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Metabolic engineering approaches for production of biochemicals in food and medicinal plants

Sarah A Wilson and Susan C Roberts

Historically, plants are a vital source of nutrients and pharmaceuticals. Recent advances in metabolic engineering have made it possible to not only increase the concentration of desired compounds, but also introduce novel biosynthetic pathways to a variety of species, allowing for enhanced nutritional or commercial value. To improve metabolic engineering capabilities, new transformation techniques have been developed to allow for gene specific silencing strategies or stacking of multiple genes within the same region of the chromosome. The 'omics' era has provided a new resource for elucidation of uncharacterized biosynthetic pathways, enabling novel metabolic engineering approaches. These resources are now allowing for advanced metabolic engineering of plant production systems, as well as the synthesis of increasingly complex products in engineered microbial hosts. The status of current metabolic engineering efforts is highlighted for the *in vitro* production of paclitaxel and the *in vivo* production of β-carotene in Golden Rice and other food crops.

Addresses
Department of Chemical Engineering, University of Massachusetts Amherst, 159 Goessman Laboratory, 686 North Pleasant Street, Amherst, MA 01003, United States

Corresponding author: Roberts, Susan C. (sroberts@ecs.umass.edu)

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Introduction

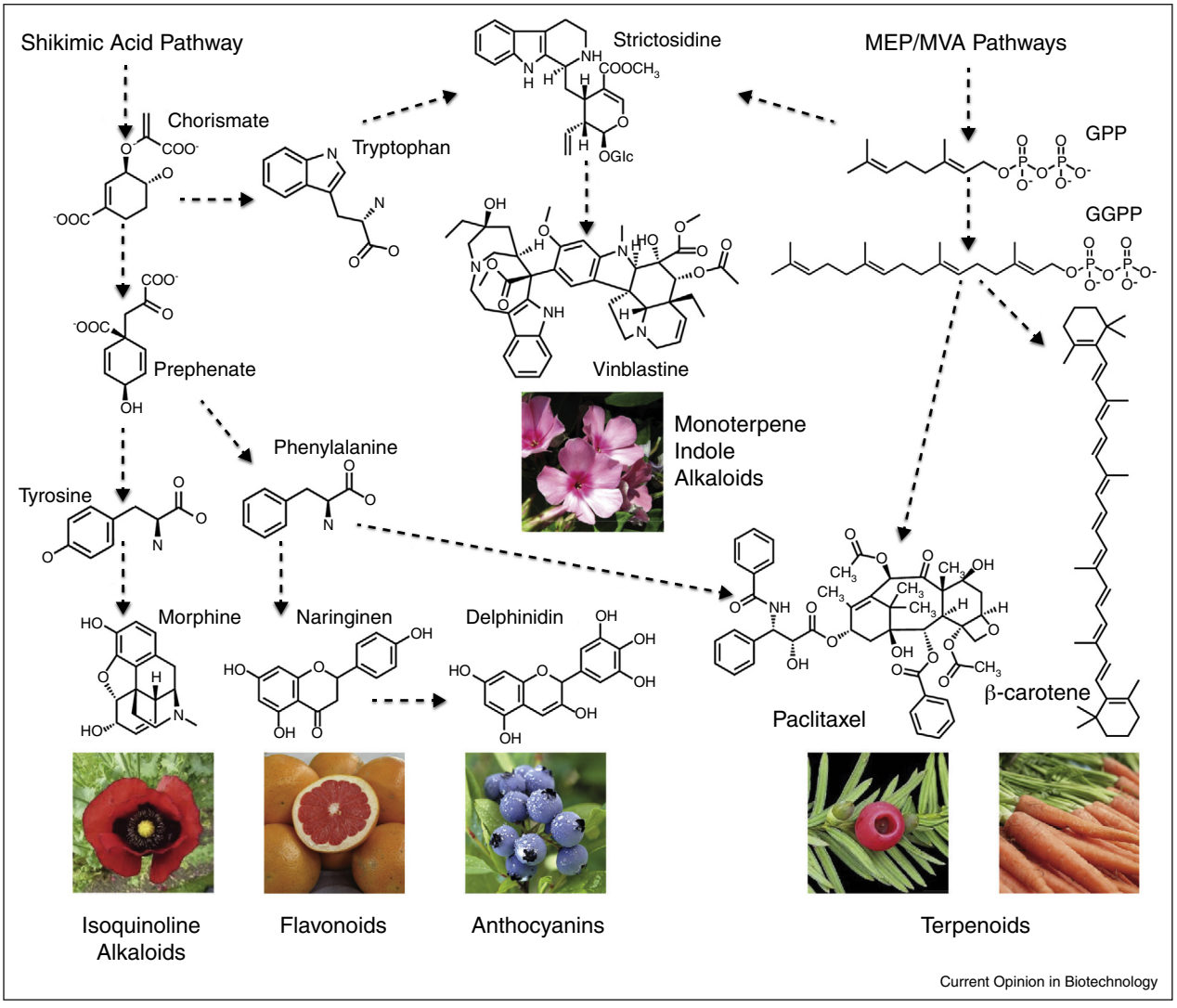
For centuries, plants have been utilized as both a food source and a source of active pharmaceutical agents. As of 2010, 75% of anti-bacterial compounds and 48.6% of anti-cancer compounds were either a natural product or natural product analog [1]. To aid in development and defense against stress, plants synthesize hundreds of thousands of compounds, many of which are produced through species-specific and complex biosynthetic pathways [2]. For instance, the shikimic acid pathway, non-mevalonate (MEP) pathway and mevalonate (MVA) pathway lead to diverse classes of compounds, which include the terpenoids, monoterpene indole alkaloids, isoquinoline alkaloids, flavonoids and anthocyanins (Figure 1). Due to their high degree of structural diversity, these compounds have significant commercial value as pharmaceuticals, nutraceuticals, dyes, fragrances, flavors and pesticides. In addition to their commercial applications, many dietary health benefits can be attributed to specific plant natural products in popular food crops. For instance, the compounds seen in Figure 1 are anti-cancer agents (vinblastine and paclitaxel), analgesics (morphine), antioxidants (naringenin and delphinidin) and provitamins (β-carotene).

Although some valuable plant natural products with simple structures are easily chemically synthesized (e.g., aspirin and ephedrine), many have complex structures with multiple chiral centers, making chemical synthesis both difficult and commercially infeasible [3]. As a result, these compounds are often produced through the exploitation of native biological pathways using natural harvest (e.g., codeine, morphine and dietary food compounds), semi-synthesis (e.g., paclitaxel), heterologous production (e.g., vanilla) or plant cell culture techniques (e.g., paclitaxel, ginseng and anthocyanins) [4]. In these biological systems, metabolic engineering can be used to manipulate flux through both primary and secondary metabolic pathways, allowing for the redirection of carbon flux towards products of interest. This review focuses on the use of metabolic engineering techniques for increasing the production of valuable plant natural products in both medicinal plants and food crops, allowing for decreased production costs and/or increased food nutritional value.

Methods for gene transfer and expression modification

To allow for metabolic engineering of a plant, the system must be amenable to stable transformation. Traditional gene transfer approaches (most commonly *Agrobacterium* transformation and particle bombardment) have advanced greatly over the last decade, allowing for the development of reliable stable transformation methods for many non-model plant species that were previously recalcitrant [5]. Moving forward, much effort has focused on advancing metabolic engineering technologies to allow for transgenics without selective markers and integration of genes in specific regions of the chromosome. Traditionally, plants are transformed with a selective marker (typically an antibiotic or herbicide resistance, such as kanamycin resistance (*npt II*) or hygromycin resistance (*hpt II*)), allowing for selection of successfully transformed

Figure 1



Examples of biosynthetic pathways leading to large classes of pharmaceutical or nutraceutical plant natural compounds. Arrows represent carbon flux through a pathway starting from primary metabolism (shikimic acid and MEP/MVA pathways) and leading to classes of secondary metabolites. Vinblastine and paclitaxel require precursors from both primary metabolic pathways as indicated by the arrows.

cells when efficiencies are low. Due to the risk of transgene migration to weeds or pathogens, selection-free transgenic systems are needed to meet regulatory and biosafety requirements for genetically modified crops. Selectable markers can be removed using techniques such as co-transformation, site-specific recombination, multi-autotransformation vectors, transposition systems and homologous recombination, as recently reviewed [6].

Zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs) have been utilized to allow for site-specific gene mutation, replacement or integration through non-homologous end joining or homologous recombination. These artificial enzymes utilize a DNA cleavage domain fused to a DNA binding domain that can be programmed to target specific regions of the genome. This enables directed mutagenesis of specific genes for silencing, transgene targeting to increase expression or stacking of multiple genes at a single loci to improve genetic transmission over multiple generations. Zinc finger technology was first demonstrated in plants with the model species *Arabidopsis* [7] and has since been used to confer herbicide resistance in both tobacco and *Zea mays* [8,9]. Recently, ZFNs were used to stack genes conferring two herbicide resistances in embryonic maize suspension cultures, allowing for co-segregation of genes to progeny [10•]. TALEN technology has been demonstrated in *Arabidopsis* [11], tobacco [12••], *Brachypodium* [13], rice [13,14], barley [15] and *Brassica* [16]. In rice, TALENs were used to confer

resistance to bacterial blight through introduction of mutations in the bacterial blight susceptibility gene *Os11N3* [14]. The high efficiency of these systems can allow for isolation of a transformant without the use of reporter or selection genes. This was demonstrated in tobacco protoplasts using TALENs, where targeted gene mutations and insertions were isolated without the use of selective markers [12••]. Whereas traditional silencing strategies (hairpin or virus-induced gene silencing vectors) often silence entire families of genes due to sequence homology, ZFNs and TALENs can be designed with enough specificity to target either single or multiple copies of a redundant gene within a genome, which was demonstrated using ZFNs in *Glycine max* [17••]. Recently, metabolic engineering strategies based on the bacterial clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated systems have been developed that offer an alternative to ZFNs and TALENs for site-specific gene modification [18•]. This system has been shown to effectively introduce targeted genome modifications in *Arabidopsis* [19], *Nicotiana benthamiana* [19,20], rice and wheat [21], and has exciting potential due to simplified genome targeting using chimeric RNA molecules.

Emerging tools for identifying metabolic engineering targets

Once a stable transformation procedure has been established, a metabolic engineering strategy must be identified. Metabolic engineering for increasing natural product yield can be approached using two primary strategies, depending upon the desired outcome. For food crops, increasing the production of an entire class of compounds is often beneficial, leading to enhanced ability of a plant to adapt to the environment (e.g., pests, temperature, drought, UV) or increasing the overall nutritional value of a food product (e.g., antioxidant capacity, vitamin production, fatty acid production). On the other hand, it is often necessary to target specific compounds within a biosynthetic pathway, allowing for increased yield of a single product for medicinal or nutraceutical applications. Typical metabolic engineering strategies for this case include upregulation or silencing of specific pathway genes, cooperative or competing pathway genes, or transcription factors, as well as introduction of heterologous genes to allow for production of non-native compounds [22]. For these strategies, at least partial characterization of the biosynthetic pathway for the compound(s) of interest is required.

Traditionally, there are several techniques that can be used to identify and characterize genes within an unelucidated pathway. These techniques include precursor feeding, gene overexpression and inhibition, mutant selection or differential gene expression studies using elicitation to create varied phenotypic states. More recently, there has been a large shift towards the use of 'omics' approaches, which take advantage of readily accessible sequencing technologies. Since the sequencing of the first plant genome, the model species *Arabidopsis*, in 2000 [23], genomic data for over 25 crop species have been published, with over half of them released since 2012 [24]. Once a reference genome has been established, high throughput sequencing technologies can be used to identify genes responsible for specific phenotypes through quantitative trait loci (QTL) and genome-wide association studies (GWAS). Due to the high degree of phenotypic variability in rice, GWAS could be used to identify putative genes responsible for 14 phenotypic traits [25] as well as flowering time and grain related traits [26].

For species where genomic data are unavailable, transcriptomic analyses can identify putative biosynthetic pathway genes through the use of differential expression studies. Next-generation sequencing can be used in RNA-sequencing (RNA-seq) experiments to collect transcriptomic data on multiple samples from different tissues, over time or undergoing different treatments, allowing for identification of key genes involved in specific cellular responses [27]. Utilizing RNA-seq data from *Catharanthus roseus*, a metabolic pathway database, CathaCyc, was generated, which allows for visualization and interpretation of complex transcriptomic data [28]. For this model, a focus was placed upon relevant secondary metabolism (e.g., biosynthetic pathway for terpene indole alkaloid (TIA) synthesis) and the metabolism of plant hormones (e.g., biosynthetic pathway for jasmonic acid, an elicitor of TIA production). The PhytoMetaSyn Project has embarked on gathering transcriptomic data for 75 relevant medicinal plant species producing terpenoids, alkaloids and polyketides [29,30]. The goal is to use these data to elucidate the biosynthetic pathways for important high value metabolites, allowing for reconstruction of complete pathways in microbial systems. To date, transcriptomic data have been collected for hundreds of non-model plant systems [30]. These data sets will be crucial to elucidation of uncharacterized metabolic pathways and also allow for discovery of enzyme variants that can be used in metabolic engineering and heterologous production approaches. Recently, co-expression analysis of transcriptomic data from *C. roseus* led to the discovery of an alternative enzyme for the cyclization of terpenes, highlighting the potential of these data sets for enzyme discovery [31••].

Utilizing data from metabolomic and proteomic analyses (recently reviewed in [32]) can enhance the knowledge gained from transcriptomic studies. For instance, analysis of hairy root and suspension cultures of *Ophiorrhiza pumila*, which differentially accumulate secondary metabolites, allowed for identification of putative genes in monoterpene indole alkaloid (i.e., the anticancer drug camptothecin) and anthraquinone biosyntheses [33]. The

Medicinal Plant Genomics Consortium embarked on an ambitious project to determine the genome, as well as transcriptome and metabolome, for 14 key medicinal plants, including *C. roseus* (source of anti-cancer agents vinblastine and vincristine) and *Camptotheca acuminate* (source of the anti-cancer agent camptothecin) (Medicinal Plant Genomics Resource; URL: http://medicinalplantgenomics.msu.edu/). Data from these studies have been publicly released, dramatically increasing the knowledge available for characterization of unelucidated biosynthetic pathways for pharmaceutical compounds.

Metabolic engineering advances for societal impact

Taxus — *in vitro* production of paclitaxel

Paclitaxel, a potent anti-cancer compound originally extracted from the bark of *Taxus brevifolia*, is currently used in the treatment of ovarian, breast and lung cancers, as well as AIDS-related Kaposi's sarcoma [34]. Although production utilizing *Taxus* plant cell culture is currently commercially sustainable, research efforts have focused on decreasing production costs through metabolic engineering of *Taxus* cell cultures for enhanced yields and heterologous expression of the paclitaxel biosynthetic pathway in microbial hosts. Through precursor feeding, hybridization probes and differential gene expression studies, many of the 19 putative steps within the paclitaxel biosynthetic pathway have been fully characterized [34,35] (Figure 2), although some key steps remain elusive (e.g., oxetane ring formation). In addition, *Taxus* culture transformation methods have been recently established [36,37], allowing for metabolic engineering efforts to proceed.

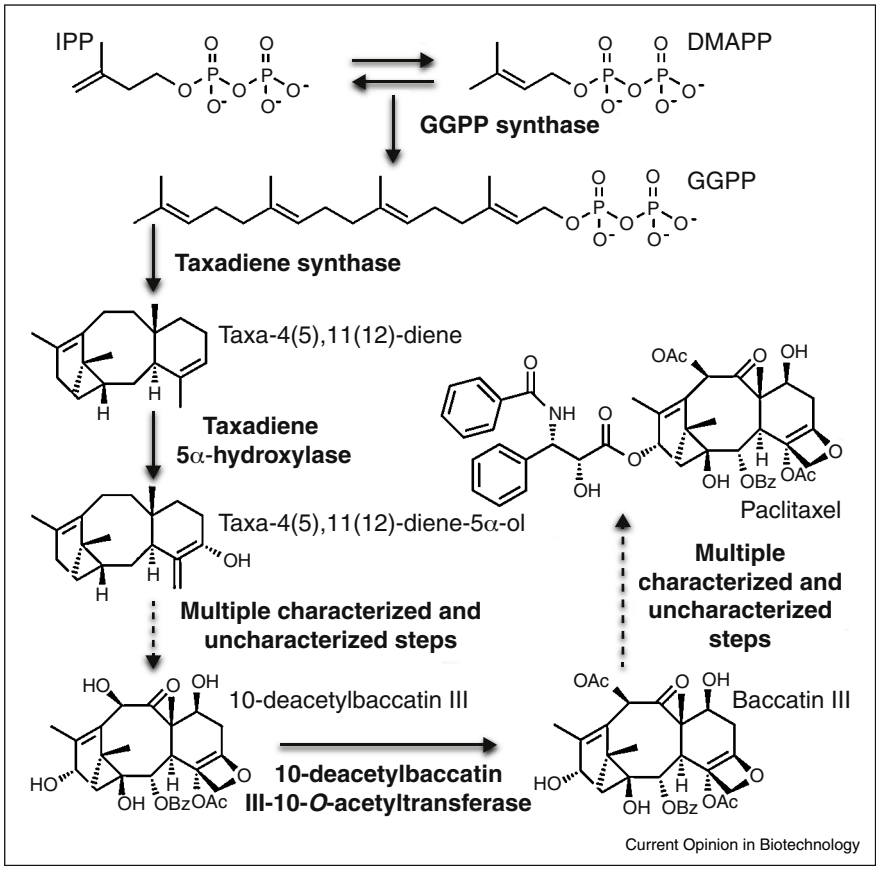
Taxadiene synthase (TASY), the first committed step in paclitaxel biosynthesis, was upregulated in hairy roots of *Taxus* × *media*, resulting in a 265% increase in paclitaxel production over untransformed cultures [38]. In *T. chinensis* suspension cultures, upregulation of 10-deacetylbaccatin III-10 β-*O*-acetyltransferase, a key enzyme in paclitaxel biosynthesis, resulted in a 1.7-fold increase in paclitaxel production [39]. Although upregulation of key pathway genes has led to an increase in paclitaxel production, studies have shown that paclitaxel accumulation is not entirely dependent upon biosynthetic pathway transcript levels within a culture [40,41]. In *Taxus* suspension cultures, differences in the expression level of known paclitaxel biosynthetic pathway genes were negligible or minor for cell populations that accumulated up to a 15-fold difference in paclitaxel [40]. These results suggest the need for studies on regulatory mechanisms outside of the biosynthetic pathway. Using particle bombardment, 9-cis-epoxycarotenoid dioxygenase, a key gene involved in abscisic acid (ABA) biosynthesis (a regulator of the plant stress response) was upregulated, resulting in a 48% increase in ABA accumulation and a 2.7-fold increase in paclitaxel accumulation [42]. To improve metabolic engineering strategies, transcriptomic analyses have been used to identify putative pathway genes, transcription factors and other genes involved in the regulation of paclitaxel biosynthesis [43–46]. Recently, a transcriptome was generated for *Taxus* × *media* cells during steady state paclitaxel production (7 days after elicitation with 200 μM methyl jasmonate) [47]. Although gene expression changes have been shown to return to basal levels within four days after elicitation with methyl jasmonate [41,48], the sequencing project was able to identify putative genes within the paclitaxel biosynthetic pathway, as well as proposed genes involved in paclitaxel transport and degradation.

To allow for further characterization of putative genes, as well as to exploit the fast growth and inexpensive substrate utilization associated with microbial systems, the first genes involved in paclitaxel biosynthesis have been introduced into microbes [49–53]. Most notably, a compartmentalized system was used to balance the precursor supply of geranylgeranyl diphosphate (GGPP) to the first product, taxadiene, reducing the inhibitory effects of indole accumulation and resulting in a yield of 1.02 g/L taxadiene [52]. This system was then used to successfully express the first cytochrome P450 involved in paclitaxel biosynthesis. After codon optimization, modification of the N-terminal of the transmembrane region of the protein and the generation of chimera enzymes fused with a reductase, a final yield of 60 mg/L of taxadiene-5α-ol was achieved [52]. To fully express the paclitaxel biosynthetic pathway in *E. coli*, the pathway must first be fully characterized or alternate enzymes to produce the same chemistries identified, which to date has not been possible. In addition, the pathway includes multiple cytochrome P450s, which require complex engineering strategies for expression in *E. coli* due to the need for a complimentary reductase, inefficient cofactor pools and improper folding, translation and insertion in the membrane. A recent study utilized transcriptome and metabolite data from 10 diterpene accumulating species to identify 46 putative diterpene synthase genes and over 400 putative terpenoid related cytochrome P450s [54•]. Although *Taxus* was not included in this study, the methods utilized could be extended to the existing transcriptome data to identify putative genes involved in the biosynthesis of diterpenes, as well as paclitaxel.

Golden Rice — *in vivo* production of β-carotene

Vitamin A deficiency affects an estimated 250 million preschool children worldwide and can lead to night blindness, xeropthalmia (dry eyes), keratomalacia, bone growth deficiencies and a weakened immune system (World Health Organization; URL: http://www.who.int/nutrition/topics/vad/en/). β-Carotene, which can be found in plant sources such as carrots, sweet potato, pumpkin and kale, is a provitamin that can yield two molecules of

Figure 2



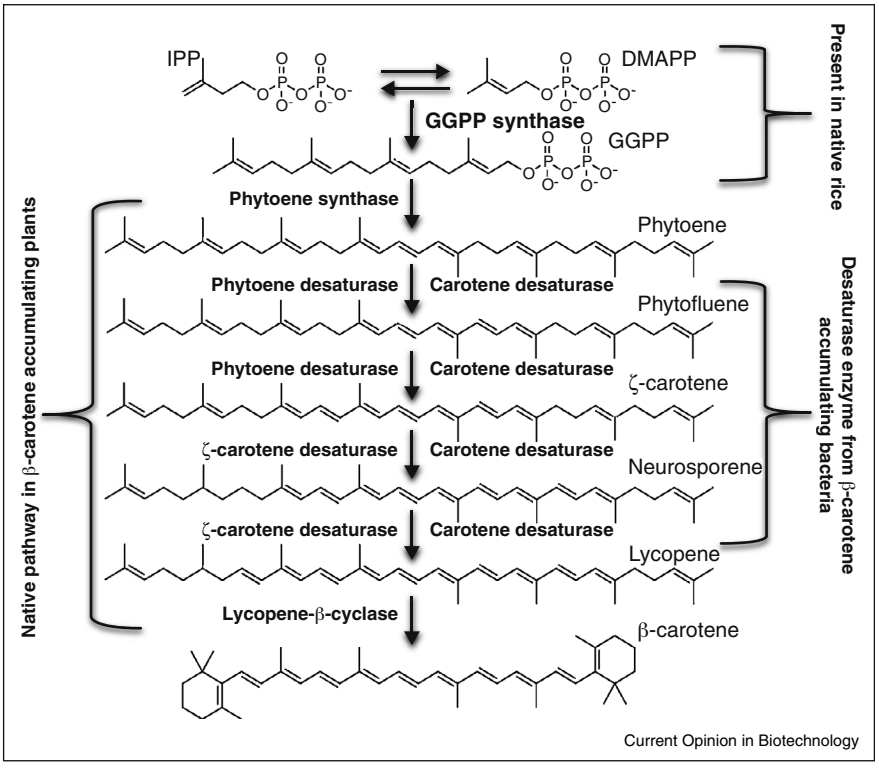
Paclitaxel biosynthetic pathway. Solid arrows represent single steps within the biosynthetic pathway that have been fully characterized. Dotted arrows represent multiple characterized and uncharacterized steps within the biosynthetic pathway.

retinoic acid (vitamin A) in the body [55]. An early intermediate to β-carotene (GGPP) is present in native rice, which, along with maize and wheat, is an important food crop and is the main staple food crop for half of the world's population [56]. However, several fully characterized genes are missing in the endogenous rice system to transform GGPP to β-carotene (Figure 3), rendering traditional breeding technologies ineffective for increasing accumulation levels in this staple food crop.

Using metabolic engineering strategies, expression of a daffodil phytoene synthase in the rice endosperm led to an accumulation of the colorless carotene phytoene [57], demonstrating the capability of rice to express genes in the β-carotene biosynthetic pathway. To further convert phytoene to the final product β-carotene, three plant enzymes are required. In bacteria, a carotene desaturase is capable of combining the activity of two of the plant enzymes, simplifying the pathway (Figure 3). Two vectors containing phytoene synthase and lycopene-β-cyclase from daffodil (*Narcissus pseudonarcissus*) as well as the bacterial carotene desaturase from *Erwinia uredovora* were expressed in the rice endosperm. This co-expression resulted in the first line of Golden Rice, which is yellow in color due to the accumulation of β-carotene [58]. To increase accumulation levels, phytoene synthase genes from multiple plant species were examined for enhanced activity over the levels of the daffodil enzyme. By replacing the phytoene synthase from daffodil with one from maize (*Zea mays*), a 23-fold increase (37 μg β-carotene per gram of rice) in carotenoid levels was achieved, with high preference for β-carotene accumulation (30 μg per gram of rice) [59].

In two separate studies, β-carotene produced in Golden Rice was shown to have high bioavailability, where a 50 g dry weight serving to children provides enough vitamin A to meet >60% of the recommended daily allowance [60,61]. This bioavailability is comparable to the bioavailability of β-carotene in oil capsules, which are often used in vitamin A supplementation strategies. In addition to the development of Golden Rice, genetic engineering has

Figure 3



β-Carotene biosynthetic pathway. Shown in brackets are: (a) the native pathway to GGPP in rice, (b) the pathway to β-carotene in plants and (c) the alternative enzyme to β-carotene found in bacteria, which combines the activity of two plant enzymes (phytoene desaturase and ζ-carotene desaturase) into carotene desaturase.

also been used to introduce β-carotene to other staple cereals (maize, wheat and sorghum), *Brassicas* (canola and cauliflower), root vegetables (carrot, potato, cassava and sweet potato) and fruits (mango, banana, pumpkin, melon, kiwifruit, and tomato) [62]. Recently, β-carotene production was increased up to 36-fold in the fruit of oranges through the silencing of the β-carotene hydroxylase gene, a gene responsible for the conversion of β-carotene to xanthophylls in the orange [63]. Additionally, rice is being engineered for increased production of folate, iron and amino acids to help combat nutritional deficiencies in developing countries [56]. The main limitations to the use of these metabolically engineered food crops for fighting malnutrition is global regulation, as well as consumer acceptance of engineered foods.

Future outlook

Metabolic engineering for the improved synthesis of plant natural products offers significant promise for decreasing production costs for commercial compounds and increasing the nutritional value of food crops. One of the major limitations to metabolic engineering of these systems is the lack of fully elucidated plant biosynthetic pathways. Although the 'omics' era has led to an enormous increase in the data available, correct annotation and functional characterization of enzymes is crucial to the successful implementation of metabolic engineering strategies. In addition, the complex interactions amongst biosynthetic pathways and sophisticated regulatory mechanisms further complicate engineering efforts. For example, in *C. roseus* hairy root cultures, an increase in the expression of a single gene (1-deoxy-D-xylulose synthase, DXS) within the TIA biosynthetic pathway resulted in mixed results; however, co-overexpression of two genes (DXS with geraniol-10-hydroxylase or anthranilate synthase α subunit) within the pathway led to a significant increase in the accumulation of multiple TIA metabolites [64]. As a result, more complex engineering strategies that manipulate expression of multiple pathway genes and/or regulators are necessary to balance flux towards the product of interest [65]. Defining these strategies requires detailed systems knowledge and sophisticated metabolic models, which are presently lacking for plant systems. Current stable transformation procedures are time consuming, making the development of high throughput transformation procedures desirable, especially as metabolic engineering schemes become more complex. Finally, for implementation of these metabolically engineered systems, it is important to have the regulatory guidelines in place. For *in vitro* systems, commercial

successes, such as paclitaxel (Phyton Biotech, Inc.; Ahrensburg, Germany) or glucocerobrosidase (Protalix, Inc.; Carmiel, Israel), will help to pave the way for future commercialization of plant cell culture production systems [4]. Similarly, successful implementation of metabolically engineered crops, such as Golden Rice, will help to standardize regulatory procedures as well as increase consumer acceptance as the global impact of these nutritionally enhanced staple foods is realized [66].

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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. Newman DJ, Cragg GM: **Natural products as sources of new drugs over the 30 years from 1981 to 2010**. *J Nat Prod* 2012, **75**:311-335.

2. DellaPenna D, O'Connor SE: **Plant gene clusters and opiates**. *Science* 2012, **336**:1648-1649.

3. De Luca V, Salim V, Atsumi SM, Yu F: **Mining the biodiversity of plants: a revolution in the making**. *Science* 2012, **336**:1658-1661.

4. Wilson SA, Roberts SC: **Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules**. *Plant Biotechnol J* 2012, **10**:249-268.

5. Barampuram S, Zhang Z: **Recent advances in plant transformation**. In *In Plant Chromosome Engineering.* Edited by Birchler JA. New York: Humana Press; 2011:1-35.

6. Tuteja N, Verma S, Sahoo RK, Raveendar S, Reddy INBL: **Recent advances in development of marker-free transgenic plants: regulation and biosafety concern**. *J Biosci* 2012, **37**:167-197.

7. Lloyd A, Plaisier CL, Carroll D, Drews GN: **Targeted mutagenesis using zinc-finger nucleases in *Arabidopsis***. *Proc Natl Acad Sci USA* 2005, **102**:2232-2237.

8. Townsend JA, Wright DA, Winfrey RJ, Fu FL, Maeder ML, Joung JK, Voytas DF: **High-frequency modification of plant genes using engineered zinc-finger nucleases**. *Nature* 2009, **459**:442-445.

9. Shukla VK, Doyon Y, Miller JC, DeKelver RC, Moehle EA, Worden SE, Mitchell JC, Arnold NL, Gopalan S, Meng XD *et al.*: **Precise genome modification in the crop species Zea mays using zinc-finger nucleases**. *Nature* 2009, **459**:437-441.

10. • Ainley WM, Sastry-Dent L, Welter ME, Murray MG, Zeitler B, Amora R, Corbin DR, Miles RR, Arnold NL, Strange TL *et al.*: **Trait stacking via targeted genome editing**. *Plant Biotechnol J* 2013, **11**:1126-1134.

Authors applied zinc finger technology to stack two herbicide resistance genes in the genome of maize embryonic suspension cultures. Stacking resulted in the cosegregation of genes to progeny, displaying the applicability of this technology for creating transgenic plants with multiple inheritable and functional transgenes.

11. Cermak T, Doyle EL, Christian M, Wang L, Zhang Y, Schmidt C, Baller JA, Somia NV, Bogdanove AJ, Voytas DF: **Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting**. *Nucleic Acids Res* 2011, **39**:e82.

12. •• Zhang Y, Zhang F, Li X, Baller JA, Qi Y, Starker CG, Bogdanove AJ, Voytas DF: **Transcription activator-like effector nucleases enable efficient plant genome engineering**. *Plant Physiol* 2013, **161**:20-27.

Authors used TALENs to transform tobacco protoplasts at a high enough effiency to recover transformants without the use of reporter or selection genes. Of the calli transformed with TALENs for targeted gene mutations, 32% tested positive for ALS mutations, whereas 4% of calli were positive when transformed for gene replacement.

13. Shan Q, Wang Y, Chen K, Liang Z, Li J, Zhang Y, Zhang K, Liu J, Voytas DF, Zheng X: **Rapid and efficient gene modification in rice and *Brachypodium* using TALENs**. *Mol Plant* 2013, **6**:1365-1368.

14. Li T, Liu B, Spalding MH, Weeks DP, Yang B: **High-efficiency TALEN-based gene editing produces disease-resistant rice**. *Nat Biotechnol* 2012, **30**:390-392.

15. Wendt T, Holm PB, Starker CG, Christian M, Voytas DF, Brinch-Pedersen H, Holme IB: **TAL effector nucleases induce mutations at a pre-selected location in the genome of primary barley transformants**. *Plant Mol Biol* 2013, **83**:279-285.

16. Sun Z, Li N, Huang G, Xu J, Pan Y, Wang Z, Tang Q, Song M, Wang X: **Site-specific gene targeting using transcription activator-like effector (TALE)-based nuclease in Brassica oleracea**. *J Integr Plant Biol* 2013, **55**:1092-1103.

17. •• Curtin SJ, Zhang F, Sander JD, Haun WJ, Starker C, Baltes NJ, Reyon D, Dahlborg EJ, Goodwin MJ, Coffman AP *et al.*: **Targeted mutagenesis of duplicated genes in soybean with zinc-finger nucleases**. *Plant Physiol* 2011, **156**:466-473.

Authors utilized zinc finger technology to target redundant genes in soy bean hairy root cultuers and whole plants. ZFNs were generated with enough specificity to target either single genes or multiple genes within a single gene family, which is a challenge for existing metabolic engineering strategies.

18. • Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E: **A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity**. *Science* 2012, **337**:816-821.

Authors demonstrated that the type II CRISPR system from *Streptococcus pyogenes* uses two RNA strands, a CRISPR RNA (crRNA) and a trans-acting RNA (tracrRNA) to guide sequence specific DNA cleavage by the Cas9 protein. Reprogramming of these RNA strands can allow for a simple and versatile system for targeted genome editing.

19. Li J-F, Norville JE, Aach J, McCormack M, Zhang D, Bush J, Church GM, Sheen J: **Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9**. *Nat Biotechnol* 2013, **31**:688-691.

20. Nekrasov V, Staskawicz B, Weigel D, Jones JDG, Kamoun S: **Targeted mutagenesis in the model plant *Nicotiana benthamiana* using Cas9 RNA-guided endonuclease**. *Nat Biotechnol* 2013, **31**:691-693.

21. Shan Q, Wang Y, Li J, Zhang Y, Chen K, Liang Z, Zhang K, Liu J, Xi JJ, Qiu J-L: **Targeted genome modification of crop plants using a CRISPR-Cas system**. *Nat Biotechnol* 2013, **31**:686-688.

22. Pickens LB, Tang Y, Chooi Y-H: **Metabolic engineering for the production of natural products**. *Annu Rev Chem Biomol Eng* 2011, **2**:211-236.

23. Kaul S, Koo HL, Jenkins J, Rizzo M, Rooney T, Tallon LJ, Feldblyum T, Nierman W, Benito MI, Lin XY *et al.*: **Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana***. *Nature* 2000, **408**:796-815.

24. Michael WB, Cristobal U: **Genomics reveals new landscapes for crop improvement**. *Genome Biol* 2013, **14**:206.

25. Huang XH, Wei XH, Sang T, Zhao QA, Feng Q, Zhao Y, Li CY, Zhu CR, Lu TT, Zhang ZW *et al.*: **Genome-wide association studies of 14 agronomic traits in rice landraces**. *Nat Genet* 2010, **42**:961-967.

26. Huang XH, Zhao Y, Wei XH, Li CY, Wang A, Zhao Q, Li WJ, Guo YL, Deng LW, Zhu CR *et al.*: **Genome-wide association study of flowering time and grain yield traits in a worldwide collection of rice germplasm**. *Nat Genet* 2012, **44**:32-39.

27. Strickler SR, Bombarely A, Mueller LA: **Designing a transcriptome next-generation sequencing project for a nonmodel plant species**. *Am J Bot* 2012, **99**:257-266.

28. Van Moerkercke A, Fabris M, Pollier J, Baart GJE, Rombauts S, Hasnain G, Rischer H, Memelink J, Oksman-Caldentey KM, Goossens A: **CathaCyc, a metabolic pathway database built from *Catharanthus roseus* RNA-seq data**. *Plant Cell Physiol* 2013, **54**:673-685.

29. Facchini PJ, Bohlmann J, Covello PS, De Luca V, Mahadevan R, Page JE, Ro DK, Sensen CW, Storms R, Martin VJJ: **Synthetic biosystems for the production of high-value plant metabolites**. *Trends Biotechnol* 2012, **30**:127-131.

30. Xiao M, Zhang Y, Chen X, Lee EJ, Barber CJS, Chakrabarty R, Desgagne-Penix I, Haslam TM, Kim YB, Liu EW *et al.*: **Transcriptome analysis based on next-generation sequencing of non-model plants producing specialized metabolites of biotechnological interest**. *J Biotechnol* 2013, **166**:122-134.

31. •• Geu-Flores F, Sherden NH, Courdavault V, Burlat V, Glenn WS, Wu C, Nims E, Cui YH, O'Connor SE: **An alternative route to cyclic terpenes by reductive cyclization in iridoid biosynthesis**. *Nature* 2012, **492**:138-142.

Authors analyzed transcriptome data to identify iridoid synthase, a novel enzyme that generates the iridoid ring scaffold from an alternative substrate, 10-oxogeranial (a linear monoterpene). Enzyme activity was confirmed through biochemical assays, gene silencing, co-expression analysis and localization studies. This enzyme offers an alternative route to iridoid biosynthesis for metabolic engineering of plant and microbial systems.

32. Higashi Y, Saito K: **Network analysis for gene discovery in plant-specialized metabolism**. *Plant Cell Environ* 2013, **36**:1597-1606.

33. Yamazaki M, Mochida K, Asano T, Nakabayashi R, Chiba M, Udomson N, Yamazaki Y, Goodenowe DB, Sankawa U, Yoshida T *et al.*: **Coupling deep transcriptome analysis with untargeted metabolic profiling in *Ophiorrhiza pumila* to further the understanding of the biosynthesis of the anti-cancer alkaloid camptothecin and anthraquinones**. *Plant Cell Physiol* 2013, **54**:686-696.

34. Vongpaseuth K, Roberts SC: **Advancements in the understanding of paclitaxel metabolism in tissue culture**. *Curr Pharm Biotechnol* 2007, **8**:219-236.

35. Croteau RB, Ketchum REB, Long RM, Kaspera R, Wildong MR: **Taxol biosynthesis and molecular genetics**. *Phytochem Rev* 2006, **5**:75-97.

36. Ketchum REB, Wherland L, Croteau RB: **Stable transformation and long-term maintenance of transgenic *Taxus* cell suspension cultures**. *Plant Cell Rep* 2007, **26**:1025-1033.

37. Vongpaseuth K, Nims E, Amand MS, Walker EL, Roberts SC: **Development of a particle bombardment-mediated transient transformation system for *Taxus* spp. cells in culture**. *Biotechnol Prog* 2007, **23**:1180-1185.

38. Exposito O, Syklowska-Baranek K, Moyano E, Onrubia M, Bonfill M, Palazon J, Cusido RM: **Metabolic responses of *Taxus* media transformed cell cultures to the addition of methyl jasmonate**. *Biotechnol Prog* 2010, **26**:1145-1153.

39. Zhang P, Li ST, Liu TT, Fu CH, Zhou PP, Zhao CF, Yu LJ: **Overexpression of a 10-deacetylbaccatin III-10 beta-O-acetyltransferase gene leads to increased taxol yield in cells of *Taxus chinensis***. *Plant Cell Tissue Organ Cult* 2011, **106**:63-70.

40. Patil RA, Kolewe ME, Normanly J, Walker EL, Roberts SC: **Contribution of taxane biosynthetic pathway gene expression to observed variability in paclitaxel accumulation in *Taxus suspension* cultures**. *Biotechnol J* 2012, **7**:418-427.

41. Onrubia M, Moyano E, Bonfill M, Ma Cusido R, Goossens A, Palazon J: **Coronatine, a more powerful elicitor for inducing taxane biosynthesis in *Taxus* media cell cultures than methyl jasmonate**. *J Plant Physiol* 2013, **170**:211-219.

42. Li S-t, Fu C-h, Zhang M, Zhang Y, Xie S, Yu L-j: **Enhancing taxol biosynthesis by overexpressing a 9-cis-epoxycarotenoid dioxygenase gene in transgenic cell lines of *Taxus chinensis***. *Plant Mol Biol Rep* 2012, **30**:1125-1130.

43. Wu QO, Sun C, Luo HM, Li Y, Niu YY, Sun YZ, Lu AP, Chen SL: **Transcriptome analysis of *Taxus cuspidata* needles based on 454 pyrosequencing**. *Planta Med* 2011, **77**:394-400.

44. Hao DC, Ge G, Xiao P, Zhang Y, Yang L: **The first insight into the tissue specific *Taxus transcriptome* via Illumina second generation sequencing**. *PLoS ONE* 2011:6.

45. Li S-T, Zhang P, Zhang M, Fu C-H, Zhao C-F, Dong Y-S, Guo A-Y, Yu L-J: **Transcriptional profile of *Taxus chinensis* cells in response to methyl jasmonate**. *BMC Genomics* 2012, **13**:295.

46. Lenka SK, Boutaoui N, Paulose B, Vongpaseuth K, Normanly J, Roberts SC, Walker EL: **Identification and expression analysis of methyl jasmonate responsive ESTs in paclitaxel producing *Taxus cuspidata* suspension culture cells**. *BMC Genomics* 2012, **13**:148.

47. Sun G, Yang Y, Xie F, Wen J-F, Wu J, Wilson IW, Tang Q, Liu H, Qiu D: **Deep sequencing reveals transcriptome re-programming of *Taxus* × media cells to the elicitation with methyl jasmonate**. *PLoS ONE* 2013, **8**:e62865.

48. Nims E, Dubois CP, Roberts SC, Walker EL: **Expression profiling of genes involved in paclitaxel biosynthesis for targeted metabolic engineering**. *Metabol Eng* 2006, **8**:385-394.

49. DeJong JM, Liu YL, Bollon AP, Long RM, Jennewein S, Williams D, Croteau RB: **Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae***. *Biotechnol Bioeng* 2006, **93**:212-224.

50. Engels B, Dahm P, Jennewein S: **Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards taxol (paclitaxel) production**. *Metab Eng* 2008, **10**:201-206.

51. Huang QL, Roessner CA, Croteau R, Scott AI: **Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol**. *Bioorg Med Chem* 2001, **9**:2237-2242.

52. Ajikumar PK, Xiao WH, Tyo KEJ, Wang Y, Simeon F, Leonard E, Mucha O, Phon TH, Pfeifer B, Stephanopoulos G: **Isoprenoid pathway optimization for taxol precursor overproduction in *Escherichia coli***. *Science* 2010, **330**:70-74.

53. Meng HL, Wang Y, Hua Q, Zhang SL, Wang XN: **In silico analysis and experimental improvement of taxadiene heterologous biosynthesis in Escherichia coli**. *Biotechnol Bioprocess Eng* 2011, **16**:205-215.

54. • Zerbe P, Hamberger B, Yuen MMS, Chiang A, Sandhu HK, Madilao LL, Nguyen A, Hamberger B, Bach SS, Bohlmann J: **Gene discovery of modular diterpene metabolism in nonmodel systems**. *Plant Physiol* 2013, **162**:1073-1091.

Authors applied tissue-specific metabolite and deep transcriptome data from 10 diterpene accumulating species to identify 46 putative diterpene synthase genes and over 400 putative terpenoid related cytochrome P450s. A library of customized target sequences was generated from previously characterized diterpene synthase and cytochrome P450s to isolate the candidates from nearly 1 million predicted transcripts. The techniques used in this study can be applied to transcriptome data from additional diterpene accumulating species and, after confirming gene function, candidates could be used in metabolic engineering strategies.

55. Martin C: **The interface between plant metabolic engineering and human health**. *Curr Opin Biotechnol* 2013, **24**:344-353.

56. Bhullar NK, Gruissem W: **Nutritional enhancement of rice for human health: the contribution of biotechnology**. *Biotechnol Adv* 2013, **31**:50-57.

57. Burkhardt PK, Beyer P, Wunn J, Kloti A, Armstrong GA, Schledz M, vonLintig J, Potrykus I: **Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis**. *Plant J* 1997, **11**:1071-1078.

58. Ye XD, Al-Babili S, Kloti 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.

59. Paine JA, Shipton CA, Chaggar S, Howells RM, Kennedy MJ, Vernon G, Wright SY, Hinchliffe E, Adams JL, Silverstone AL *et al.*: **Improving the nutritional value of Golden Rice through increased pro-vitamin A content**. *Nat Biotechnol* 2005, **23**:482-487.

60. Tang G, Qin J, Dolnikowski GG, Russell RM, Grusak MA: **Golden Rice is an effective source of vitamin A**. *Am J Clin Nutr* 2009, **89**:1776-1783.

61. Tang G, Hu Y, Yin S-A, Wang Y, Dallal GE, Grusak MA, Russell RM: **Beta-carotene in Golden Rice is as good as beta-carotene in oil at providing vitamin A to children**. *Am J Clin Nutr* 2012, **96**:658-664.

62. Bai C, Twyman RM, Farre G, Sanahuja G, Christou P, Capell T, Zhu C: **A golden era-pro-vitamin A enhancement in diverse crops**. *In Vitro Cell Dev Biol Plant* 2011, **47**:205-221.

63. Pons E, Alquézar B, Rodríguez A, Martorell P, Genovés S, Ramón D, Rodrigo MJ, Zacarías L, Peña L: **Metabolic engineering of β-carotene in orange fruit increases its in vivo antioxidant properties**. *Plant Biotechnol J* 2013, **12**:17-27.

64. Peebles CAM, Sander GW, Hughes EH, Peacock R, Shanks JV, San K-Y: **The expression of 1-deoxy-D-xylulose synthase and geraniol-10-hydroxylase or anthranilate synthase increases terpenoid indole alkaloid accumulation in *Catharanthus roseus* hairy roots**. *Metab Eng* 2011, **13**:234-240.

65. Morandini P: **Control limits for accumulation of plant metabolites: brute force is no substitute for understanding**. *Plant Biotechnol J* 2013, **11**:253-267.

66. Potrykus I: **"Golden Rice", a GMO-product for public good, and the consequences of GE-regulation**. *J Plant Biochem Biotechnol* 2012, **21**:S68-S75.