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Cytochrome P450-mediated metabolic engineering: current progress and future challenges☆

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Cytochromes P450 catalyze a broad range of regiospecific, stereospecific and irreversible steps in the biosynthetic routes of plant natural metabolites with important applications in pharmaceutical, cosmetic, fragrance and flavour, or polymer industries. They are consequently essential drivers for the engineered bioproduction of such compounds. Two ground-breaking developments of commercial products driven by the engineering of P450s are the antimalarial drug precursor artemisinic acid and blue roses or carnations. Tedious optimizations were required to generate marketable products. Hurdles encountered in P450 engineering and their potential solutions are summarized here. Together with recent technical developments and novel approaches to metabolic engineering, the lessons from this pioneering work should considerably boost exploitation of the amazing P450 toolkit emerging from accelerated sequencing of plant genomes.

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Introduction

Cytochromes P450 (P450s) constitute the most extensively studied class of enzymes with more than 69 000 articles published at the end of 2013 (http://www.ncbi.nlm.nih.gov/queried 20/12/2013 with 'P450' or 'P-450') according to PubMed count. An initial strong interest was fostered by the essential roles of several P450s in mammalian drug metabolism with wide-ranging implications in medicine and pharmacology [1]. It now appears that they also form by far the largest superfamily of enzymes, essentially due to the expansion of the enzyme family in plants. As of December 2013, the count of annotated plant P450s, not including allelic variants, is 7512 (David Nelson, personal communication). It is expected that this number will increase in parallel with the exponential increase in sequencing information resulting from ongoing large-scale plant genome/transcriptome sequencing efforts (e.g. www.onekp.com). This rapidly expanding repository of P450 sequences could hold the key to bioengineering and emerging synthetic biology, when developed into an amazing functional toolkit.

It has been estimated that plants produce more than 200 000 metabolites as signalling, allelochemicals or defence compounds, to interact with and adapt to their environment. These metabolites have the potential to provide a major source of new drugs and bioactive compounds for cosmetics and biopesticides [2,3]. Cytochromes P450 catalyze most of the rate-limiting (with turnovers rates typically in the ten to a few hundreds per minute range) and completely irreversible reactions in their biosynthetic pathways, usually regio-specific and stereospecific oxygenations or oxidations, but also more complex reactions such as dealkylation, deamination, decarboxylation, C–C cleavage, ring expansion, ring opening, ring migration, ring coupling and dehydration [4,5].

Extraction of most bioactive compounds from plants is achieved with low yield in a costly, season-dependent and time consuming manner, and is therefore not sustainable. Moreover, many plants containing pharmaceutically interesting compounds are not cultivable, or are at risk of extinction in their natural habitat. Chemical synthesis of complex plant metabolites is excessively expensive. Metabolic engineering in microbial or plant hosts offers a more cost-effective alternative for production of compounds marketable as 'natural' products. We summarize here recent advances involving a combination of gene function discovery and complex metabolic engineering that demonstrate how P450s can be exploited for drug production or to confer valuable traits to plants for various applications. We also discuss specific limitations and pitfalls associated with this class of enzymes, as well as new trends and technical advances expected to significantly speed-up P450 exploitation in biotechnology.

Potential applications for P450s in metabolic engineering

P450s contribute to the biosynthesis and/or catabolism of all phytohormones, and of many biopolymer (e.g. lignin, cutin, suberin, sporopollenin) subunits, pigments, fragrances, flavours, antioxidants, allelochemicals and defence compounds, among which are many compounds of commercial interest. Oxidations are critical steps for the cross-linking of biopolymers and for the formation of bioactive compounds, and are most often a prerequisite for further decorations or modifications essential for bioactivity. For example, 97% of the 46 000 current recorded plant terpenoids (one of the major classes of bioactive compounds: Dictionary of Natural Products (http://dnp.chemnetbase.com) accessed February, 2014; DNP 22.2 Taylor & Francis Group) are oxygenated [6,7]. When displaying relaxed substrate specificity, P450s can also participate to the activation or catabolism of xenobiotics, such as industrial pollutants or pesticides [8]. Herbicide detoxifying plant P450s can be exploited as selectable markers for plant transformation [9], whereas bacterial or human P450 enzymes have been expressed in plants for the purpose of bioremediation and the removal of pollutants, including explosives or pesticides [10•,11,12]. Conversely, suppression of herbicide (nicosulfuron) detoxification in corn was recently developed as a tool for controlling the spread of transgenes [13•]. So far, only a very small part of the huge potential provided by plant P450s has been exploited. A few examples will be illustrated below.

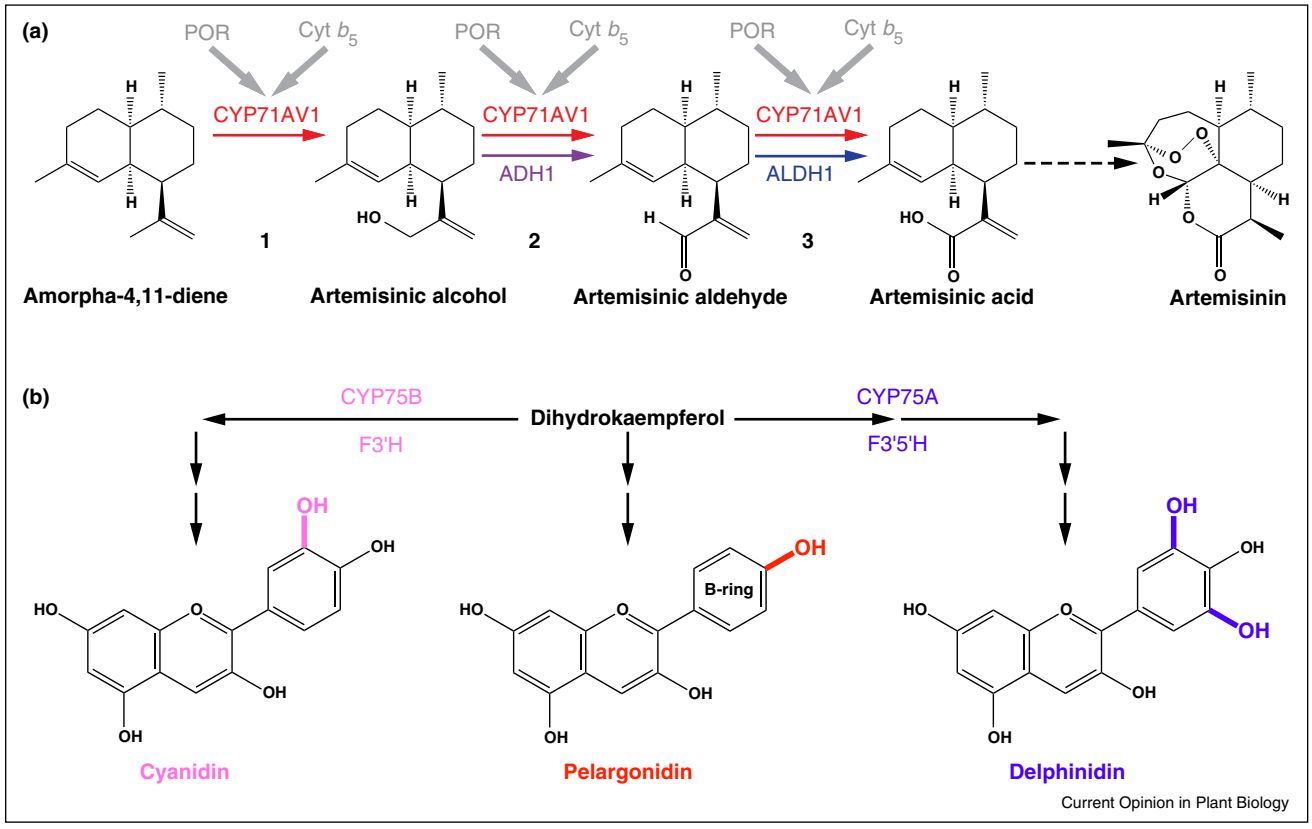
Major achievements: from engineered production of artemisinic acid to flower colour modification

Numerous P450s are already present in the metabolic engineering toolbox with 2880 US issued patents related to the use of P450s (source: www.google.com/patents; query: 'cytochrome P450' + 'metabolic engineering'), of which around 25% are associated with plants (query: 'cytochrome P450' + 'metabolic engineering' + 'plant'). Few of those, however, have led to commercial applications.

The most prominent story in synthetic biology for drug production was recently reported by Paddon and coworkers [14••]. Artemisinin, a sesquiterpenoid originally sourced from *Artemisia annua*, is used in combination therapy for malaria. A stable supply of inexpensive artemisinin is essential in the developing world, where Malaria is a leading cause for death and disease. The effort for process development was thus supported by the Bill & Melinda Gates foundation. It was initially found that a single P450 expressed in the trichomes of *A. annua*, CYP71AV1, catalyzes the three successive oxidations steps leading from amorpha-4,11-diene to artemisinic acid [15,16]. CYP71AV1 was thus a prime target for engineered semisynthetic production of artemisinin (Figure 1a). Commercially relevant yield (25 g/L) was achieved in baker's yeast after multiple steps of optimization [14••]. The first steps led to optimized production of amorphadiene in yeast via expression of the amorphadiene synthase from *A. annua*, but also up regulation and fine-tuning of all enzymes of the mevalonate pathway and down regulation of *ERG9* (encoding squalene synthase and involved in biosynthesis of sterols) which competes with the introduced pathway [17,18]. The next stage required optimization of electron transfer to CYP71AV1, via moderate expression of the NADPH dependent P450 oxidoreductase (POR) and introduction of *A. annua* cytochrome b_5, preventing the observed formation of reactive oxygen and the accumulation of toxic intermediates that interfered with yeast viability. The third stage utilized acceleration of the formation and channelling of final oxidation products by co-expression of an artemisinic aldehyde dehydrogenase and an alcohol dehydrogenase, both highly represented in *A. annua* trichome ESTs (Figure 1a). Final improvements were obtained via optimized yeast cultivation and extractive fermentation processes [14••]. This outstanding work provides a thorough and instructive example of metabolic engineering whose lessons will undoubtedly help speed up future engineering attempts. It also reveals that although some oxidation cascades can be achieved by P450s alone, additional oxidoreductases can be critical to drive fluxes compatible with viable pathways.

Long and sustained efforts were also required to bring to the market flowers with modified colours using CYP75 enzymes [19••], which are the only CYP-engineered plants marketed so far. Anthocyanidins determine the colour of flowers, which shifts from orange via red to violet and blue hues with increasing hydroxyl groups on the B-ring. This hydroxylation pattern is determined by two P450 enzymes of the CYP75 family: the flavonoid 3′-and 3′,5′-hydroxylase (Figure 1b: CYP75B and CYP75A, respectively). The corresponding genes were initially identified in petunia and have subsequently been identified and isolated from a range of different plants [19••]. The top-selling cut-flowers such as rose, carnation or chrysanthemum never show blue shades in nature due to a CYP75A deficiency. Whereas plant engineering towards blue flowers seemed initially straightforward, complex and often empirical strategies had to be implemented to obtain marketable products. In addition to overexpression of a CYP75A gene, these strategies included promoter optimization (e.g. promoter from a snapdragon chalcone synthase), selection of suitable genetic background, including silencing of competing pathways, and choice of most efficient CYP75A gene (with pansy CYP75A showing highest efficiency owing to better mRNA stability). Further colour improvements were achieved via coexpression of a 4-dihydroflavanol reductase (DFR) with appropriate substrate specificity, and of the flavone synthase II CYP93B, yet another

Figure 1



Cytochrome P450 functions in the engineered artemisinin and anthocyanin pathways. **(a)** Cytochrome P450-catalyzed reactions in the artemisinin pathway: hydroxylation of amorpha-4,11-diene to produce artemisinic alcohol (1) is catalyzed by the cytochrome P450 CYP71AV1. Artemisinic alcohol is then synergistically oxidized (2) by CYP71AV1 and ADH1 (artemisinic alcohol dehydrogenase 1) to produce artemisinic aldehyde, which is also synergistically converted into artemisinic acid (3) by CYP71AV1 and ALDH1 (artemisinic aldehyde dehydrogenase 1). Optimal CYP71AV1 activity requires two electron donors POR (P450:oxidoreductase) and Cyt b_5 (cytochrome b_5). **(b)** Structure and colour of the main types of flower anthocyanidins. Members of the CYP75B subfamily are flavonoid 3′-hydroxylases (F3′H), catalyzing B-ring 3′-hydroxylation to produce cyanidin-derived red/magenta pigments. Members of the CYP75A subfamily are flavonoid 3′,5′-hydroxylases (F3′5′H), catalyzing both B-ring 3′-hydroxylation and 5′-hydroxylation leading to delphinidin-derived violet/blue pigments. Plants deficient in both CYP75A and CYP75B proteins accumulate pelargonidin-derived red/orange pigments.

flavonoid hydroxylase, leading to a deepening and increase of the blue colour through formation of co-pigments [19••]. Other parameters such as vacuolar pH and metal ion content required control. Optimal electron transfer was found to be important in some carnation varieties, requiring overexpression of cytochrome b_5 for accumulation of the blue pigment delphinidin. This is most likely related to the previously reported requirement of cytochrome b_5 for CYP75A, but not for CYP75B activity in petunia [20]. A similar strategy with further promoter enhancement recently led to the generation of violet/blue chrysanthemums [21].

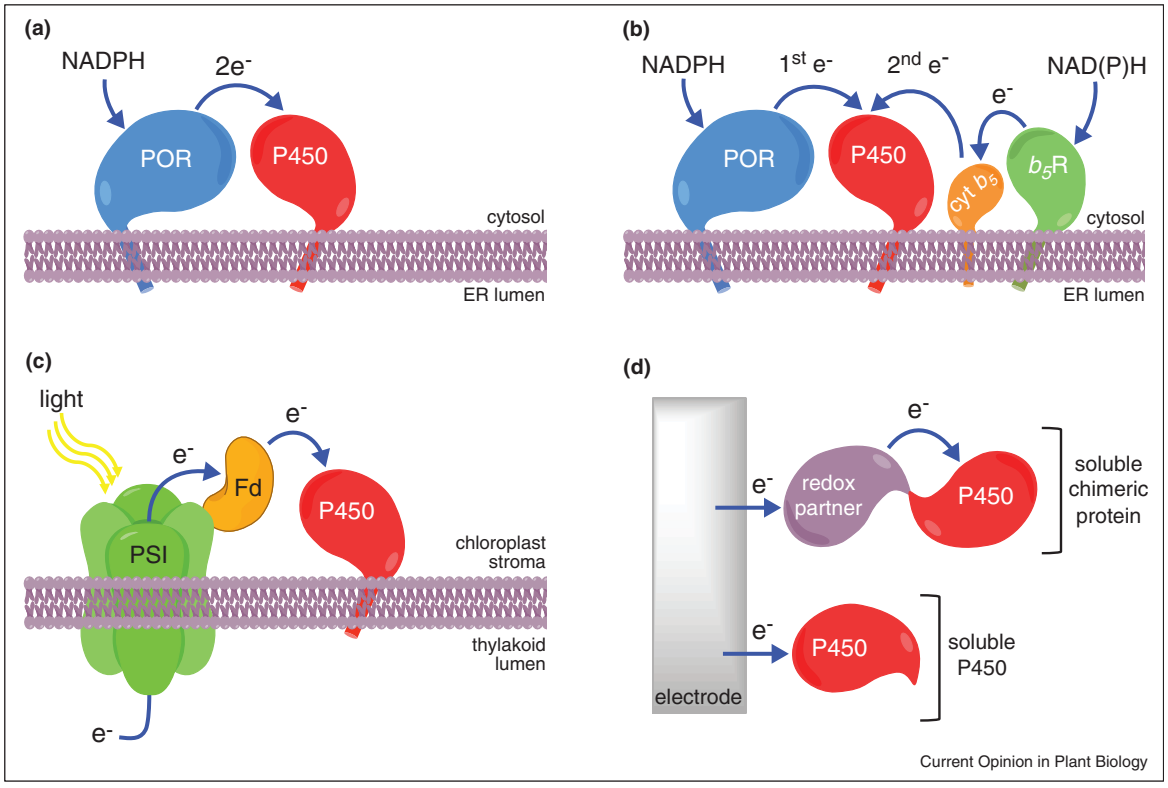
Conversely, efforts for engineering pink flowers from native blue cultivars via suppression of CYP75B and CYP75A and overexpression of a heterologous *DFR* gene from geranium led to pink torenia flowers [22], whereas CYP75A suppression was sufficient to shift cyclamen flowers to orange-red [23].

Both these success stories illustrate that engineering of industrially relevant processes or products requires more than P450 expression in a heterologous organism. Optimization of both metabolic fluxes and electron transfer is required.

The challenge of electron source and transfer

Oxygen activation by P450s requires two electrons (for an update on P450 mechanism see [24]), which are provided *in vivo* via different donor proteins or short protein cascades [25]. Those depend on organism and subcellular compartment. While diversified electron donor systems have evolved in microorganisms, plant P450 enzymes normally require a POR as minimal electron donor, and

Figure 2



Main natural and engineered sources of electrons exploited for P450-driven reactions. In a natural context, the two electrons required for P450 reactions are most often transferred from an N-terminally membrane-anchored P450:oxidoreductase (POR) **(a)**; natural or artificial soluble P450-POR proteins fusions (as shown in (d)) can be found or generated. In some instances, a C-terminally membrane anchored cytochrome b_5 (Cyt b_5) is required to provide the second electron, Cyt b_5 being itself reduced by a cytrochrome b_5 reductase (b_5R) **(b)**. Recent work demonstrates that it is possible to use photosystem I (PSI)-derived electrons to reduce P450 enzymes via ferredoxin (Fd) *in vitro* and *in vivo* **(c)**. Electrode-driven reduction of soluble P450 is possible *in vitro*, especially when fused to a redox partner **(d)**.

both P450 and POR co-localize on the membrane of endoplasmic reticulum (or in a few cases on the plastid envelope) anchored via their N-terminus (Figure 2a). Achieving high plant P450 activity in microbial cells usually requires co-expression of a plant POR [26,27]. However, cytochrome b_5 might favour (or even be required for) reduction of some P450 enzymes (Figure 2b) [14••,19••,28]. Excess expression of POR is toxic for the cells since it leads to electron transfer uncoupling and the generation of chemically generated reactive oxygen species (see e.g. [14••,29]). Probably for this reason, P450s are usually found in large excess to POR in plant or animal cells. Optimization of electron flux to P450s is thus an important aspect of process development. Some microorganisms have solved this problem by evolving highly catalytically efficient soluble P450-POR fusion proteins, the most famous example of which is $P450_{BM3}$ from *Bacillus megaterium*. Artificial P450-POR fusions were generated with some success in *Escherichia coli* where POR and ER membranes are absent. For example the CYP725A4 from Pacific yew, which catalyses the first oxidation step in the biosynthesis of the anticancer compound paclitaxel, was found to be active in *E. coli* as a fusion with yew POR [30]. Likewise, plant P450s have been fused to the bacterial POR domain from *Rhodoccocus*, generating active enzymes [31]. However, the arbitrary linkage and protein interactions in such artificial constructs is not optimized by evolution, and could therefore lead to poorly performing enzymes, uncoupling of electron transfer and other deleterious cellular effects. Optimization of electron transfer via directed saturation mutagenesis of the electron donor interface of a bacterial enzyme has been recently reported [32].

The fact that ferredoxins mediate electron transfer in mitochondrial and some bacterial P450 systems, and also function as electron carriers in the photosynthetic chain, has inspired attempts to replace NADPH by a light-generated source of electrons in P450 catalyzed biosynthesis of natural products (Figure 2c). Isolated photosystem I membrane complexes in combination with ferredoxin as an electron carrier were shown to support

P450 based mono-oxygenations *in vitro*, supporting the concept of engineering light-driven biosynthesis in synthetic biology [33]. The same team delivered demonstration for an entire biosynthetic pathway elegantly in an *in vivo* system, effectively rerouting photosynthetic reducing equivalents into production of a bioactive natural compound [34••]. The key to their strategy was to exploit the highly compartmentalized nature of plant pathways, breaking the separation of photosynthetic energy generation in chloroplasts and P450 based biosynthetic routes localized to the endoplasmic reticulum. This was achieved by re-targeting three enzymes to the chloroplast. These were two P450s and one glycosyl-transferase, constituting the entire biosynthetic pathway of the plant defence compound dhurrin, a cyanogenic glucoside found in *Sorghum bicolour*. It was demonstrated that reduced ferredoxin generated by photosystem I provided the electrons for the P450 reactions directly, effectively circumventing involvement of NADPH and the POR [34••]. Alternative approaches aiming at bypassing NADPH dependence such as coupling of the P450 with photosensitisers to generate the electrons [35], or non-enzymatic NADPH regeneration with artificial electron donors and mediators [36] are in their infancy and have so far only been tested with the soluble bacterial $P450_{BM3}$ and complex polypyridin-ruthenium compounds. While interesting from a technological perspective, the practical and economic feasibility, and transferability to true production systems of these technologies remain to be demonstrated.

Pitfalls of P450-driven metabolic engineering and potential solutions

P450-mediated metabolic engineering is usually not a straightforward process. The main challenges and their possible solutions are summarized in Table 1. The main limitation for pathway reconstruction in microorganisms is quite often the level of expression of P450 enzymes. About 40% of plant P450s are poorly expressed, if at all, in yeast (from a sample of more than 250 from different plant species: author's lab statistics). For enzyme functional characterization, expression in insect cells or plant tissues can provide alternate strategies, which are not necessarily relevant for industrial production. Recoding to match yeast codon usage is not often helpful, although partial recoding [37] or reducing RNA structure might provide a more useful approach [38]. A common problem encountered with heterologously produced bioactive products is their inherent toxicity when accumulated as aglycones. In particular, isoprenoid oxygenated products resulting from P450-mediated decoration are usually more toxic than the corresponding olefins and exert this toxicity via accumulation in cellular membranes as has been recently shown for nootkatol [39]. In addition, alcohol intermediates may inhibit P450-catalyzed reactions via direct coordination to the heme iron in the active site [39]. Plants provide excellent core metabolism platforms to graft specialized pathways, but also present a major challenge because of their propensity for further conversion of the desired activated

Table 1

Pitfalls, challenges of P450 engineering and possible solutions

	References
Expression level/protein stability and activity	
Codon optimization	[38,41]
Secondary structure optimization	[38]
Decrease expression of POR	[14••]
Co-express other POR	[26,27,42]
Use other more stable ortholog	[19••,28]
Change expression system	[14••,52]
Expensive co-factors	
Whole-cell biotransformation	[14••,18,39,53•]
Co-factor regenerating system	[36,54,55]
Light-driven electron transfer	[33,34••,56]
Electrode-driven electron transfer	[57–59]
Toxicity of substrate/intermediates/product	
Biphasic bioreactor	[14••,41]
Anion-exchange products trapping	[60]
Conjugation of products	[39,61]
Compartmentation of products	[50,51]
Boost flux to final non-toxic products	[14••,30]
Lack of specificity/efficiency/alternative enzyme	
Screen natural enzyme collections	[39,46,47,59]
Directed evolution	[62]
Active site design	[45••,63,64]
Metabolic flux control	
Host engineering: expression of upstream/supporting enzymes or silencing of side-branch pathways	[14••,17,18,28,30,41,42]
Use of more appropriate natural genetic background	[14••,28]
Co-express optimal protein sets/modular pathway reconstitution/Expression level control	[14••,21,28,30,42,65]

products into conjugates or other derivatives due to the activity of the host plant enzymes (see e.g. [40]). Via insertion of hydroxyl groups on hydrophobic hydrocarbon structures, P450s provide grips to oxidoreductases and conjugations enzymes present in the host organism. Fast channelling of activated metabolic intermediates to downstream products is thus required.

New targets and future developments

The main targets for P450-mediated engineered production are high-value metabolites with applications in fragrance/flavour, cosmetics or pharmaceutical industries. With the development of next generation sequencing, those are now low-hanging fruits. There are recent reports of partial reconstruction of pathways for the production of the diterpenoid taxol [30], triterpenoid ginsenosides [41] and norditerpenoids tashinones [42], all for pharmaceutical applications. The natural pathways will be exploited, but the availability of large gene collections also pave the way for combinatorial assembly of non-natural enzyme sequences for generation of novel compounds by a synthetic biology approach [43•,44], thus expanding the already huge repertoire of plant metabolites. Moreover, natural enzyme structures will be redesigned to permit non-natural reactions as recently elegantly illustrated by the replacement of the heme axial cysteine ligand with a serine in the bacterial $P450_{BM3}$ which generated a C–C bound forming enzyme catalyzing olefin cyclopropanation [45••].

Beside tapping the immense resource for natural enzymes, major innovations are expected from technical developments such as novel nanoscale high-throughput functional screening systems [46,47], enzyme trapping and stabilization in membrane substitutes such as lipid nanodiscs [48] or amphipols [49]. The use of new convenient plant hosts amenable to homologous recombination and easily grown in controlled environment, such as algae or moss, or of specialized plant cell types providing suitable metabolic backgrounds and allowing product compartmentation to alleviate toxicity, such as trichomes [50,51], will open new avenues for plant supported production of valuable compounds.

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