Within and beyond organelle engineering: strategies for increased terpene production in yeasts and plants

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Abstract

Synthetic biology programs for the increased production of bioactive plant-derived terpenes initially focused on linear aspects of their biosynthetic pathways. Yet, the spatial organization of terpene pathways, typically across multiple cellular compartments, seriously encumbers engineering success. Here, we discuss the recent advances in the endoplasmic reticulum, peroxisome and other organellar engineering and illustrate how this is being applied to increase terpene pathway performances in plants and yeasts. We also discuss how specialized transporters could present potent novel tools to connect cellular compartments. Altogether, these new perspectives demonstrate how synthetic biology can offer real-world solutions for the efficient and sustainable production of high-value terpenes and eventually address the shortcomings of extraction from natural resources.

Introduction

Terpenes comprise a large group of chemically diverse compounds that are mostly found within the plant kingdom [1]. The vast array of structures and physiochemical features of these molecules are reflected in their multiple biological activities finding numerous applications for human health [2,3]. However, because large-scale and sustainable production of plant-derived terpenes is hindered by the often low in planta metabolite levels [4], plant engineering is required [5, 6, 7]. Alternatively, the introduction of terpene biosynthetic pathways in robust microbial hosts, such as Escherichia coli and Saccharomyces cerevisiae, is a frequently pursued substitute. However, plant terpene pathways are typically highly compartmentalized; they involve numerous enzymes operating in multiple subcellular compartments [4,8]. Consequently, microbial hosts are often not readily suitable to host the heterologous terpene pathway [6]. Concomitantly, the engineering of superior plant lines remains a valid option for the sustainable production of numerous high-value plant terpenes [5, 6, 7,9].

Classical metabolic engineering efforts for terpene production generally focus on boosting metabolic fluxes, both in microbes and plants. However, more recently, strategies focusing on subcellular compartmentalization and organellar engineering are demonstrating exciting potentials. In this review, we will, therefore, discuss the recent advances in organellar engineering and how these are expanding the toolkit for synthetic biology programs for plant terpenes, in particular in yeasts and plants, as production hosts. All examples and strategies are schematically summarized and visualized in Figure 1, Figure 2.

Localization matters: pathway compartmentalization

Ensuring an adequate cell environment for terpene production is crucial for optimized production. Modulating substrate availability in particular subcellular compartments or redirecting enzymes to a specific organelle, different from its native environment, can promote precursor flow and enzyme activity. Furthermore, spatial segregation of biosynthetic pathways may allow to reduce competition for substrates and cofactors between endogenous and heterologous path-ways to concentrate substrates and increase reaction kinetics and reduce the chances of cell toxicity by intermediates [4,10].

As such, targeting the expression of integrated terpene pathways to mitochondria in S. cerevisiae was shown to create an efficient way for the dedicated conversion of precursors into specialized mono- and sesquiterpenes [11,12], presumably in part because the mitochondrial precursor pool could not be consumed by the endogenous, cytosolic ergosterol pathway. A more comprehensive review on harnessing yeast mitochondria for chemical production was published recently [13].

Similarly, given that the physiological roles of peroxisomes seem dispensable in yeast [14], peroxisomes represent a favorable location for metabolic engineering. Indeed, targeting among others, the precursor mevalonate (MVA) pathway to the peroxisome allowed to increase squalene production more than a hundredfold [15]. Interestingly, inspiration for this approach partly came from the observation that squalene overproduced in the cytoplasm of engineered yeast cells was distributed in oil droplet-like structures, which turned out to be ‘inflated’ peroxisomes, indicating that yeast peroxisomes could be dynamic terpene depots. Subsequent dual modulation of cytoplasmic and peroxisomal engineering led to squalene levels of over 10 g/L in fed-batch fermentation [15]. Likewise, a combination of peroxisomal and cytoplasmic engineering enhanced the accumulation of the sesquiterpene α-humulene in yeast [16]. Peroxisomes were also shown to be excellent microfactories for monoterpene production, providing additional advantages for specialized reactions catalyzed by cytochrome P450s, for example, needed for the synthesis of precursors of monoterpene indole alkaloids and menthol [17]. Targeting pathways to the peroxisome is also particularly useful when dealing with enzymes or products that are toxic for heterologous organisms. This was elegantly

illustrated in yeast engineered for the production of plant alkaloids targeting norcoclaurine synthase to the peroxisomes increased the cellular titer of its product (S)-reticuline and alleviated its toxicity when expressed in the cytosol [18].

Many of a plant's defense metabolites, including terpenes, are often as toxic for themselves as for other organisms. Therefore, compartmentalized production of plant-specialized metabolites is often a strategy of self-protection [19]. Accordingly, redirection of metabolic pathways into non-native plant compartments may result in growth imbalances or undesired pathway reprograming, thereby decreasing the titers of the target metabolite. Nonetheless, successful examples of targeted compartmentalization for plant-based terpene production have been reported [20, 21, 22∗]. For instance, the production of the antimalarial sesquiterpene artemisinin in tobacco leaves was optimized by the dual introduction of the MVA precursor and the committed artemisinin biosynthetic pathway genes in the chloroplast and nuclear genomes, respectively [20]. Likewise, targeting the three committed enzymes for synthesis of the sorghum cyanogenic glycoside dhurrin to the chloroplast thylakoid membrane in Nicotiana benthamiana leaves resulted in a fivefold increase in dhurrin production and a concomitant decrease of side-products [21]. Conversely, rerouting of the diterpene biosynthesis pathway from the chloroplast to the cytosol increased titers of the diterpene momilactone B in N. benthamiana, as it enabled connecting to the cytosolic high-flux cytosolic MVA pathway that provides the building blocks for and caused a 10-fold increase in momilactone production when compared with endogenous production in rice [22].

Remodeling the biosynthetic factories: engineering subcellular organelles

The squalene example [15] illustrates that providing the appropriate environment for the accumulation of hydrophobic terpenes can boost their production. In this regard, lipid droplets (LDs), which are endoplasmic reticulum (ER)-derived organelles, have attracted a lot of attention. LDs are predominantly involved in lipid metabolism and offer a hydrophobic phase environment for lipid storage [23]. As such, they have been successfully targeted to anchor terpene biosynthetic enzymes both in yeasts and plants [24]. Moreover, combinatorial strategies in which pathway fluxes are coupled with LD size engineering have been explored. For instance, deletion of FLD1, the ortholog of the mammalian LD size regulator seipin in S. cerevisiae, in combination with the overexpression of a fatty acid desaturase, enabled the increased accumulation of lycopene in LDs [25]. Similarly, increased flux through the MVA pathway coupled with improved production of LDs led to several hundredfold increases in squalene production in S. cerevisiae [26]. In another example, targeting a normally ER-located cytochrome P450 enzyme involved in ginsenoside biosynthesis to the LD membrane increased the ginsenoside production in S. cerevisiae [27]. However, combining this enzyme retargeting with LD proliferation affected the enzyme's efficiency, indicating much remains to be learned about engineering natural organelles.

Also, in plants, the potential of LD engineering has been explored. Several approaches, including overexpression of lipid biosynthesis enzymes, of oleosins (proteins embedded in the LD membrane) targeted to the chloroplast or plant terpene enzymes fused to an LD surface protein from microalgae were successfully used in N. benthamiana leaves to enhance the production of distinct terpenes [28, 29, 30∗]. Cytosolic LD formation in N. benthamiana was achieved by the production of a key regulator of plastid fatty acid biosynthesis and an LD surface protein from Nannochloropsis oceanica with analogous functions as plant oleosins [30].

Also, the ER itself is a popular organellar engineering target. The ER is central to the synthesis of a wide diversity of plant metabolites. In yeast, it was demonstrated that ER expansion increases its metabolic capacity. Loss of function of the enzyme phosphatidic acid phosphatase (PAH1), which is involved in membrane lipid synthesis, stimulated the accumulation of recombinant ER-localized plant terpene

biosynthesis enzymes and ultimately boosted in vivo plant terpene accumulation in engineered S. cerevisiae strains [31]. Similarly, overexpression of INO2, encoding an ER size regulation factor, in S. cerevisiae led to increased production of squalene and ginsenosides [32]. In a recent study, a synergistic approach was used to increase β-carotene yields in S. cerevisiae. Overexpression of β-carotene biosynthetic enzymes was coupled with the deletion of several phosphatidate phosphatase genes, including PAH, which resulted in increased β-carotene production, and was accompanied by elevated levels of phospholipids and triacylglycerols, the principal component of LDs [33].

Unlike in yeast, only few attempts toward ER engineering in plants have been reported to date. In the model plant Arabidopsis, phosphatidylcholine synthesis was also shown to steer ER proliferation, making this pathway an equally relevant potential target for ER engineering. Indeed, Arabidopsis PAH seemingly regulates phospholipid synthesis in an analogous way as described in yeast [34] because the loss of function of PAH causes ER proliferation. As a downside though, the pah mutation has a strong negative impact on plant growth and viability. Also different from yeast, in Arabidopsis, the disruption of PAH leads to an accumulation of its substrate, phosphatidic acid, which, in turn, stimulates phosphocholine-cytidylyl transferase (CCT), a key regulatory enzyme in the phosphatidylcholine biosynthesis pathway [35]. Notably, overexpression of a constitutively active, truncated version of CCT, devoid of the C-terminal lipid-binding domain, largely replicates the pah phenotype. The utility of such an engineered enzyme for plant metabolic engineering remains to be determined. Other peculiar plant ER-derived organelles are the ER bodies, which are presumed to be specific to the order Brassicales, and accumulate β-glucosidases responsible for the conversion of inactive glucosinolates into toxic compounds in stress conditions [36,37]. A recent study demonstrated that ER body formation can also be induced in other plant taxa through overexpression of Arabidopsis ER body regulators [38]. Whether targeting such ER bodies for heterologous terpene (or other metabolites) biosynthesis is feasible and whether they could be a potential target for organelle engineering in plants needs further investigation.

Finally, also engineering the expansion of other organelles, such as peroxisomes in yeasts and chloroplasts in plants, is being considered in synthetic biology programs. For instance, increased peroxisome capacity in S. cerevisiae can be achieved, by manipulating transcription factors controlling peroxisome size, more particularly by deleting their regulatory domains. Overexpression of such truncated transcription factors, in combination with the peroxisomal pathway compartmentalization strategy mentioned above further increased the benzylisoquinoline alkaloid titer in yeast cells [18]. It should be noted that such peroxisome engineering also has a negative impact on yeast growth. An attempt to increase chloroplast size in Solanum tuberosum (potato), by overexpressing a tubulin-like GTPase gene from Arabidopsis, was recently reported [39]. This approach has yet to be combined with metabolic pathway engineering. In the longer term, strategies for organelle expansion could be integrated with synthetic biology programs aiming to expand the diversity of terpene structures. Particularly, noteworthy for terpene engineering is the potential to expand terpene building blocks beyond the classical five-carbon (C5) units, which was recently enforced by the discovery that engineering of a plant monoterpene synthase capacitated the production of C11 terpene compounds [40]. This kind of approach widens the potential array of intrinsically diverse terpene structures considerably.

Beyond the organelle

In nature, complex biogeochemical cycles are carried out by an immense mosaic of diverse communities of organisms that are metabolically linked to each other [41]. Metabolic interconnection is achieved either indirectly when organisms feed on each other after cell death or directly when organisms are able to export certain classes of metabolites to the surroundings that become the energy source for other microorganisms [42]. Different from nature, heterologous production of natural

products in microbial hosts mainly relies on engineered host cells in a monoculture fermentation. Recently, the engineering of microbial communities by splitting heterologous pathways into individual biosynthetic modules expressed in different microbial hosts has been shown to be a promising approach to improve the biosynthesis of complex natural products [43,44]. Modular co-culture engineering has the potential to reduce the metabolic burden on individual microbial hosts, increase flexibility and thus resilience of the system, and fine-tune the final metabolic output simply by controlling ratios of different strains within the population. Moreover, it allows the production of new chemicals simply by mixing strains that harbor different biosynthetic modules. The adoption of engineered microbial consortia has been proven successful by several pioneering studies for a variety of natural compounds, such as phenylpropanoids, terpenes, and alkaloids, was recently reviewed [45].

In this regard, the exploitation of (specific) metabolite transporters for synthetic biology may become crucial. In the above context, transporters may metabolically link different microbial strains and thereby increase the titer of the desired metabolite. Conversely, transporters may increase the biosynthetic performance at a single-cell level by correctly distributing substrates among different cell compartments. This is especially relevant when introducing complex plant pathways in unicellular hosts that lack the transport systems endogenously present in plants. Indeed, heterologous expression in yeast of multiple plant alkaloid transporters demonstrated how to correct distribution of pathway intermediates and products across different cell compartments can be critical for efficient production of complex plant natural products [46,47]. This strategy allowed to exploit six different subcellular locations, that is, the cytosol, peroxisome, mitochondrion, ER, vacuole, and the vacuolar membranes for the production of tropane alkaloids. Efficacy for such approaches for terpene production in either plant or yeast host remains to be determined, but it stands beyond doubt that they serve as thoughtful inspiration. Specialized metabolite transporters in plants are still limitedly characterized, however [48]; hence their potential exploitation for synthetic biology and metabolic engineering remains largely untapped. It is plausible that the advances in powerful gene discovery tools such as transcriptome and genome sequencing [49] and/or protein–metabolite interactomics technologies will empower scientists to identify metabolite-binding or metabolite-transporting proteins [50]. By doing so, the knowledge gained could be used to assemble metabolite transporter sets not only to enable export out of the cell or between specific cellular compartments but also with the innovative perspective of physically linking transporters with enzymes that metabolize the transported substrates into a ‘membrane transport metabolon’ [51]. By fusing a hexose transporter to a xylose isomerase, the latter strategy has been adopted to increase substrate utilization, reduce the production of side products, and ultimately increase ethanol production in yeast [52].

Conclusions

In addition to understanding the linear design of terpene and other metabolic pathways, successful engineering strategies must consider a third dimension comprising spatiotemporal regulation of pathways and organellar capacity. Here, we have highlighted some recent successful efforts that focused on organellar compartmentalization and engineering the production of plant-derived terpenes. However, many challenges related to maintaining the complex balance between subcellular specific pathways and inter-compartment transportation of intermediates remain to be addressed. Better knowledge of, for example, subcellular localization and abundance of key enzymes in terpene production, cross-talk between cytosolic and plastidial terpene biosynthetic pathways in plants, or organellar permeability of terpenes and their precursors will be essential to improve pathway compartmentalization strategies. Clearly, expansion of organellar capacities represents a promising engineering approach that will not only be complementary to but also likely may even go beyond the conventional pathway engineering efforts. In this regard, the discovery of (novel) regulators of

organelle generation, morphology, structure, and proliferation are a must. For example, increased understanding of processes tightly involved in ER homeostasis, such as the unfolded protein response, ER-associated protein degradation, or ER autophagy, will undoubtedly provide novel targets for ER engineering [53,54]. These mechanisms are still poorly studied in plants, but recent findings in other organisms have demonstrated their importance in maintaining organellar size [55,56]. Recently, the importance of ER homeostasis was also illustrated by the finding that safeguarding correct folding of ER enzymes during metabolically demanding times, more specifically by pairing ER-resident chaperones and P450s from plants in yeast, allows to enhance heterologous terpene synthesis [57].

Furthermore, other subcellular dynamics, such as those occurring at membrane contact sites (MCS), could become key in organellar engineering programs. MCS has been reported to occur between distinct organelles, also in plants [58,59]. MCS can provide a cellular environment with an increased local concentration of particular enzymes or a more efficient exchange of compounds, thereby enabling cooperation between organelles without shifting the general cytosolic environment [60]. Increased understanding of MCS events and functions will not only generate exciting new fundamental insights but, ultimately, also novel potent tools for synthetic biology.

Finally, the perhaps ultimate level of compartmentalization may involve the development of artificial organelles for the tailored design of metabolic micro-compartments. A switchable, light-harvesting synthetic organelle, able to reproduce complex homeostasis behaviors, has been recently designed to optically control ATP synthesis [61]. Such techniques could potentially be used to create biosynthetic modules for homologous or heterologous hosts, where optimal characteristics for terpene production can be combined. Likewise, the construction of synthetic organelles from free-living bacteria or even synthetic membrane-less organelles has been presented as alternatives to create organisms or systems with an increased metabolic capacity [62,63].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURES

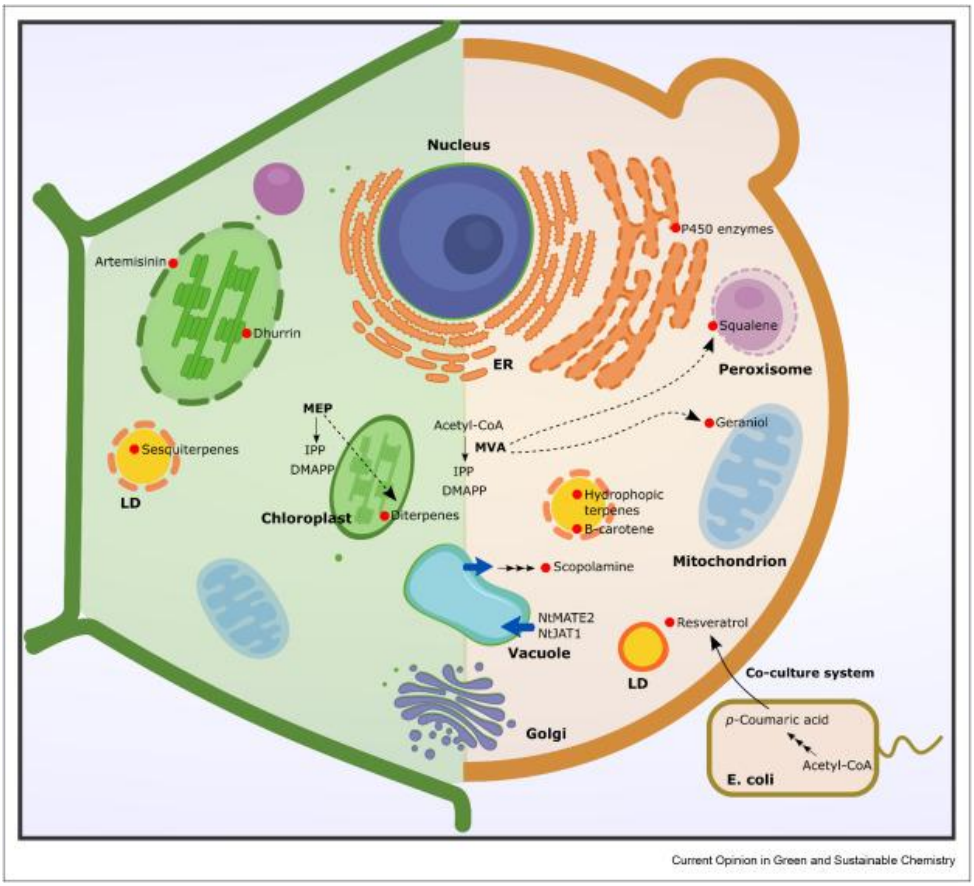

Figure 1. Overview of recent advances in organellar engineering in yeasts and plants as production hosts. Compartmentalization strategies are illustrated by black dashed arrows that indicate the relocalization of pathways to other subcellular compartments to increase the production of metabolites and/or enzymes of interest (indicated by red dots). Organelles such as the endoplasmic reticulum (ER), peroxisomes, lipid droplets (LD), and chloroplasts that have been reported to be successfully expanded for increased metabolic capacity are displayed in colored dashed lines. The blue arrows in the yeast vacuole indicate recently described transporters that contribute to the production of tropane alkaloids. Metabolic interconnection is illustrated by modular co-culture engineering to produce the phenolic compound resveratrol using *E. coli* as co-host. NtJAT1, jasmonate-inducible alkaloid transporter 1; NtMATE2, multidrug and toxin extrusion 2, both from Nicotiana tabacum. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Organelles	Key Features	Target terpenes	Strategy	
			Yeast	Plant
 Endoplasmic Reticulum	• Phospholipid bilayer allows tethering enzymes • Oxidative environment • Endogenous supply of C30 molecules	• Sesquiterpenes • Sterols • Triterpenes	• ER expansion increased biosynthetic capacity: pah1 loss-of-function [31], INO2 overexpression [32]	• Arabidopsis pah loss-of-function stimulates key enzyme in the phosphatidylcholine biosynthesis [35] linked to ER expansion
 Golgi	• Sorting compartment • High supply of UDP- sugars for glycosylation	• Saponins		
 Mitochondrion	• High endogenous Acetyl-CoA supply • Abundance of cofactors and ATP • High pH reducing environment	• Monoterpenes • Sequiterpenes	• Targeted expression of integrated terpene pathways to mitochondria increase conversion of precursors into mono- and sesquiterpenes [24,25]	
 Chloroplast	• Endogenous MEP pathway • Endogenous supply of IPP	• Mono-, di-, and tetraterpenes		• Production of sesquiterpene artemisinin in tobacco leaves: MVA biosynthetic pathway compartmentalized in chloroplast [20] • Targeting cyanogenic glycoside biosynthetic enzymes from Sorghum to the thylakoid membrane in Nicotiana benthamiana [22]
 Peroxisome	• Dispensable to cell viability • Abundant pool of Acetyl-CoA by fatty acid oxidation • Single layer membrane may allow passive diffusion of small molecules	• Mono-, and sesquiterpenes • Squalene	• Monoterpene biosynthetic pathways targeted to peroxisomes [17] • Peroxisome compartmentalization avoid self-toxicity of alkaloid norcoclaurine synthase and increased titer of product (S)-reticuline [30]	
 Lipid Droplets	• ER derived, rich in neutral lipids • Hydrophobic core • Outer membrane allow enzymes tethering	• Sterols • Sesqui-, di-, and tetraterpenes	• Deletion of FLD1 and concomitant overexpression of fatty acid desaturase, increased accumulation of lycopene in lipid droplets [25] • Increased squalene production via increased MVA flux coupled with improved lipid droplets production [26] • Targeting of ER-located cytochrome P450 enzyme to the lipid droplet membrane increased ginsenoside titer [27]	• Plant terpene enzymes fused to lipid droplet surface protein from algae adopted for enhanced terpenes production in tobacco [28-30]
 Vacuole	• Storage of toxic compounds • Low pH	• Storage of saponins and soluble terpenes	• Expression of JA-inducible alkaloid transporter (NtJAT1) and two MATEs (NtMATE1, NtMATE2) from tabacco to yeast increased titer of tropane alkaloids [40]	

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Figure 2. Key features and target terpenes for plant and yeast subcellular organelles. When no relevant studies are known for terpenes, examples from other classes of specialized metabolites have been included.