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New developments in engineering plant metabolic pathways

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Plants contain countless metabolic pathways that are responsible for the biosynthesis of complex metabolites. Armed with new tools in sequencing and bioinformatics, the genes that encode these plant biosynthetic pathways have become easier to discover, putting us in an excellent position to fully harness the wealth of compounds and biocatalysts (enzymes) that plants provide. For overproduction and isolation of high-value plant-derived chemicals, plant pathways can be reconstituted in heterologous hosts. Alternatively, plant pathways can be modified in the native producer to confer new properties to the plant, such as better biofuel production or enhanced nutritional value. This perspective highlights a range of examples that demonstrate how the metabolic pathways of plants can be successfully harnessed with a variety of metabolic engineering approaches.

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Introduction

Plants provide a seemingly inexhaustible pool of structurally diverse chemicals. *In planta*, the biosynthesis of these compounds is a response to external or environmental cues, and therefore plays a crucial role in shaping the interdependencies and diversity of plant ecosystems. These chemicals impact how effectively plants can be used as food and energy sources. Moreover, many chemicals that are produced by plants promote human health, and numerous plant metabolites are isolated for use in the pharmaceutical industry. Despite the importance of plant metabolites, the biosynthetic processes for only a small fraction of these complicated molecules are known, indicating that the immense diversity of plant metabolism has not been explored. The recent advances in next-generation sequencing technologies, along with the continuous development of new algorithms for bioinformatic analysis of these sequence data, has greatly expedited the process of plant metabolic gene discovery. By extension, these discoveries have allowed advancements in the engineering of plant metabolism.

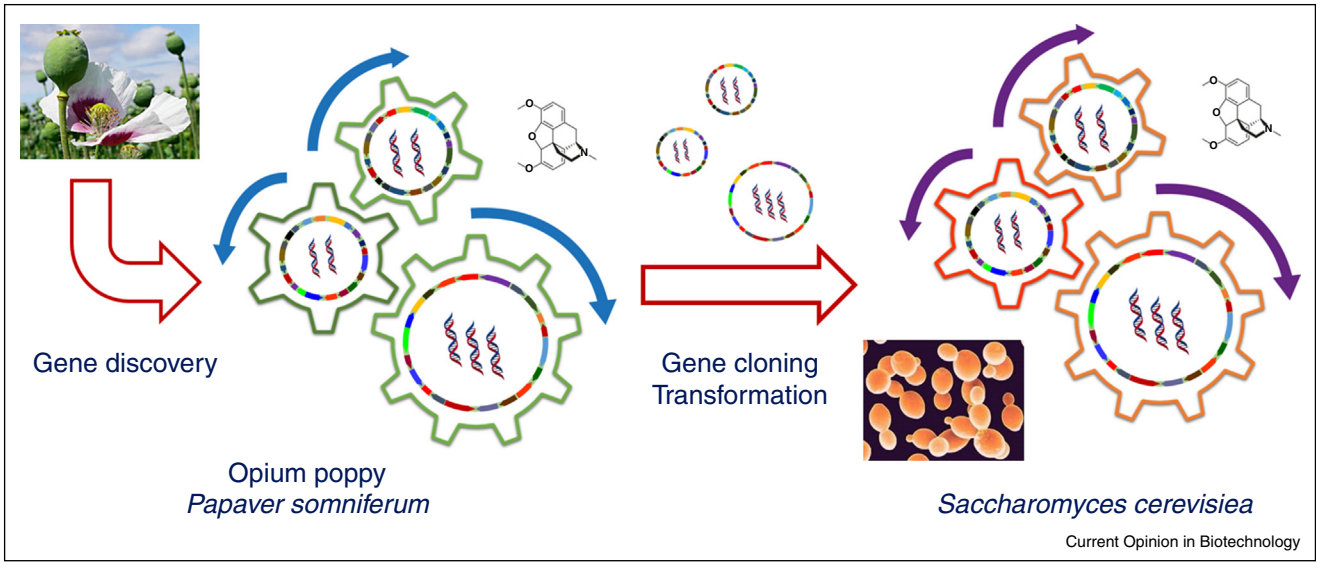
It is of great importance to elucidate and engineer the plant metabolic pathways that construct complex metabolites from simple building blocks. An understanding of these pathways will allow us to fully harness the wealth of compounds and biocatalysts that plants provide. In this perspective, we highlight several important recent examples of metabolic engineering with plant metabolic pathways. These examples demonstrate the wide range of engineering approaches that can be applied to plant pathways, and also illustrate the range of problems that can be addressed by plant metabolic engineering. Collectively, these examples demonstrate the progress that we are making to fully harness the metabolic power of plants.

Heterologous reconstitution of plant metabolic pathways

One approach to harness plant metabolic pathways is to reconstitute the biosynthetic genes into a heterologous organism [1] (Figure 1). Microbial (e.g. *Saccharomyces cerevisiea* and *Escherichia coli*) and plant (e.g. *Nicotiana benthamiana*) hosts can be used, with each system having advantages and disadvantages. For example, plants, which utilize photosynthesis, do not require exogenous carbon feedstocks [2••]. Many plants such as *Nicotiana tabacum* (tobacco) and *N. benthamiana* can generate large amounts of biomass quickly and cheaply [2••,3], making them a robust, sustainable, and scalable platform for large-scale terpene production. On the other hand, microbial hosts can be genetically manipulated in a rapid fashion, are fast growing, and the infrastructure required for microbial production is well established [4]. Below are two representative examples, one utilizing the plant host *N. tabacum* to overproduce high value triterpenoids, and the other using *S. cerevisiea* to produce the plant derived opiate morphine. Other examples using *Nicotiana* [5–7] and *Saccharomyces* [8–12] have also been recently reported in the literature.

Linear, branch-chained triterpenes that are generated by the green alga *Botryococcus braunii* are increasingly recognized as important chemical and biofuel feedstocks [13]. However, the slow-growing *B. braunii* is an impractical production system for large-scale isolation of these compounds [14]. In a recent study, high levels of the *B. braunii*

Figure 1



Heterologous reconstitution of plant pathways in yeast, as exemplified by reconstitution of opiate biosynthetic pathways in yeast. The genes responsible for biosynthesis of opiates were cloned from opium poppy and introduced into the appropriate vectors for expression of enzymes in yeast.

triterpene botryococcene (Figure 2) were produced in *N. tabacum* plants by the overexpression of an avian farnesyl diphosphate synthase along with two versions of botryococcene synthases in the chloroplast [2••]. High yields of methylated botryococcene derivatives could also be obtained when triterpene methyltransferases were expressed in the chloroplast. While approximately 90% of the triterpenes were converted to methylated derivatives when all enzymes were targeted to the chloroplasts, less than 15% of triterpenes were methylated when this metabolic pathway was expressed in the cytoplasm, highlighting the enormous impact that enzyme localization can have on metabolic engineering. Chloroplasts, which have a high flux of carbon passing through the MEP (2-*C*-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate) pathway, appear to be particularly suited for expression of terpenes [2••]. While the plants in this study accumulated 0.2–1.0 mg triterpene per gram of plant fresh weight, the authors of this study pointed out that previously reported engineering efforts with sesquiterpene and monoterpene pathways in plants often resulted in much lower production levels, perhaps because different terpene compounds may have differing effects on physiological homeostasis and growth.

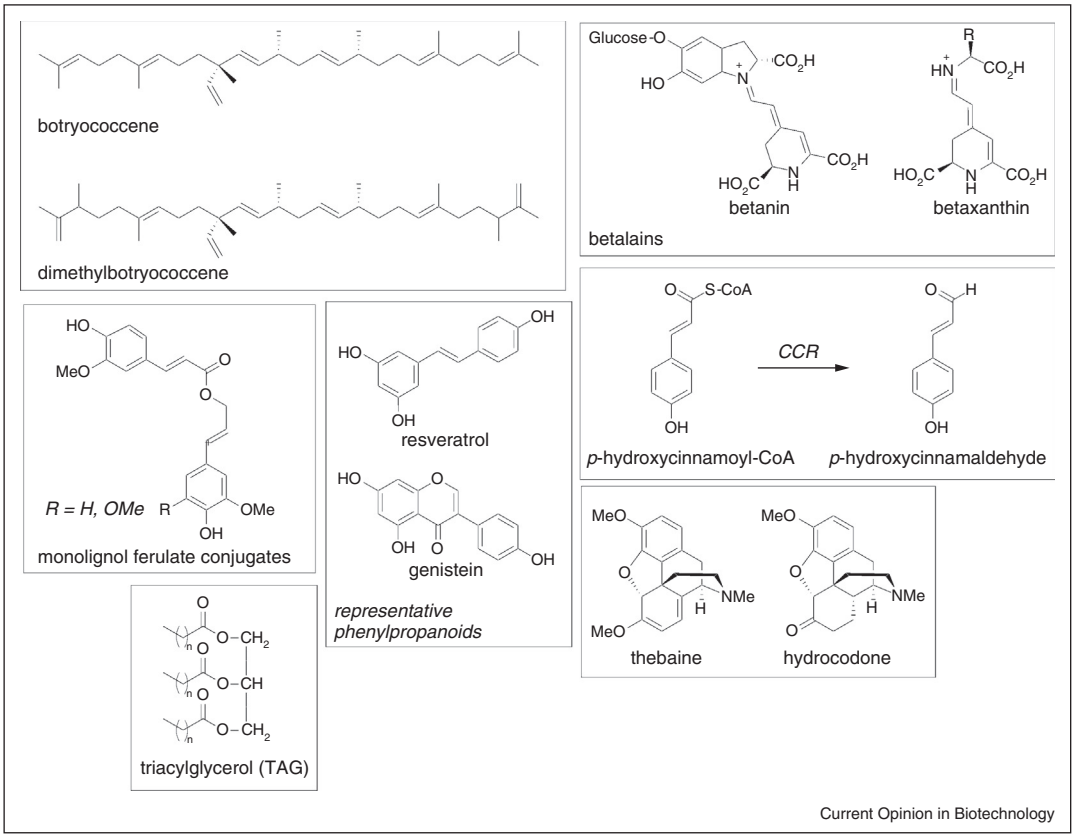
Opioids such as thebaine, codeine and morphine are widely used around the globe to treat pain [15]. Currently, farming of opium poppies and isolation of opiates from the poppy latex is the only commercial source of these compounds. However, in a recent study, yeast (*S. cerevisiea*) was engineered to produce the opiates thebaine and hydrocodone (Figure 2) de novo from an exogenous sugar carbon source [16••]. The resulting strains expressed 21 genes for thebaine production and 23 genes for hydrocodone production. While yields were low (<1 μg/L), this study provides a dramatic proof-of-principle that complex opiates can be produced in yeast. Notably, this work was made possible by the recent discovery of an opiate biosynthetic gene, reticuline epimerase, which researchers had struggled to identify for decades [16••,17••,18••].

Engineering plant pathways to create better biofuels

A major challenge of the modern era is the transition to a bio-based economy. Biofuels are a key part of this landscape, but challenges to efficiently and cost-effectively produce biofuels still remain [19,20]. Bioethanol is currently the major biofuel in use, and it is produced by the easily accessible sugars of sugar cane and corn. However, as food security becomes an increasing concern in an ever-expanding population, other approaches for producing biofuels must be considered [21]. A promising source for next generation biofuels are those produced from lignocellulosic biomass that originates from the residual biomass of crops, such as wheat, corn and sugarcane. Alternatively, the biomass from crops such as poplar and switchgrass that can be grown on marginal land are also possibilities for fuel production [22].

The presence of lignin in plant cell walls undermines the ability to access the polysaccharides of biomass by enzymatic degradation. This biomass must therefore be subjected to hydrolysis under acidic or alkaline conditions to

Figure 2



Summary of chemical structures of plant products produced by metabolic engineering strategies discussed in this review.

break the bonds between lignin and hemicellulose, before subsequent enzymatic degradation can take place. Therefore, there has been a substantial effort on metabolic engineering to reduce lignin content in plants, since it is the major limiting factor of conversion of biomass to fermentable sugars. One recent study exploited a key enzyme in lignin biosynthesis, cinammoyl-CoA reductase (CCR), which catalyzes the conversion of hydroxycinnamoyl-CoA esters to the corresponding aldehydes [23•] (Figure 2). Field trials on poplar plants have shown that biomass from transgenic plants with downregulation of CCR is more easily processed to production of bioethanol. Although downregulation of CCR results in reduced amounts of biomass due to a lower growth rate, the overall yield of sacharification suggests that this strategy could lead to more efficient biofuel production [23•]. Another attempt to design plants with cell walls more susceptible to chemical depolymerization was based on the discovery of the enzyme monolignol ferulate transferase (MFT) [24••]. The introduction of MTF into transgenic poplar plants alters the pool of monolignols, with an increase of monolignol ferulate conjugates (Figure 2). Since the ferulate conjugates are capable of introducing readily cleavable ester bonds into the lignin backbone without affecting the plant development lignification process, this proved to be a highly innovative metabolic engineering approach to produce biomass more susceptible to hydrolysis. However, it is important to note that altering the structure of the lignin polymer often has an impact on the growth and fitness of the resulting plant. A recently published perspective on the challenges of altering plant lignin content discusses some of these issues [25].

An excellent example of a systems approach for improving saccharification yields of lignin was performed on *Arabidopsis thaliana* plants [26•]. The authors restricted lignin biosynthesis to vessels while also increasing secondary cell wall thickening to generate healthy plants with increased sugar yield upon saccharification. The authors noted that reduction in lignin usually correlates with a loss of integrity in tissues responsible for water and nutrient distribution from roots to above ground tissues (vessels) [27,28]. The first step was to redirect lignin biosynthesis only to vessels by controlling expression of C4H, a key enzyme in lignin biosynthesis. This control was performed by replacing the promoter of C4H with the vessel specific promoter of transcription factor VND6. Additionally, the authors engineered the increase of

secondary wall thickening by an artificial positive feedback loop. The NST1 transcription factor is key to secondary wall regulation by controlling all the genes that are involved in biosynthesis of cellulose, hemicelluloses, and lignin polymers. A new copy of NST1 was expressed that was under control of its downstream-induced promoters to enhance the overall expression. The results of saccharification of this combinatorial approach has shown that sugar release was 2.5 times higher than the wild type and similar to plants with C4H expression switched off. This lignin rewiring approach could potentially be transferred to crop plants for enhanced bioethanol production (Figure 3).

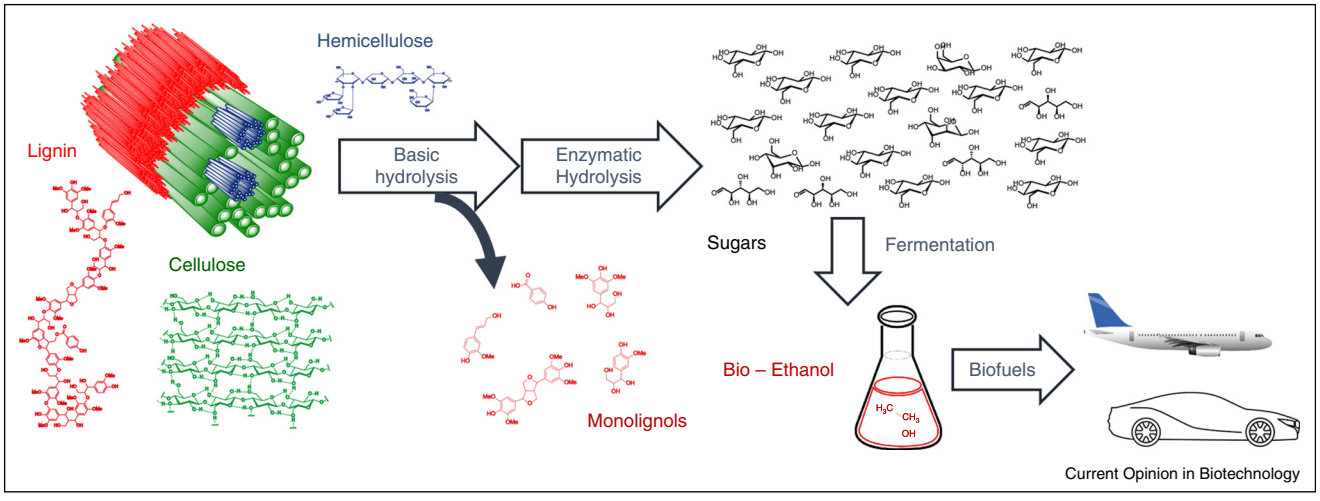
Storage lipids in plants, triacylglycerols (TAGs) (Figure 2), which are one of the most abundant and energy-rich forms of reduced carbon in nature, can be readily converted to biofuels. A second strategy to improve access to biofuels is to increase the content of TAGs in plant vegetative tissues [29]. A variety of genes that enhance TAG accumulation levels have been identified: the transcription factor WRINKLED1, the TAG biosynthetic gene diacylglycerol acyltransferase1-2 (*DGAT*1-2) and a gene encoding a structural protein oleosin1 (*OLE*1) that impacts oil body formation. Moreover, it has been shown that silencing an enzyme involved in starch biosynthesis, ADP-glucose pyrophosphorylase (AGPase), diverts carbon away from starch and into TAG biosynthesis, and silencing of the peroxisomal ABC transporter1 (PXA1) prevents fatty acids from being oxidized in the mitochondria. In this study, the authors combined all of this knowledge into a single metabolic engineering experiment. WRINKLED1, *DGAT*1-2 and *OLE*1 were expressed in sugar cane, while AGPase and PXA1 were silenced. The result was transgenic sugar cane plants that accumulated TAGs at 95-fold and 43-fold higher levels in leaves and stems compared to wild type plants.

Engineering new traits into crops by engineering plant metabolism

With the population of the planet currently at 7 billion and rising, food security is a tremendously important issue: as land becomes limiting, it becomes more important to obtain the maximum nutritional value from the crops that are grown. Many plant metabolites have important nutritional and health benefits, so crops can be made more nutritionally dense by upregulating these pathways. In particular, phenylpropanoid and terpenoid compounds have important nutritional roles in the human diet [30]. Therefore, metabolic engineering of these pathways in crop plants have the potential to dramatically impact food security.

Phenylpropanoids are plant metabolites that act as antioxidant agents, and therefore have essential health promoting properties [30]. Engineering the increase in the levels of these compounds in edible parts of crop plants could positively impact human nutrition. Tomato has been subjected to some outstanding engineering efforts to improve the production levels of various metabolites [31–33]. In one very recent example, phenylpropanoid production was substantially upregulated in tomato fruits by introducing fruit-specific expression of the *A. thaliana* transcription factor AtMYB12 [34•]. AtMYB12 increases phenylpropanoid levels by transcriptionally activating the biosynthetic genes of these pathways. However, this transcription factor also appears to direct carbon flux towards aromatic amino acid biosynthesis, which in turn increases the supply of substrate for phenylpropanoid metabolism. While the content of aromatic amino acids increased significantly in

Figure 3



The general process for production of biofuels. Plant biomass is subjected to chemical hydrolysis (basic and/or acidic), followed by enzymatic hydrolysis and then fermentation of the resulting sugars for production of biofuels (primarily bioethanol). During chemical hydrolysis, the lignin macromolecule is deconstructed to monolignols. By the use of suitable pectinolytic enzymes, cellulose and hemicellulose macromolecules are hydrolysed to simple carbohydrates. The carbohydrates are the 'food' for ethanol producing yeast during fermentation.

AtMYB12 tomatoes — 10% of fruit dry weight existed as flavonols and hydroxycinnamates (Figure 2) — the levels of major sugars simultaneously decreased, suggesting that carbon flux is being redirected to the shikimate and aromatic amino acid pathways. In contrast, other transcription factors that are known to upregulate anthocyanin biosynthesis do not upregulate the shikimate pathway that leads to aromatic amino acids. Reprogramming carbon flux to the shikimate pathway represents a systems based approach to enhance phenylpropanoid production in plants.

The biosynthesis of betalains (Figure 2), which are tyrosine-derived red-violet and yellow pigments, remains unsolved. Betalains are widely used as natural food colorants and dietary supplements [35], and L-DOPA, a betalain pathway intermediate is widely used for treatment of Parkinson's disease [36]. Most notably, the first committed step in the pathway, 3-hydroxylation of tyrosine to form L-3,4-dihydroxyphenylalanine (L-DOPA) is not characterized. Transcriptome analysis of the betalain-producing plants red beet (*Beta vulgaris*) and four o'clocks (*Mirabilis jalapa*) was used to identify a novel, betalain-related cytochrome P450-type gene, CYP76AD6 that exhibits tyrosine hydroxylase activity [37]. This discovery enabled metabolic engineering of entirely red-pigmented tobacco plants through heterologous expression of three genes taking part in the fully decoded betalain biosynthetic pathway.

Metabolic engineering approaches are also used to address environmental problems such as heavy metal toxicity [38,39]. For example, cadmium binds to the thiol groups of proteins and coenzymes and displaces endogenous metal cofactors from native binding partners [40]. Phytochelatins are peptides that protect plants from heavy metal toxicity by binding tightly to these metals. By engineering the biosynthesis of these peptides, plants could potentially be used to remediate soils contaminated with heavy metals. In a recent study, the phytochelatin synthase from *A. thaliana* (AtPCS1) was subjected to directed evolution [41••]. Surprisingly, mutants that conferred the desired tolerance phenotype in *Arabidopsis*, *Brassica juncea* or yeast were catalytically inferior to the wild type enzyme. It was hypothesized that transformation with AtPCS1 decreases the levels of the phytochelatin precursors upon exposure to cadmium, while the selected mutant enzymes do not. By maintaining the presence of phytochelatin precursors, redox homeostasis is improved. However, the attenuated biochemical activity of the mutant enzyme still supports phytochelatin synthesis during cadmium exposure. This work is a beautiful example of how the biochemical properties of an enzyme must be assessed within the context of the entire metabolic pathway to achieve the desired biological outcome.

The next generation of engineering plant metabolic pathways

While metabolic engineering of plant pathways has made substantial leaps in the last several years, new approaches to manipulate plant pathways are continually emerging. Perhaps most notably, the CRISPR/Cas9 genome engineering system has become an important new genome-editing tool for plant biologists due to this system's efficiency and specificity [42••]. While CRISPR/Cas9 studies in plants have been largely confined to proof of concept studies [42••], the approach has been implemented in a number of economically important crop plants such as rice [43], wheat [44], maize [45], soybean [46], tomato [47], potato [48] and poplar [49]. In one notable example, mutations in the MILDEW-RESISTANCE LOCUS (MLO) proteins in hexaploid wheat have been engineered through a combination of transcription activator-like effector nuclease (TALEN) and CRISPR/Cas9 technologies to confer resistance to powdery mildew [44]. While these studies are, for the most part, still in the early stages, the stage is set for CRISPR/Cas9 to dramatically impact crop trait improvement.

The use of genetically engineered plants as a food source has been a controversial topic. For example, as a mechanism to prevent blindness caused by vitamin A deficiency, a strain of rice was genetically engineered to express three heterologous genes that enabled production of vitamin A [50,51]. The controversy surrounding the use of this resulting Golden Rice highlights the challenges of reconciling public perception and genetically engineered food crops. The introduction of gene editing, which can be used to make highly targeted and controlled changes to the plant genome, has raised the question of whether certain gene-edited plants can be considered separately from 'standard' GM plants. Additionally, plants can be gene-edited with constructs that are composed exclusively of DNA sequence derived from the same or similar plant species [52]. These so-called cisgenic plants (as opposed to transgenic plants), which could in principle be obtained through standard breeding practices, may be more readily accepted by the public and regulatory bodies [52].

Despite the controversy associated with genetically modified plants, biotech crop hectarage continues to grow, with 18 million farmers in 28 countries planting more than 181 million hectares in 2014 [53]. Given the impact that plant metabolism has on health and food security, metabolic engineering of these pathways is a crucial part of our future.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. O'Connor SE: **Engineering of secondary metabolism**. *Annu Rev Genet* 2015, **49**:71-94.

2. •• Zuodong J, Chase K, Caroline JB, Eric N, Chappell J: **Engineering triterpene and methylated triterpene production in plants provides biochemical and physiological insights into terpene metabolism**. *Plant Phys* 2016, **170**:702-716.
This manuscript details how plants can be engineered to produce high levels of unusual triterpenes that have excellent promise as biofuels. The authors use several innovative approaches, including targeting of the pathway to the chloroplast, to achieve high yields.

3. Schillberg S, Fischer R, Emans N: **Molecular farming of recombinant antibodies in plants**. *Cell Mol Life Sci* 2003, **60**:433-445.

4. Li Y, Pfeifer BA: **Heterologous production of plant-derived isoprenoid products in microbes and the application of metabolic engineering and synthetic biology**. *Curr Opin Plant Biol* 2014, **19**:8-13.

5. Crocoll C, Mirza N, Reichelt M, Gershenzon J, Halkier BA: **Optimization of engineered production of the glucoraphanin precursor dihomomethionine in *Nicotiana benthamiana***. *Front Bioeng Biotechnol* 2016, **4**:14.

6. Polturak G, Breitel D, Grossman N, Sarrion-Perdigones A, Weithorn E, Pliner M, Orzaez D, Granell A, Rogachev I, Aharoni A: **Elucidation of the first committed step in betalain biosynthesis enables the heterologous engineering of betalain pigments in plants**. *New Phytol* 2016, **210**:269-283.

7. Geisler K, Hughes RK, Sainsbury F, Lomonossoff GP, Rejzek M, Fairhurst S, Olsen CE, Motawia MS, Melton RE, Hemmings AM, Bak S, Osbourn A: **Biochemical analysis of a multifunctional cytochrome P450 (CYP51) enzyme required for synthesis of antimicrobial triterpenes in plants**. *Proc Natl Acad Sci U S A* 2013, **110**:E3360-E3367.

8. Zhuang X, Chappell J: **Building terpene production platforms in yeast**. *Biotechnol Bioeng* 2015, **112**:1854-1864.

9. Ignea C, Athanasakoglou A, Ioannou E, Georgantea P, Trikka FA, Loupassaki S, Roussis V, Makris AM, Kampranis SC: **Carnosic acid biosynthesis elucidated by a synthetic biology platform**. *Proc Natl Acad Sci U S A* 2016, **113**:3681-3686.

10. Ignea C, Ioannou E, Georgantea P, Trikka FA, Athanasakoglou A, Loupassaki S, Roussis V, Makris AM, Kampranis SC: **Production of the forskolin precursor 11β-hydroxy-manoyl oxide in yeast using surrogate enzymatic activities**. *Microb Cell Fact* 2016, **15**:46.

11. Qu Y, Easson ML, Froese J, Simionescu R, Hudlicky T, De Luca V: **Completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline and its assembly in yeast**. *Proc Natl Acad Sci U S A* 2015, **112**:6224-6229.

12. Brown S, Clastre M, Courdavault V, O'Connor SE: **De novo production of the plant-derived alkaloid strictosidine in yeast**. *Proc Natl Acad Sci U S A* 2015, **112**:3205-3210.

13. Hillen LW, Pollard G, Wake LV, White N: **Hydrocracking of the oils of *Botryococcus braunii* to transport fuels**. *Biotechnol Bioeng* 1982, **24**:193-205.

14. Niehaus TD, Okada S, Devarenne TP, Watt DS, Sviripa V, Chappell J: **Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii***. *Proc Natl Acad Sci U S A* 2011, **108**:12260-12265.

15. Ziegler J, Facchini PJ: **Alkaloid biosynthesis: metabolism and trafficking**. *Ann Rev Plant Biol* 2008, **59**:735-769.

16. •• Galanie S, Thodey K, Trenchard IJ, Filsinger IM, Smolke CD: **Complete biosynthesis of opioids in yeast**. *Science* 2015, **349**:1095-1100.
This study highlights how the complete pathways for high-value plant-derived opioids can be expressed in yeast. The opioids can be produced de novo from simple sugars.

17. •• Winzer T, Kern M, King AJ, Larson TR, Teodor RI, Donninger SL, Li Y, Dowle AA, Cartwright J, Bates R, Ashford D, Thomas J, Walker C, Bowser TA, Graham IA: **Morphinan biosynthesis in opium poppy requires a P450-oxidoreductase fusion protein**. *Science* 2015, **349**:309-312.
This study used a genetic approach to identify the missing enzyme of morphine biosynthesis as an unusual fusion of a reductase and P450.

18. •• Farrow SC, Hagel JM, Beaudoin GA, Burns DC, Facchini PJ: **Stereochemical inversion of (S)-reticuline by a cytochrome p450 fusion in opium poppy**. *Nat Chem Biol* 2015, **11**:728-732.
These authors identified the missing enzyme of morphine biosynthesis (P450-reductase fusion) through biochemical and gene silencing approaches.

19. Tyner WE: **Biofuels and agriculture: a past perspective and uncertain future**. *Intl J Sustain Dev World Ecol* 2012, **19**:389-394.

20. Taheripour TMF, Zhuang Q, Tyner WE, Lu X: **Biofuels, cropland expansion, and the extensive margin**. *Energy Sustain Soc* 2012, **2**:25.

21. Healey AL, Lee DJ, Furtado A, Simmons BA, Henry RJ: **Efficient eucalypt cell wall deconstruction and conversion for sustainable lignocellulosic biofuels**. *Front Bioeng Biotechnol* 2015, **3**:190.

22. Evans SG, Ramage BS, Dirocco TL, Potts MD: **Greenhouse gas mitigation on marginal land: a quantitative review of the relative benefits of forest recovery versus biofuel production**. *Environ Sci Technol* 2015, **49**:2503-2511.

23. • Van Acker R, Leplé JC, Aerts D, Storme V, Goeminne G, Ivens B, Légée F, Lapierre C, Piens K, Van Montagu MC, Santoro N, Foster CE, Ralph J, Soetaert W, Pilate G, Boerjan W: **Improved saccharification and ethanol yield from field-grown transgenic poplar deficient in cinnamoyl-CoA reductase**. *Proc Natl Acad Sci U S A* 2014, **111**:845-850.
The authors showed that when the lignan biosynthetic enzyme cinnamoyl-CoA reductase was downregulated in poplar, more ethanol could be obtained after processing the wood. They note that downregulation of this gene could be a strategy to improve ethanol production.

24. •• Wilkerson CG, Mansfield SD, Lu F, Withers S, Park JY, Karlen SD, Gonzales-Vigil E, Padmakshan D, Unda F, Rencoret J, Ralph J: **Monolignol ferulate transferase introduces chemically labile linkages into the lignin backbone**. *Science* 2014, **344**:90-93.
This manuscript describes the discovery of the enzyme monolignol ferulate transferase, which can introduce more chemically labile ester linkages into the lignin structure. Transformation of this gene into poplar resulted in tissue that could be more readily decomposed.

25. Doblin MS, Johnson KL, Humphries J, Newbigin EJ, Bacic A: **Are designer plant cell walls a realistic aspiration or will the plasticity of the plant's metabolism win out?** *Curr Opin Biotechnol* 2014, **26**:108-114.

26. • Yang F, Mitra P, Zhang L, Prak L, Verhertbruggen Y, Kim JS, Sun L, Zheng K, Tang K, Auer M, Scheller HV, Loqué D: **Engineering secondary cell wall deposition in plants**. *Plant Biotechnol J* 2013, **11**:325-335.
The authors describe a systems-wide approach to metabolic engineering of cell wall biosynthesis in plants. By enhancing lignan biosynthesis in the vessels, the authors improved saccharification yields while maintaining biomass.

27. Boyce CK, Zwieniecki MA, Cody GD, Jacobsen C, Wirick S, Knoll AH, Holbrook NM: **Evolution of xylem lignification and hydrogel transport regulation**. *Proc Natl Acad Sci U S A* 2004, **101**:17555-17558.

28. Dejardin A, Laurans F, Arnaud D, Breton C, Pilate G, Lepl J-C: **Wood formation in angiosperms**. *C R Biol* 2010, **333**:325-334.

29. Zale J, Jung JH, Kim JY, Pathak B, Karan R, Liu H, Chen X, Wu H, Candreva J, Zhai Z, Shanklin J, Altpeter F: **Metabolic engineering of sugarcane to accumulate energy-dense triacylglycerols in vegetative biomass**. *Plant Biotechnol J* 2016, **14**:661-669.

30. Korkina LG: **Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health**. *Cell Mol Biol* 2007, **53**:15-25.

31. Butelli E, Titta L, Giorgio M, Mock HP, Matros A, Peterek S, Schijlen EG, Hall RD, Bovy AG, Luo J, Martin C: **Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors**. *Nat Biotechnol* 2008, **26**:1301-1308.

32. Giorio G, Yildirim A, Stigliani AL, D'Ambrosio C: **Elevation of lutein content in tomato: a biochemical tug-of-war between lycopene cyclases**. *Metab Eng* 2013, **20**:167-176.

33. Gutensohn M, Nguyen TT, McMahon RD, Kaplan I, Pichersky E, Dudareva N: **Metabolic engineering of monoterpene biosynthesis in tomato fruits via introduction of the non-canonical substrate neryl diphosphate**. *Metab Eng* 2014, **24**:107-116.

34. • Zhang Y, Butelli E, Alseekh S, Tohge T, Rallapalli G, Luo J, Kawar PG, Hill L, Santino A, Fernie AR, Martin C: **Multi-level engineering facilitates the production of phenylpropanoid compounds in tomato**. *Nat Commun* 2015, **6**:8635.
The authors discovered that a transcription factor from *Arabidopsis* enhanced not only the levels of phenylpropanoids in tomato, but also impacted the metabolic flux driving carbon into the upstream shikimate pathway.

35. Azeredo HMC: **Betalains: properties, sources, applications, and stability — a review**. *Intl J Food Sci Technol* 2009, **44**:2365-2376.

36. Nagatsu T, Sawada M: **L-Dopa therapy for Parkinson's disease: past, present, and future**. *Parkinsonism Relat Disord* 2009, **15**:S3-S8.

37. Polturak G, Breitel D, Grossman N, Sarrion-Perdigones A, Weithorn E, Pliner M, Orzaez D, Granell A, Rogachev I, Aharoni A: **Elucidation of the first committed step in betalain biosynthesis enables the heterologous engineering of betalain pigments in plants**. *New Phytol* 2015 http://dx.doi.org/10.1111/nph.13796.

38. Mohanty M, Patra HK: **Attenuation of chromium toxicity by bioremediation technology**. *Rev Environ Contam Toxicol* 2011, **210**:1-34.

39. Thévenod F, Lee WK: **Toxicology of cadmium and its damage to mammalian organs**. *Met Ions Life Sci* 2013, **11**:415-490.

40. Salt DE, Smith RD, Raskin I: **Phytoremediation**. *Annu Rev Plant Physiol Plant Mol Biol* 1998, **49**:643-668.

41. •• Cahoon RE, Lutke WK, Cameron JC, Chen S, Lee SG, Rivard RS, Rea PA, Jez JM: **Adaptive engineering of phytochelatin-based heavy metal tolerance**. *J Biol Chem* 2015, **290**:17321-17330.
This study uses directed evolution of a phytochelatin biosynthetic enzyme to improve the heavy metal tolerance of *Arabidopsis*. The work unexpectedly shows that catalytic activity of a single enzyme must be considered within the context of the entire metabolic pathway to achieve the desired biological trait.

42. •• Schaeffer SM, Nakata PA: **CRISPR/Cas9-mediated genome editing and gene replacement in plants: transitioning from lab to field**. *Plant Sci* 2015, **240**:130-142.
This excellent review provides an up to date summary of the progress that has been made in applying next generation gene0editing methods to crop plants. The review also provides insight into the challenges, advantages and limitations of this technology.

43. Zhou H, Liu B, Weeks DP, Spalding MH, Yang B: **Large chromosomal deletions and heritable small genetic changes induced by CRISPR/Cas9 in rice**. *Nucleic Acids Res* 2014, **42**:10903-10914.

44. Wang Y, Cheng X, Shan Q, Zhang Y, Liu J, Gao C, Qiu JL: **Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew**. *Nat Biotechnol* 2014, **32**:947-951.

45. Liang Z, Zhang K, Chen K, Gao C: **Targeted mutagenesis in *Zea mays* using TALENs and the CRISPR/Cas system**. *J Genet Genomics* 2014, **41**:63-68.

46. Jacobs TB, LaFayette PR, Schmitz RJ, Parrott WA: **Targeted genome modifications in soybean with CRISPR/Cas9**. *BMC Biotechnol* 2015, **15**:16.

47. Brooks C, Nekrasov V, Lippman ZB, van Eck J: **Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system**. *Plant Physiol* 2014, **166**:1292-1297.

48. Wang S, Zhang S, Wang W, Xiong X, Meng F, Cui X: **Efficient targeted mutagenesis in potato by the CRISPR/Cas9 system**. *Plant Cell Rep* 2015, **34**:1473-1476.

49. Fan D, Liu T, Li C, Jiao B, Li S, Hou Y, Luo K: **Efficient CRISPR/Cas9-mediated targeted mutagenesis in Populus in the first generation**. *Sci Rep* 2015, **5**:12217.

50. Moghissi AA, Pei S, Liu Y: **Golden rice: scientific, regulatory and public information processes of a genetically modified organism**. *Crit Rev Biotechnol* 2015, **15**:1-7.

51. Ye X, Al-Babili S, Klöti A, Zhang J, Lucca P, Beyer P, Potrykus I: **Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm**. *Science* 2000, **287**:303-305.

52. Schouten HJ, Krens FA, Jacobsen E: **Cisgenic plants are similar to traditionally bred plants**. *EMBO Rep* 2006, **7**:750-753.

53. James C: **Global status of commercialized biotech/GM crops**. *International Service for the Acquisition of Agri-biotech Applications*. 2014.