a squalene accumulating line.

Supplemental Figure S3. (6E,10R,11E,13R,16E)-botryococcene (C30 botryococcene) isolated from plants 1H-NMR (400 MHz).

Supplemental Figure S4. ^{13}C -NMR (100 MHz) of botryococcenes isolated from plants.

Supplemental Table S1. Screen of T0 transgenic lines targeting select TMT activities to the chloroplast or the cytoplasm of high squalene (tpSQS+tpFPS) and botryococcene (tpSSL1-3+tpFPS) accumulating lines for their methylated triterpene content.

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Z.J. and J.C. conceived the research plan; Z.J., C.K., C.B. and S.E.N. performed the experiments; Z.J., C.K. and J.C. wrote the article.

Table I. Chemical assessment of T0 transgenic lines for their botryococcene content¹

Construct	Expression type	# of lines evaluated	# of lines having botryococcene	Young (µg/g)			Mature (µg/g)			# of lines exhibited phenotype
				Min	Max	Ave	Min	Max	Ave	
tpSSL1-3+tpFPS	Constitutive	60	34	0.2	275	91.4	0.9	544	269	25
tpSSL1-3M+tpFPS	Constitutive	75	24	5	110	48	1.1	202	131	19
SSL1-3M+FPS	Constitutive	38	17	0.5	5.4	1.4	1.0	16.4	5.8	0
SSL1-3+FPS	Constitutive	20	6	0.5	7.1	2.6	0.5	6.3	3.5	0
e2tpSSL1-3M+e2tpFPS	Trichome	62	19	0.6	53.8	16.2	1.1	105	32.8	15
e2tpSSL1-3+e2tpFPS	Trichome	37	9	0.9	75	22	3.2	8.5	5.3	6
e2SSL1-3M+e2FPS	Trichome	60	18	0.7	2.3	1.7	0.5	8.1	3.1	0
e2SSL1-3+e2FPS	Trichome	29	5	1.0	6.7	2.1	0.5	14.7	4.1	0

¹Wild type tobacco (*Nicotiana tabacum* accession 1068) was transformed with each indicated constructs consisting of chimeric botryococcene synthase gene (SSL1-3 or SSL1-3M) and the avian farnesyl diphosphate (FPS) gene, inserted downstream of constitutive promoters (cassava vein mosaic viral promoter and cauliflower mosaic viral promoter, respectively), or enhanced trichome specific promoters (two 35S enhancers fused to cembratrienol synthase and hydroxylase promoters, respectively) (Ennajdaoui et al., 2010; Erming et al., 2002). More than 20 independent lines for each indicated construct were generated and grown under greenhouse conditions. The first fully expanded leaf from each plant was sampled for botryococcene content after 1 month (young) and 6 months (mature). Botryococcene levels were analyzed by GC-FID and the average (ave), as well as the minimum (min) and maximum (max) reported. The transgenic lines under constitutive promoters having crinkle leaf and dwarf phenotypes (Fig 8. A left, B and C), and those under the trichome specific promoters scored as chlorotic and dwarf are noted (Fig. 4A right and B).

Table II. Chemical assessment of T0 transgenic lines targeting TMT activity to the chloroplast or the cytoplasm of high squalene (tpSQS) or botryococcene (tpSSL1-3; tpSSL1-3M) accumulating lines for their methylated triterpene contents¹

Construct	Parental line	# of lines evaluated	# of lines having methylated triterpene	triterpene/total (ave)			Conversion: (C31+C32)/total					Total triterpene ave (µg/g)
				C30	C31	C32	# <10%	# 10%-50%	# >50%	highest	ave	
tpTMT-1	tpSQS+tpFPS #5	75	47	34%	25%	41%	2	26	19	91%	65%	222
tpTMT-2	tpSQS+tpFPS #5	69	36	50%	31%	18%	2	17	17	82%	51%	301
TMT-1	tpSQS+tpFPS #5	36	11	96%	0	4%	11	0	0	7%	4%	251
TMT-2	tpSQS+tpFPS #5	33	12	96%	0	4%	12	0	0	6%	4%	241
tpTMT-3	tpSSL1-3M+tpFPS #31	76	18	46%	17%	37%	3	3	12	87%	54%	131
TMT-3	tpSSL1-3M+tpFPS #31	40	19	94%	3%	3%	15	4	0	14%	6%	126
tpTMT-3	tpSSL1-3+tpFPS #10	69	29	65%	10%	25%	0	23	6	66%	35%	283
TMT-3	tpSSL1-3+tpFPS #10	40	17	97%	1%	2%	16	1	0	10%	3%	294
	tpSQS+tpFPS #5	4	0	100%	0	0	4	0	0	0	0	234
	tpSSL1-3+tpFPS #10	3	0	100%	0	0	3	0	0	0	0	237
	tpSSL1-3M+tpFPS #31	3	0	100%	0	0	3	0	0	0	0	125

¹More than 30 independent lines were generated for each transformation construct consisting of one of the three TMT genes targeting triterpene methyltransferase activity to the chloroplast (with tp) or the cytoplasm (without tp) of the indicated parental lines. The squalene (tpSQS+tpFPS-5) or the botryococcene accumulating lines (tpSSL1-3+tpFPS-10, or tpSSL1-3M+tpFPS-31) were transformed with the indicated TMT construct and evaluated after 4 months by GC-FID/GC-MS for methylated triterpenes. The number of transgenic lines accumulating methylated squalene or botryococcene were scored, along with the average (ave) percentage of non-methylated (C30), monomethylated (C31) or dimethylated (C32) triterpenes relative to total triterpene (C30+C31+C32) content. Percentage of methylated triterpene (C31+C32) to total triterpene is denoted as conversion, and the number of plants with less than 10% (<10%), between 10% and 50% (10%- 50%), and above 50% (>50%) conversion for each construct were counted accordingly. The highest amount of conversion as well as the average conversions are noted. The average total triterpene content for each line is also noted, as is the average triterpene content for three or four plants for each of the parental (control) lines.

Table III. Chemical assessment of T0 transgenic lines targeting TMT activity to the chloroplast or the cytoplasm of wild type plants for their methylated squalene contents¹

Constructs	Parental line	# of lines evaluated	# of lines having methylsqualene	triterpene/total			Conversion					Total triterpene (ave)
				C30	C31	C32	# <10%	# 10%-50%	# >50%	highest	ave	
tpTMT-1	Wild type	9	2	65%	0	35%	0	1	1	61%	41%	8.9
tpTMT-2	Wild type	7	0	100%	0	0	0	0	0	0	0	6.3
TMT-1	Wild type	14	3	24%	10%	66%	0	0	3	82%	72%	36
TMT-2	Wild type	5	2	38%	11%	50%	0	1	1	85%	67%	16.8
	Wild type	3	0	100%	0	0	0	0	0	0	0	8.2

¹Independent lines were generated for each of the indicated constructs consisting either TMT-1 or TMT-2 targeted to the chloroplast (with tp) or the cytoplasm (without tp) of wild type plants. T0 antibiotic-selected transgenic plants were propagated in the greenhouse for up to 5 months before their triterpene and methylated triterpene contents was determined by GC-MS. The number of transgenic plants accumulating methylated squalene were scored and their average (ave) percentage of the non-methylated (C30), mono- (C31) or di- (C32) methylated squalenes determined relative to the total squalenes (C30+C31+C32). Percentage of methylated squalene (C31+C32) to total triterpene is denoted as conversion, and the number of plants with less than 10% (<10%), between 10% and 50% (10%- 50%), and above 50% (>50%) conversion for each construct were counted accordingly. The highest amount of conversion as well as the average conversions are noted. The average total squalene content for each line is also noted, as is the average squalene content for three of the wild type control plants.

Figure Legends

Figure 1. A depiction of the catalytic roles of novel squalene synthase-like enzymes (SSL) and triterpene methyltransferases (TMT) in *Botryococcus braunii* race B and their putative contributions to the triterpene constituents (Niehaus et al., 2011). SSL-1 catalyzes the condensation of two farnesyl diphosphate molecules (FPP) to pre-squalene diphosphate (PSPP), which is converted to either squalene or botryococcene by SSL-2 or SSL-3, respectively. Squalene can also be synthesized directly from the condensation of two FPP molecules catalyzed by squalene synthase (SQS). TMT-1 and TMT-2 transfer the methyl donor group from AdoMet (SAM) to squalene to form mono- and di-methylated squalene, whereas TMT-3 acts on botryococcene to form mono- and di-methylated botryococcene (Niehaus et al., 2012).

Figure 2. Triterpene content of independent T0 transgenic lines transformed for novel botryococcene synthesis. Schematic outline of the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways operating in the cytoplasm and chloroplast compartments, respectively, and conceptual strategies to divert carbon flux from these two pathways for the biosynthesis of botryococcene by heterologous expression of avian FPP synthase (FPS) and a chimeric botryococcene synthase (SSL1-3) targeted to membranes or not with a carboxy-terminal membrane spanning domain (M) (**A**). *Nicotiana tabacum* accession KY 1068 was transformed with the indicated gene constructs (**B**), consisting of the chimeric botryococcene synthase: squalene synthase-like enzymes SSL-1 fused to SSL-3 via a linker peptide with a membrane associating domain (M) (SSL1-3M) or without (SSL1-3) (Niehaus et al., 2011), coupled with the avian farnesyl diphosphate synthase (FPS) gene. Expression of both engineered genes were under the direction of strong, constitutive viral promoters (cassava vein mosaic viral promoter, Pcv; cauliflower mosaic viral promoter, Pca, respectively) (Benfey et al., 1990; Verdaguer et al., 1998). A plastid targeting signal sequence (tp) was fused to the 5' end of the respective genes where indicated. Thus, the constructs with tp will target the enzymes to the chloroplasts, and those without tp will express the enzyme in the cytoplasm. Antibiotic selected T0 lines propagated in the greenhouse were assessed from botryococcene accumulation at a relatively young age (1 month old, light green) and mature age (6 month old, green) by GC-MS (**C**). Three representative, independent elite transgenic lines from each engineered construct were chosen to illustrate their capacity for botryococcene production.

Figure 3. Triterpene accumulation in different tissues of transgenic lines. Select transgenic lines and wild type plants were grown in the tissue culture for 3 months. The plant materials from different tissues (root, stem, vein and leaf) were then sampled for their triterpene contents. The gene construct and the accumulating triterpene monitored are indicated.

Figure 4. Phenotype of a transgenic plant expressing trichome specific, plastid targeted botryococcene biosynthesis (tpSSL1-3+tpFPS) (right, panel A) in comparison to wild type control plant (left, panel A). The control plant was initiated from seed while the transgenic line is a T0 generation plant transferred from the tissue culture regeneration process. The plants were grown under greenhouse conditions for approximately 2 months. Panel B is a close-up of the transgenic line in panel A.

Figure 5. Methylated triterpene content in independent transgenic lines targeting TMT activity to the chloroplast or the cytoplasm of high triterpene accumulating lines. Conceptual strategies to convert triterpenes accumulated in transgenic plants where the carbon flux from MEP pathway is diverted to novel triterpene (C30) biosynthesis by heterologous expressed terpene synthase (TS) and farnesyl diphosphate synthase (FPS), then methylated by triterpene methyltransferase (TMT) targeted to the chloroplast or cytoplasm (**A**). The gene constructs harboring indicated TMT genes targeting to the chloroplast (with tp) or the cytoplasm (without tp) (**C**) were transformed into parental lines accumulating high levels of squalene or botryococcene (**B**). The antibiotic selected T0 lines propagated in the greenhouse were assessed for their triterpene and methylated triterpene content at their 4 months old by GC-MS and GC-FID. The level of triterpene and methylated triterpene accumulation of three elite independent lines was shown as non-methylated (C30, green) mono (C31, blue) and dimethyl (C32, orange) methylated triterpene (**C**).

Figure 6. Accumulation of total triterpene content and the fraction of triterpene converted to methylated triterpenes over a time course of leaf development. Transgenic lines directing squalene (tpSQS+tpFPS) biosynthesis and squalene methylation by TMT-1 or TMT-2 to the chloroplast compartment, or directing botryococcene (tpSSL1-3+tpFPS) biosynthesis and botryococcene methylation by TMT-3 to the chloroplast compartment were grown under greenhouse conditions for 4 months. Leaves at the noted positions relative to the apex of the plant were collected and their triterpene and methylated triterpene contents determined by GC-MS. Absolute levels of total triterpenes (C30, C31 and C32) are reported in the histogram (blue), while the fraction of triterpene converted to methylated forms are denoted by the scatter plot (red).

Figure 7. Methylated triterpene content in independent transgenic lines targeting TMT activity to the chloroplast or cytoplasm of wild type plants. Conceptual strategies how endogenous squalene biosynthesized by wild type plants in the cytoplasm might be methylated by methyltransferases (TMT-1, TMT-2) directed to the chloroplast or cytoplasm (**A**). Gene constructs harboring TMT genes targeting the triterpene methyltransferase to the chloroplast (with tp) or the cytoplasm (without tp) were transformed into wild type plants (**B**), and the antibiotic selected T0 lines propagated in the greenhouse were assessed for their triterpene content (4 months old) by GC-MS. The level of squalene (C30, green) and methylated squalenes (C31, blue; C32, orange) accumulating in three independent lines for each construct was determined in the first fully mature leaf of each line by GC-MS (**C**).

Figure 8. Phenotypes of transgenic lines expressing botryococcene and squalene biosynthetic genes. Some transgenic lines targeting botryococcene biosynthesis to the chloroplast (tpSSL1-3+tpFPS) and hence accumulating high levels of botryococcene are dwarfed (**A**, left plant; right, wild type plant) and exhibit chlorotic, mottled, and wrinkled leaf morphologies (**B** and **C**). Addition of triterpene methyltransferase activity targeted to the chloroplasts of these botryococcene accumulating lines (tpTMT-3→tpSSL1-3+tpFPS) did not result in a wild type phenotype (**D**, left plant; right, wild type plant). In contrast, plants targeting squalene biosynthesis to the chloroplast (tpSQS+tpFPS) and accumulating high levels of squalene did not exhibit such phenotypes, nor when triterpene methyltransferases were also targeted to the chloroplasts (tpTMT-1 or tpTMT-2→tpSQS+tpFPS, left and middle plants in panel **E**, respectively; wild type control on right).

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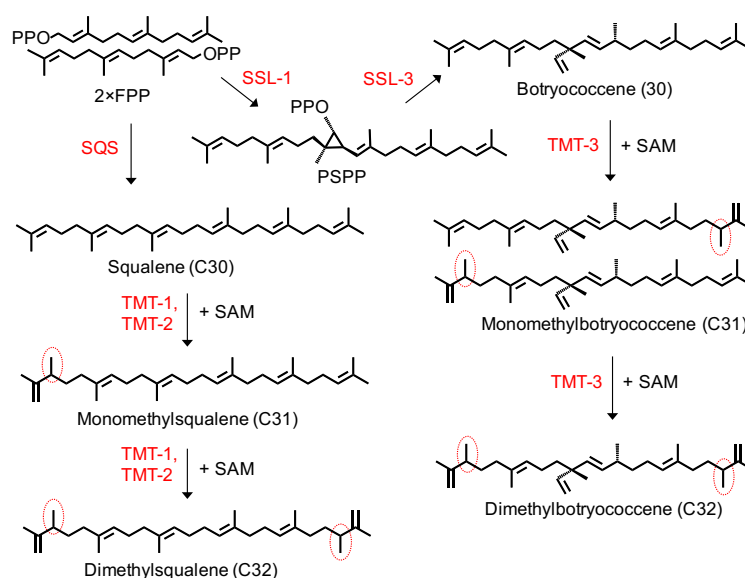


Figure 1. A depiction of the catalytic roles of novel squalene synthase-like enzymes (SSL) and triterpene methyltransferases (TMT) in *Botryococcus braunii* race B and their putative contributions to the triterpene constituents (Niehaus et al., 2011). SSL-1 catalyzes the condensation of two farnesyl diphosphate molecules (FPP) to pre-squalene diphosphate (PSPP), which is converted to either squalene or botryococcene by SSL-2 or SSL-3, respectively. Squalene can also be synthesized directly from the condensation of two FPP molecules catalyzed by squalene synthase (SQS). TMT-1 and TMT-2 transfer the methyl donor group from AdoMet (SAM) to squalene to form mono- and di-methylated squalene, whereas TMT-3 acts on botryococcene to form mono- and di-methylated botryococcene (Niehaus et al., 2012).

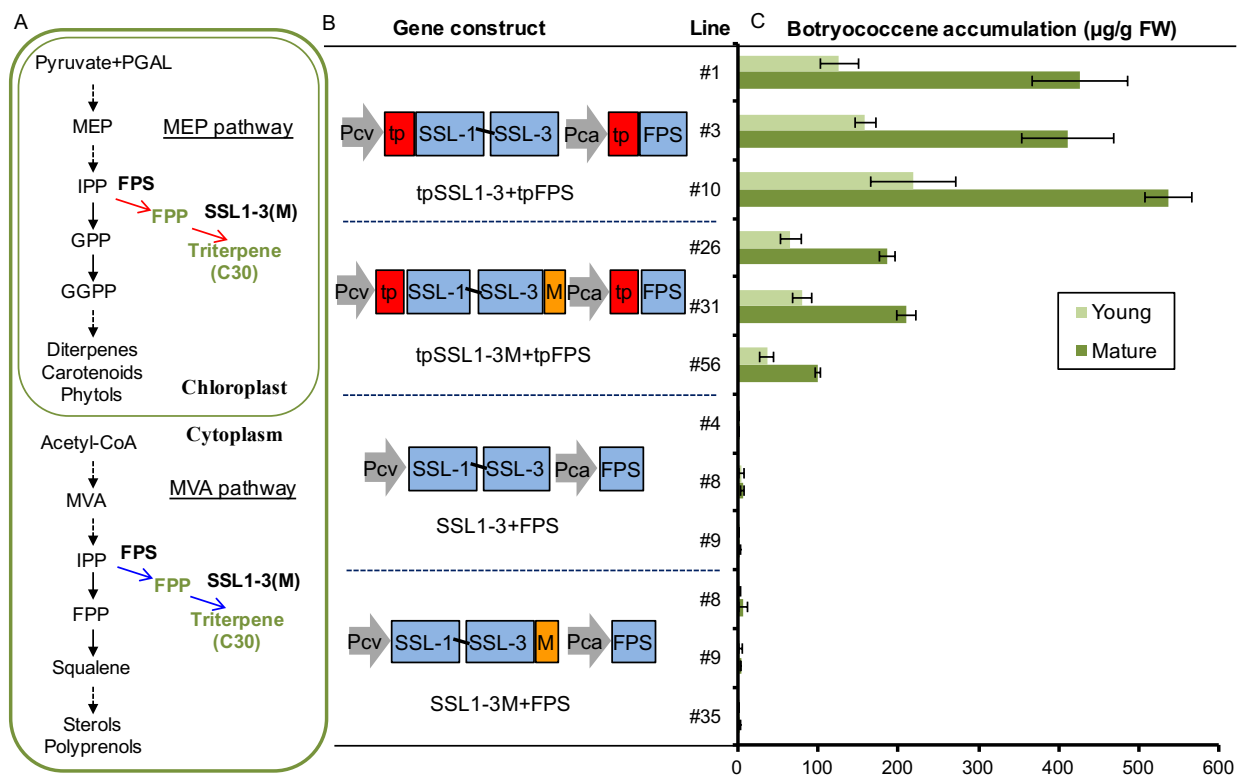


Figure 2. Triterpene content of independent T0 transgenic lines transformed for novel botryococcene synthesis. Schematic outline of the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways operating in the cytoplasm and chloroplast compartments, respectively, and conceptual strategies to divert carbon flux from these two pathways for the biosynthesis of botryococcene by heterologous expression of avian FPP synthase (FPS) and a chimeric botryococcene synthase (SSL1-3) targeted to membranes or not with a carboxy-terminal membrane spanning domain (M) (**A**). *Nicotiana tabacum* accession KY 1068 was transformed with the indicated gene constructs (**B**), consisting of the chimeric botryococcene synthase: squalene synthase-like enzymes SSL-1 fused to SSL-3 via a linker peptide with a membrane associating domain (M) (SSL1-3M) or without (SSL1-3) (Niehaus et al., 2011), coupled with the avian farnesyl diphosphate synthase (FPS) gene. Expression of both engineered genes were under the direction of strong, constitutive viral promoters (cassava vein mosaic viral promoter, Pcv; cauliflower mosaic viral promoter, Pca, respectively) (Benfey et al., 1990; Verdaguer et al., 1998). A plastid targeting signal sequence (tp) was fused to the 5' end of the respective genes where indicated. Thus, the constructs with tp will target the enzymes to the chloroplasts, and those without tp will express the enzyme in the cytoplasm. Antibiotic selected T0 lines propagated in the greenhouse were assessed from botryococcene accumulation at a relatively young age (1 month old, light green) and mature age (6 month old, green) by GC-MS (**C**). Three representative, independent elite transgenic lines from each engineered construct were chosen to illustrate their capacity for botryococcene production.

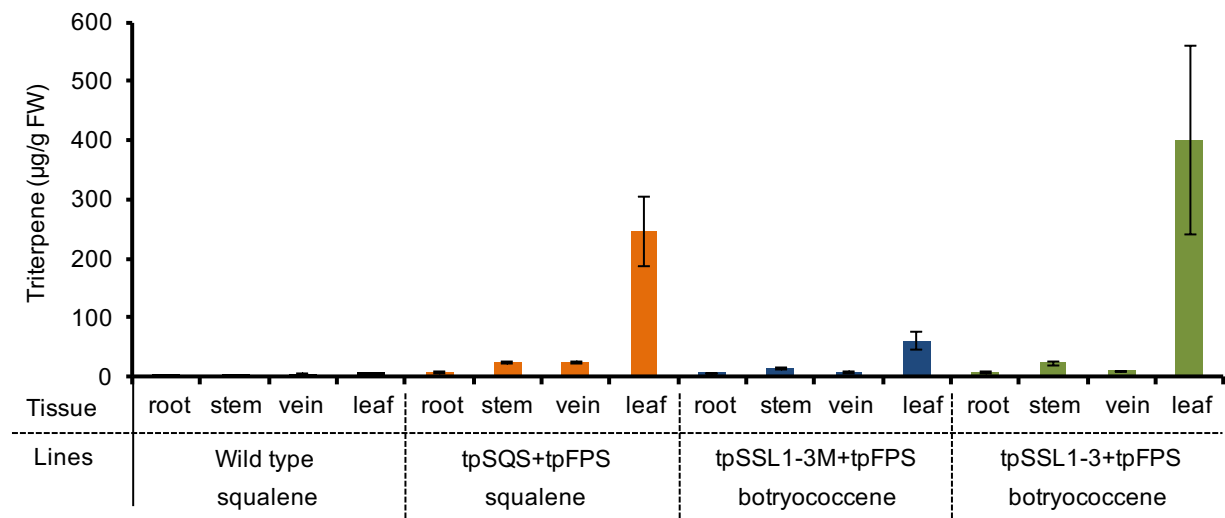


Figure 3. Triterpene accumulation in different tissues of transgenic lines. Select transgenic lines and wild type plants were grown in the tissue culture for 3 months. The plant materials from different tissues (root, stem, vein and leaf) were then sampled for their triterpene contents. The gene construct and the accumulating triterpene monitored are indicated.

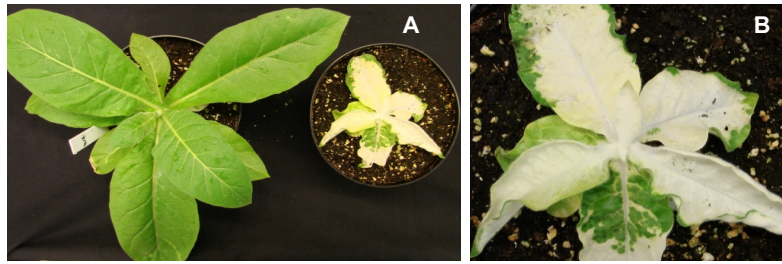


Figure 4. Phenotype of a transgenic plant expressing trichome specific, plastid targeted botryococcene biosynthesis (tpSSL1-3+tpFPS) (right, panel A) in comparison to wild type control plant (left, panel A). The control plant was initiated from seed while the transgenic line is a T0 generation plant transferred from the tissue culture regeneration process. The plants were grown under greenhouse conditions for approximately 2 months. Panel B is a close-up of the transgenic line in panel A.

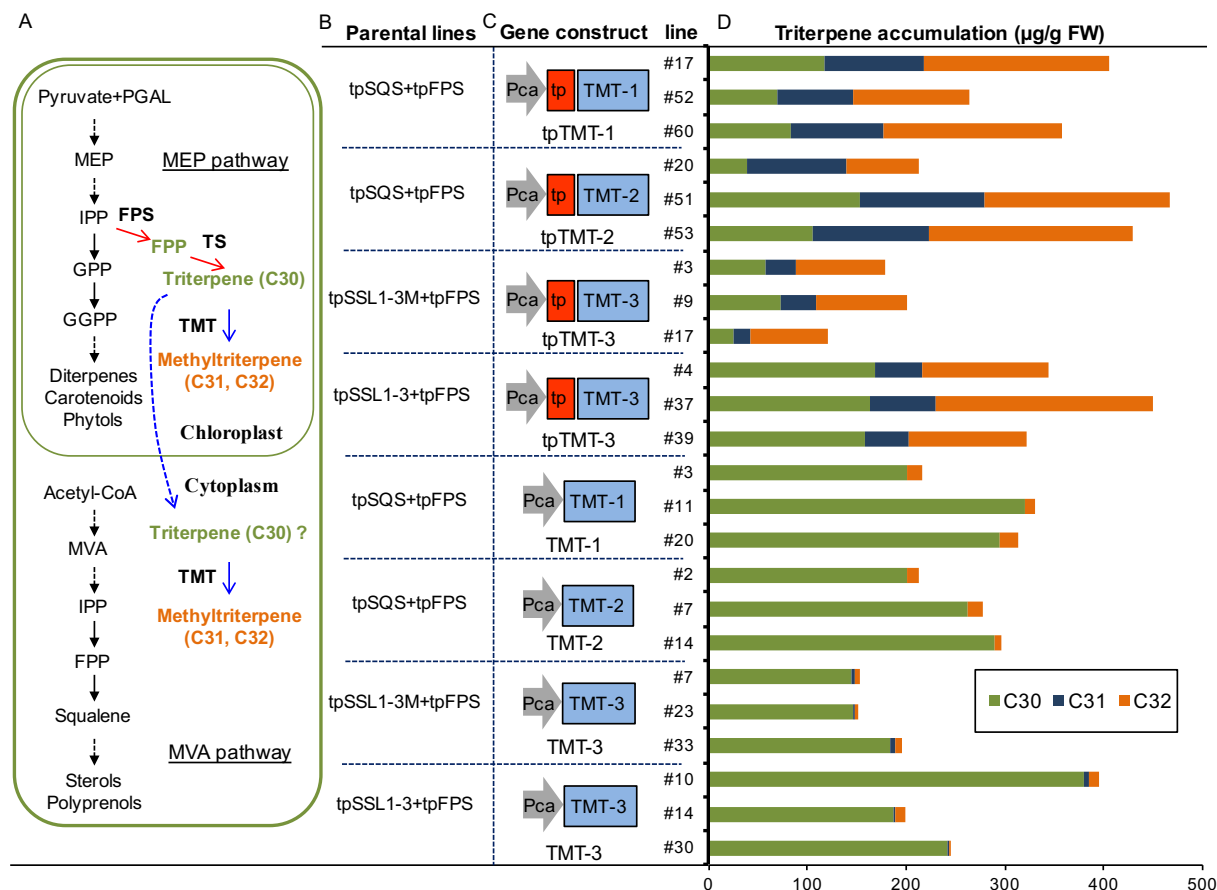


Figure 5. Methylated triterpene content in independent transgenic lines targeting TMT activity to the chloroplast or the cytoplasm of high triterpene accumulating lines. Conceptual strategies to convert triterpenes accumulated in transgenic plants where the carbon flux from MEP pathway is diverted to novel triterpene (C30) biosynthesis by heterologous expressed terpene synthase (TS) and farnesyl diphosphate synthase (FPS), then methylated by triterpene methyltransferase (TMT) targeted to the chloroplast or cytoplasm (**A**). The gene constructs harboring indicated TMT genes targeting to the chloroplast (with tp) or the cytoplasm (without tp) (**C**) were transformed into parental lines accumulating high levels of squalene or botryococcene (**B**). The antibiotic selected T0 lines propagated in the greenhouse were assessed for their triterpene and methylated triterpene content at their 4 months old by GC-MS and GC-FID. The level of triterpene and methylated triterpene accumulation of three elite independent lines was shown as non-methylated (C30, green) mono (C31, blue) and dimethyl (C32, orange) methylated triterpene (**C**).

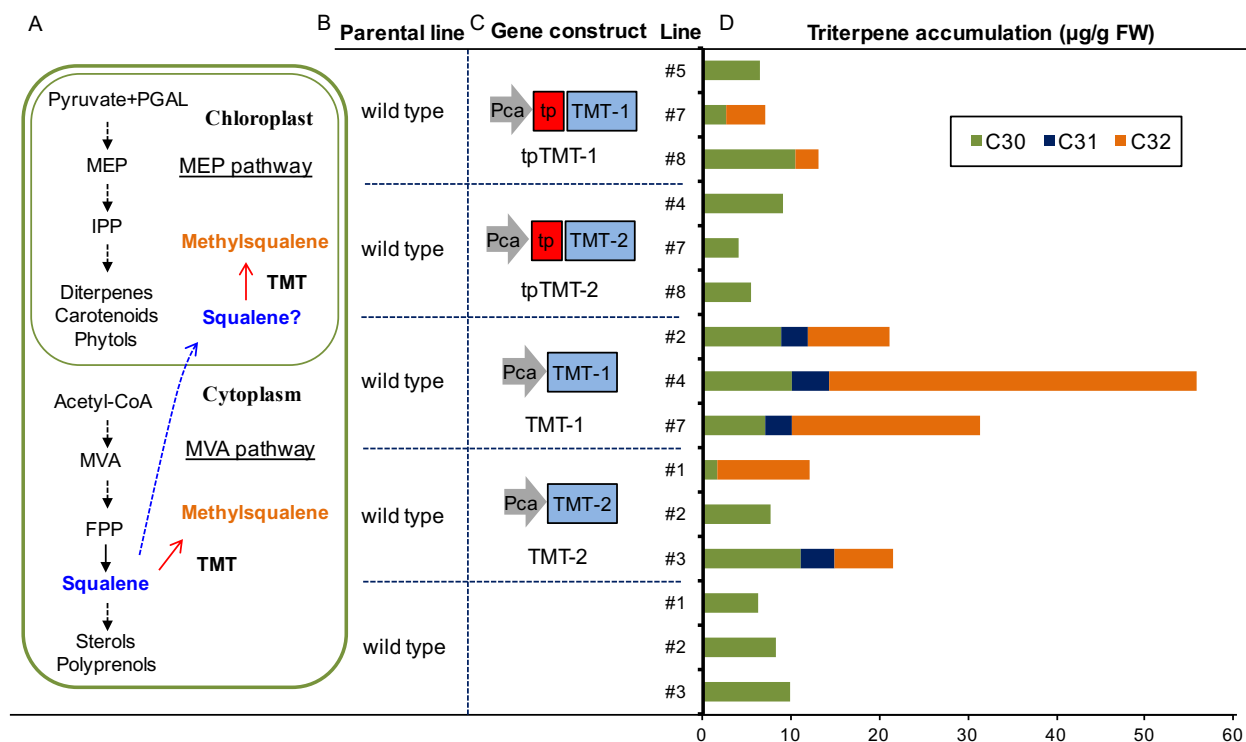


Figure 6. Accumulation of total triterpene content and the fraction of triterpene converted to methylated triterpenes over a time course of leaf development. Transgenic lines directing squalene (tpSQS+tpFPS) biosynthesis and squalene methylation by TMT-1 or TMT-2 to the chloroplast compartment, or directing botryococcene (tpSSL1-3+tpFPS) biosynthesis and botryococcene methylation by TMT-3 to the chloroplast compartment were grown under greenhouse conditions for 4 months. Leaves at the noted positions relative to the apex of the plant were collected and their triterpene and methylated triterpene contents determined by GC-MS. Absolute levels of total triterpenes (C30, C31 and C32) are reported in the histogram (blue), while the fraction of triterpene converted to methylated forms are denoted by the scatter plot (red).

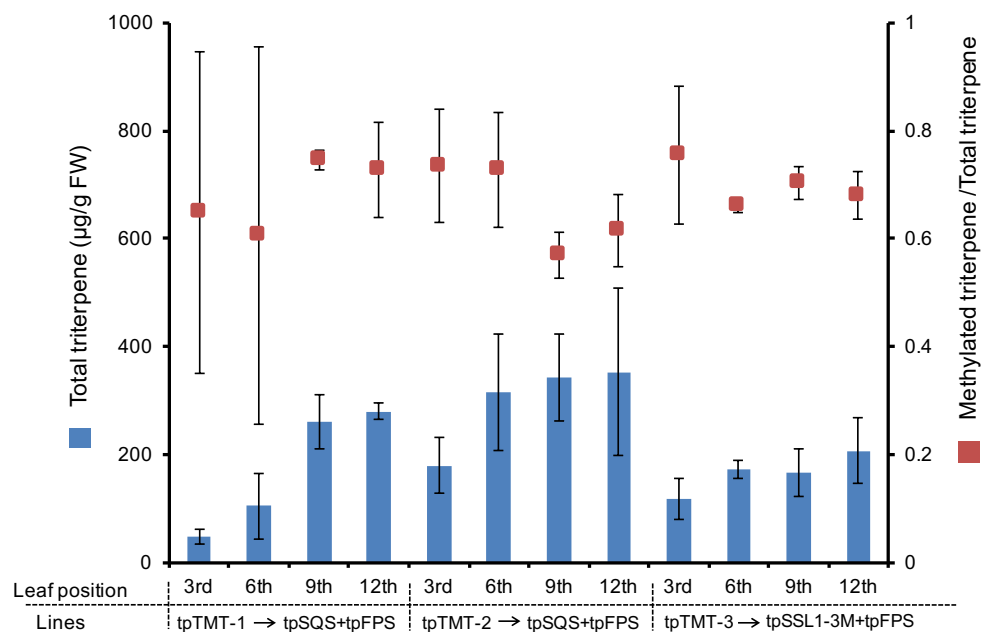


Figure 7. Methylated triterpene content in independent transgenic lines targeting TMT activity to the chloroplast or cytoplasm of wild type plants. Conceptual strategies how endogenous squalene biosynthesized by wild type plants in the cytoplasm might be methylated by methyltransferases (TMT1, TMT2) directed to the chloroplast or cytoplasm (A). Gene constructs harboring TMT genes targeting the triterpene methyltransferase to the chloroplast (with tp) or the cytoplasm (without tp) were transformed into wild type plants (B), and the antibiotic selected T0 lines propagated in the greenhouse were assessed for their triterpene content (4 months old) by GC-MS. The level of squalene (C30, green) and methylated squalenes (C31, blue; C32, orange) accumulating in three independent lines for each construct was determined in the first fully mature leaf of each line by GC-MS (C).

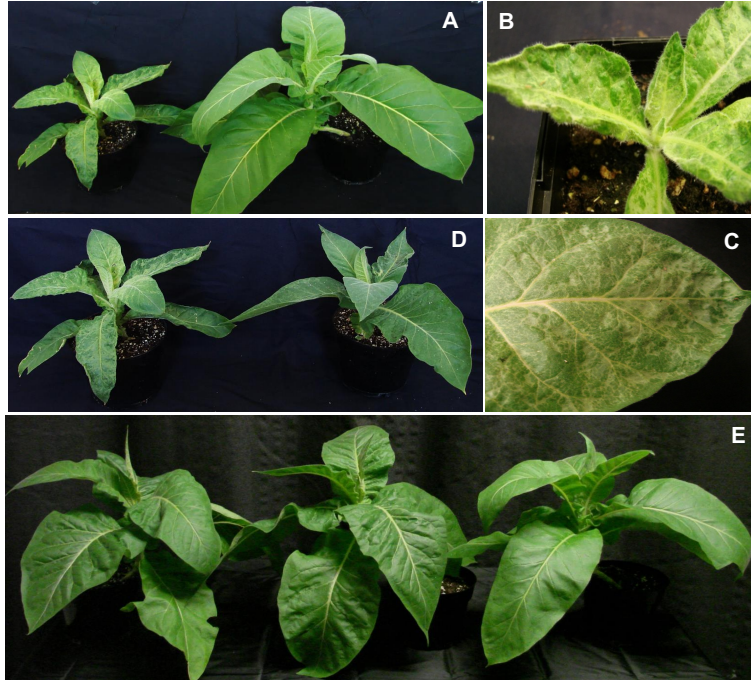


Figure 8. Phenotypes of transgenic lines expressing botryococcene and squalene biosynthetic genes. Some transgenic lines targeting botryococcene biosynthesis to the chloroplast (tpSSL1-3+tpFPS) and hence accumulating high levels of botryococcene are dwarf (A, left plant; right, wild type plant) and exhibit chlorotic, mottled, and wrinkled leaf morphologies (B and C). Addition of triterpene methyltransferase activity targeted to the chloroplasts of these botryococcene accumulating lines (tpTMT3 + tpSSL1-3+tpFPS) did not rescue the wild type phenotype (D, left plant; right, wild type plant). In contrast, plants targeting squalene biosynthesis to the chloroplast (tpSQS+tpFPS) and accumulating high levels of squalene did not exhibit such phenotypes, nor when triterpene methyltransferases were also targeted to the chloroplasts (tpTMT1 or tpTMT2 + tpSQS+tpFPS, left and middle plants in panel E, respectively; wild type control on right).

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