Over the past few years, there has been a growing realization that metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level, and that even the simplest modifications can send ripples throughout the entire system. Attention has therefore shifted away from reductionist, single-gene engineering strategies and towards more complex approaches involving the simultaneous overexpression and/or suppression of multiple genes. The use of regulatory factors to control the abundance or activity of several enzymes is also becoming more widespread. In combination with emerging methods to model metabolic pathways, this should facilitate the enhanced production of natural products and the synthesis of novel materials in a predictable and useful manner.

As a result, it has been realized that the manipulation of single genes is of only limited value in metabolic engineering, and attention has shifted towards more complex and sophisticated strategies in which several steps in a given pathway are modified simultaneously to achieve optimal flux. In this review, we look at recent examples of single and multiple gene metabolic engineering in primary and secondary metabolism that demonstrate how this new knowledge of metabolic systems is being applied in plants.

Strategies for metabolic engineering in plants
There are three basic goals of metabolic engineering in plants [4]: the production of more of a specific desired compound, the production of less of a specific unwanted compound, and the production of a novel compound (i.e. a molecule that is produced in nature, but not usually in the host plant, or a completely novel compound). Strategies for achieving these goals include the engineering of single steps in a pathway to increase or decrease metabolic flux to target compounds, to block competitive pathways or to introduce short cuts that divert metabolic flux in a particular way (Figure 1a). However, this approach has only limited value, because the effects of modulating single enzymatic steps are often absorbed by the system in an attempt to restore homeostasis. Targeting multiple steps in the same pathway could help to control metabolic flux in a more predictable manner. This might involve upregulating several consecutive enzymes in a pathway, upregulating enzymes in one pathway while suppressing those in another competing pathway, or using regulatory genes to establish multi-point control over one or more pathways in the cell (Figure 1b). Examples are provided below to show how such strategies are applied to manipulate the production of different classes of compound.

Engineering primary metabolic pathways
Carbohydrate metabolism
Carbohydrate metabolism in plants centers on the reversible conversion of sugars, the direct products of photosynthesis, into storage and structural carbohydrates such as starch and cellulose. Starch is a storage carbohydrate that accumulates transiently in leaves and stably in seeds, tubers and roots. It is a staple source of dietary carbohydrate for animals and also has a large number of industrial uses. Metabolic engineering has been used in an attempt to increase starch yields and to modify the properties of starch by changing the relative proportions of its two components, amylose and amylopectin. There has also been some interest in producing novel starches using
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bacterial enzymes to change the nature and frequency of branching [5]. Recent examples of metabolic engineering to increase the quantity of starch include overexpressing the potato sucrose transporter in transgenic pea seeds to accelerate sugar uptake [6] and boosting the adenylate pool in potato tubers, resulting in a 60% increase in the level of starch in combination with an increased tuber yield [7]. Smidansky and colleagues [8,9] have shown that wheat and rice plants transformed with a modified form of the maize gene for ADP-glucose pyrophosphorylase (shrunken2) utilize their spare capacity for starch biosynthesis and produce 40% and 20% more seed biomass, respectively, than wild-type controls.

Many of the enzymes that control starch synthesis and branching have been modulated in potatoes to produce high-amylose starch (e.g. for improved frying or for industrial use as gelling agents and thickeners) and high-amylopectin starch (e.g. for improved freeze thaw characteristics or for improved paper quality or adhesive manufacture). More recently, similar experiments have been carried out in other starch-producing crops.
example, a low-amylose rice grain has been produced by expressing an antisense *Waxy* gene [10] and rice grains with modified amylopeptin have been produced by antisense inhibition of the gene encoding isoamylase [11]. Several novel strategies for starch metabolic engineering have been explored, including the use of starch-binding domains to target recombinant enzymes to starch granules during starch biosynthesis [12].

The cellulose biosynthetic pathway is also a useful target for metabolic engineering, because cellulose is valuable as pulp, fiber and as a starting point for the synthesis of commercially important polymers. Although the cellulose biosynthesis pathway is not fully understood, several enzymes required for cellulose synthesis have been identified, including sucrose synthase, the CesA family of cellulose synthases, and Korrigan cellulases. The inhibition of sucrose synthase in cotton has been shown recently to inhibit fiber formation [13], whereas antisense and dominant negative experiments involving *CesA* genes in *Arabidopsis* have also demonstrated roles for several of the CesA proteins in primary and secondary cell-wall formation [14,15].

The fructans are another group of storage carbohydrates that are important targets for metabolic engineering, as they have a similar texture to fats and can substitute for them in many foods, yet contain far fewer calories. At the present time the only commercially available fructan is inulin from chicory, but bacterial and plant enzymes for fructan biosynthesis have been introduced into several crops to increase fructan levels to levels suitable for extraction [16]. Another use envisaged for fructans is the protection of plants from abiotic stress. This application is shared with sugars such as trehalose, as well as other small-molecule compounds like glycine betaine, mannitol and sorbitol, all of which have been subject to metabolic engineering to produce improved plant varieties [17].

**Amino acid and polyamine metabolism**

Amino acid synthesis pathways have been targets for metabolic engineering predominately to increase the abundance of essential amino acids (lysine, threonine, methionine and tryptophan) in food and feed crops [18]. Other amino acids, such as proline, are important in plant stress responses and their modulation provides a way to engineer stress tolerance in transgenic plants [17]. Recent examples of amino acid pathway engineering demonstrate the impact of RNA interference (RNAi) on metabolic engineering and the value of multiple point intervention. A new version of the maize *opaque2* mutant has been generated by RNAi, using a construct targeting the 22 kDa zein genes [19]. Like the original mutant, the new version of *opaque2* has an increased lysine content, but unlike the original it behaves in a dominant rather than recessive manner. The lysine content of *Arabidopsis* seeds can be increased either by expressing a bacterial, feedback-insensitive dihydrodipicolinate synthase (DHPS) transgene or by knocking out the lysine catabolism pathway, resulting in twelve- to fifteenfold gains in lysine, respectively. However, where both the transgene and knockout are combined in the same plant line, an 80-fold increase over wild-type levels can be achieved [20].

Polyamines are small aliphatic amines that are derived from the amino acids ornithine and arginine by decarboxylation. They have several important roles in plant physiology and development. The three major polyamines found in plants are putrescine, spermidine and spermine. Recent analysis of the polyamine biosynthesis pathway has focused on tobacco and rice, with many enzymatic steps being studied by overexpression or antisense suppression. Rice plants expressing 8-adenosylmethionine decarboxylase (SAMDC) from carrot showed increased levels of the higher polyamines (spermine and spermidine) in seeds, while only spermine levels were affected in leaves [21]. When a yeast SAMDC gene was expressed in tomato, higher levels of spermine and spermidine were detected in fruits, as well as higher levels of lycopene, increasing the nutrient value of the tomato juice [22]. Inhibition of the arginine decarboxylase (ADC) gene in rice using an oat antisense ADC construct reduces the levels of putrescine and spermine in transgenic plants, but does not effect downstream genes in the pathway [23].

**Lipid metabolism**

The modification of lipid metabolism to change the quantity and quality of fatty acids in plants has important applications in the food industry, as well as in the production of detergents, fuels, lubricants, paints and plastics [24,25]. The metabolic engineering of seed oils is attractive, because even extensive modifications do not affect the growth and development of the vegetative parts of the plant [26]. Recent notable studies include experiments in which oilseed rape (*Brassica napus*) was transformed with multiple cDNAs encoding enzymes for the desaturation and functional modification of long-chain fatty acids. Liu *et al.* [27] expressed 18:1 Δ12 desaturase either alone or in combination with 18:2 Δ6 desaturase. With the 18:1 Δ12 desaturase alone, the seeds produced up to 46% linoleic acid, whereas with both enzymes the seeds produced up to 43% γ-linolenic acid. Han *et al.* [28] expressed a combination of β-ketoacyl-CoA synthase and 22:1 acyl-CoA/lysophosphatidic acid acyltransferase in oilseed rape, yielding 60% erucic acid. More recently, the ζ-linolenic acid content of rice grains has been increased tenfold by expressing the soybean *FAD3* gene encoding Ω3 fatty acid desaturase [29]. Cahoon *et al.* [30], expressing a cytochrome P450 isolated from seeds of *Euphorbia lagascae*, have produced up to 7% epoxy-fatty acids without inhibiting oleate desaturation.
Engineering secondary metabolic pathways

Alkaloids
The alkaloids are perhaps the largest group of secondary products synthesized by plants and the pathways, enzymes and regulatory genes involved in their synthesis have been extensively reviewed [31]. Several distinct types of alkaloids can be distinguished, including the isoquinoline, indole and tropane classes. Park et al. [32] have recently demonstrated antisense suppression of the berberine bridge enzyme in poppy cells, reducing the amount of benzophenanthridine alkaloids but increasing the levels of several amino acids. There have been many attempts to modulate the indole alkaloid biosynthetic pathway, which produces important pharmaceutical compounds such as the anticancer drugs vinblastine and vincristine [33].

Terpenoids
Terpenoids (isoprenoids) are structurally diverse polymers constructed from the basic building block isoprene. They are classified according to the number of isoprene units they contain, which increase by fives. Thus, hemiterpenes contain five isoprene units, monoterpene contain 10, sesquiterpenes contain 15 and diterpenes contain 20. Carotenoids, another important group of plant secondary metabolites, are terpenoids with 40 isoprene units. The largest polymers contain thousands of units and include natural products such as rubber. Despite their diversity, all terpenoids are synthesized from the common precursors dimethylallyl pyrophosphate and isopentyl pyrophosphate. This occurs through two distinct pathways, the mevalonate-independent pathway and the mevalonate pathway, both of which have been targets for metabolic engineering [34].

Thus far, there have been few examples of monoterpene metabolic engineering, but see Update. In one study, petunia was transformed with the (S)-limonene synthase gene from Clarkia breweri resulting in the production of linalool, which repels aphids [35]. In another, the same gene was introduced into tomato, under the control of a fruit-specific promoter, resulting in the accumulation of linalool in tomato fruits [36].

Much more attention has been paid to the carotenoid metabolic pathway, which produces pigments with roles in light harvesting and photoprotection, and which form vital components of human and animal diets. In plants, the first step in carotenoid biosynthesis is the coupling of two geranyl phosphate molecules to yield geranylgeranyl pyrophosphate (GGPP). This is converted into phytoene by the enzyme phytoene synthase and then to \( \beta \)-carotene by enzymatic steps catalyzed by \( \alpha \)-cyclase and \( \beta \)-cyclase [37].

The principal example of carotenoid metabolic engineering in plants is of course the synthesis of \( \beta \)-carotene in rice endosperm (Golden Rice), which normally accumulates GGPP but lacks the subsequent enzymes in the pathway. Since publication of the original reports, further ‘Golden Rice’ lines have been generated containing only two foreign enzymes (the daffodil phytoene synthase and a bacterial phytoene desaturase [38]). Hoa et al. [39] reported the production of indica Golden Rice varieties that are amenable to deregulation and also assure freedom to operate in developing countries due to the removal of contraints brought about by intellectual property and regulatory issues.

Transgenic tomatoes have also been described expressing phytoene desaturase or phytoene synthase [40]. In the former case, there was no overall increase in carotenoid content, although \( \beta \)-carotene levels were three times higher than normal; in the latter case, the total carotenoid content was up to fourfold normal levels [40].

Flavonoids
The flavonoids are phenolic compounds derived ultimately from phenylalanine, and thus share a common metabolic source with lignins and lignans (see below). The first committed step in flavonoid biosynthesis is the conversion of the precursor 4-coumaroyl-CoA into chalcone by the enzyme chalcone synthase. Chalcone is then derivatized in a series of enzymatic steps to produce a variety of molecules that act as pigments, defense chemicals (phytoalexins) and regulatory molecules [41]. Flavonoids have been a favorite target for metabolic engineering since the early 1990s, because the result of different modifications can be determined through alterations in flower color [42]. As for other pathways, the number of experiments involving multiple gene transfer strategies is beginning to increase. A recent example is the stacking of dihydroflavonol 4-reductase and anthocyanidin synthase transgenes in forsythia by sequential transformation [43]. Increasing the levels of flavonoids in food plants can provide health benefits, as these molecules often have antioxidant activity. This has been demonstrated in the case of tomatoes by overexpressing the petunia gene for chalcone isomerase, leading to an 80-fold increase in the flavonoid content of the tomato peel and a corresponding 20-fold increase in the flavonoid level in tomato paste [44]. In addition, chalcone synthase and flavonol synthase transgenes were found to act synergistically to upregulate flavonol biosynthesis significantly in the flesh of tomato fruits [45].

Lignins
Lignins, like flavonoids, are derived from phenylalanine and can be classed as phenylpropanoid compounds. They
are complex polymers composed of caffeic, ferulic and sinapic acid monomers joined together and to the cell wall. The metabolic engineering of lignins has been reviewed comprehensively very recently [46–48] and we will only mention here a few notable examples that demonstrate contemporary metabolic engineering approaches. One interesting example is the use of the LIM regulatory protein from tobacco to control several of the metabolic enzymes involved in the lignification pathway coordinately [49]. Similarly, two Myb-like transcription factors have been isolated from Eucalyptus gunnii, which are expressed in the xylem and recognize sites in the hydroxycinnamoyl-CoA reductase (CCR) and cinna- myl alcohol dehydrogenase (CAD) genes which encode sequential enzymes in the synthesis of p-coumaryl alcohol [48]. Recent reports have also demonstrated the feasibility of multiple gene transfer to modify several lignin characteristics simultaneously in trees [50∗] and the simultaneous suppression of multiple lignin biosynthetic genes in tobacco [51,52∗].

**Quinones and other benzoic acid derivatives**

Quinones are cyclic compounds derived from benzoic acid and often act as electron transporters in plants. Thus far, three major metabolic pathways that begin with benzoic acid have been modified in plants — those leading to salicylic acid, shikonin and γ-tocopherol (vitamin E) production. Vitamin E synthesis in plants has obvious implications for the nutritional value of foods and recent work has aimed to increase the levels of this molecule in the model plant Arabidopsis thaliana. For example, Savidge et al. [53] have overexpressed A. thaliana homogentisate prenyltransferase, producing twice the level of vitamin E found in normal seeds. A very recent study [54∗∗] described the expression of the A. thaliana VTE3 and VTE4 genes, encoding 2-methyl-6-phytylbenzoquinol methyltransferase and γ-tocopherol methyltransferase, in transgenic soybean. The transgenic seeds showed a greater than eightfold increase in γ-tocopherol and a fivefold increase in vitamin E levels. Other strategies for increasing vitamin E levels in transgenic plants are discussed in a recent review [55].

**Engineering novel metabolic pathways**

Although metabolic engineering is often used to modify or extend existing pathways in the host plant, there are some examples where multigene engineering has been used to introduce completely novel pathways and thus to produce completely alien products. In one such experiment two multifunctional cytochrome P450 enzymes and a uridine diphosphate glucose (UDPG)-glucosyltransferase were transferred from sorghum (Sorghum bicolor) into A. thaliana, resulting in the production of the cyanogenic glucoside dhurrin (which confers resistance to the flea beetle Phyllothis nemorum) [56]. These investigators also generated transgenic plants carrying one or both of the cytochrome P450 enzymes, but lacking the UDPG-glucosyltransferase, and demonstrated the accumulation of appropriate metabolic intermediates.

Another example of this general approach is the production of polyhydroxalkanoate (PHA) polymers in transgenic plants [57]. PHAs are polyesters of hydroxyacids synthesized by a range of bacteria, which can be used as biodegradable plastics. The best-studied PHA, poly(3-hydroxybutyrate) (PHB), can be made to accumulate in the plastids of transgenic A. thaliana plants at levels exceeding 40% dry shoot weight. This is achieved through the introduction of three bacterial genes, encoding the enzymes 3-ketothiolase, acetacetyl-CoA reductase and PHA synthase [58]. PHB is a brittle plastic with few potential uses. The copolymer poly(3-hydroxybutyrate-co-3-hydroxyvalerate) is much more useful, and is marketed under the trade name Biopol. This has been produced in A. thaliana and rapeseed through the coordinated expression of four bacterial genes: the three mentioned above plus Escherichia coli threonine deaminase [59]. Although high-level accumulation of these plastics is generally detrimental to plant growth (by draining the pools of acetyl- and propionyl-CoA), both plastics have recently been produced at relatively high levels in alfalfa with no adverse effects [60∗].

**Conclusions**

Multipoint metabolic engineering is now beginning to supersede single-point engineering as the best way to manipulate metabolic flux in transgenic plants. As discussed above, several points in a given metabolic pathway can be controlled simultaneously either by overexpressing and/or suppressing several enzymes of through the use of transcriptional regulators to control several endogenous genes. Applied genomics, proteomics and metabolomics are continuing to expand our knowledge of metabolic pathways, while advances in systems biology help us to model the impact of different modifications more accurately. We are fast approaching a time when sophisticated strategies for the multipoint manipulation of metabolic pathways can be modeled and implemented by multiple gene transfer, therefore facilitating the production of desirable molecules in transgenic plants.

**Update**

Since this article was written, Lucker et al. [61] have reported the transformation of tobacco plants with three different monoterpane synthases from lemon. When the three transgenes were stacked in one transgenic line, the tobacco plants produced more terpenoids and displayed a different terpenoid profile to wild-type plants. Incidentally, this is also the first report of transgenic plants expressing multiple foreign enzymes competing for the same substrate.
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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
• of special interest
**of outstanding interest


• The authors demonstrate that by increasing the activity of plastidial adenylyl kinase in transgenic potato plants, a larger pool of adenylates becomes available that can be used to fuel several metabolic pathways. Not only was there an increase in starch levels, but there was also a two- to fourfold elevation in the levels of several amino acids.


• The authors describe an interesting study in which a starch-binding domain from potato granule-bound starch synthase I was used to target luciferase to the inside of the starch granule. This is a promising method for directing enzymes into the starch granule for starch metabolic engineering.


• This study demonstrates multipoint engineering of lysine metabolism, combining the overexpression of a bacterial enzyme resistant to lysine inhibition together with the knockout of the lysine catabolism pathway, resulting in much higher lysine levels than produced by either strategy alone.


• A yeast gene encoding S-adenosylmethionine decarboxylase was expressed in tomato under the control of the ripening-inducible E8 promoter to increase levels of the polyamines spermidine and spermine specifically in tomato fruit during the ripening process. This resulted in the enhanced production of lycopene, a longer vine life and nutritionally improved fruit juice.


In this study, the investigators transformed aspen with an antisense construct against 4-coumarate-CoA ligase (4CL) and a sense construct encoding coniferaldehyde 5-hydroxylase (CAD5H). Plants expressing each gene alone, and both genes together, were recovered. The effects of the two constructs were additive. In the dual transgenic plants, there was half as much lignin, a 64% higher syringyl/guaiacyl ratio, and 30% more cellulose than in wild-type controls.


A new strategy is described in which three different genes are suppressed using a single chimeric antisense construct incorporating partial sequence for all three genes. The authors show that this strategy gives more predictable and consistent results than the use of three separate antisense constructs.


This report describes the map-based cloning of an Arabidopsis gene affecting the ratio of γ- and α-tocopherol in seeds and the identification of its biochemical function. The transformation of soybean with this gene in combination with another gene active in the same metabolic pathway was shown to increase the amount of α-tocopherol in soy oil from 10% to over 90%.


In this report, the expression of multiple bacterial enzymes in alfalfa resulted in the accumulation of polyhydroxybutyrate granules at levels up to 1.8 g kg⁻¹ dry weight in transgenic leaves, with apparently no adverse effects on plant growth or development.