

Plant Metabolic Engineering

Eleanore T. Wurtzel

Department of Biological Sciences, Lehman College, The City University of New York, Bronx, New York, U.S.A.

Erich Grotewold

Department of Plant Cellular and Molecular Biology and Plant Biotechnology Center, The Ohio State University, Columbus, Ohio, U.S.A.

INTRODUCTION

The advent of recombinant DNA technologies over the past 20–30 yr has resulted in an impressive emergence of metabolic engineering as a powerful complement to classical chemical synthesis for the production of specialized pharmaceuticals and industrial compounds. The simplicity and low cost of growing plants makes them ideal systems for metabolic engineering. Here, we intend to provide a perspective of the advantages of plants for metabolic engineering, highlighting the significant hurdles that still need to be overcome. This entry begins by introducing plant metabolic engineering and by providing an overview of the main systems available, whole plants or plant cells in culture. We then describe the different approaches on hand for increasing flux through existing metabolic pathways and end by providing a perspective of the future of plants as chemical factories for the production of industrial materials and pharmaceuticals, or for removing pollutants from the environment.

PLANTS: THE NATURAL FACTORIES

Plant biotechnology is rapidly gaining momentum as a serious alternative to other biotechnological approaches for the production of fine chemicals, pharmaceuticals, and industrial products, for the cleaning of the environment through bioremediation while creating novel opportunities in agriculture. Plants are very attractive for biotechnological applications in large part because of the cheap availability of the main requirements for efficient plant growth, energy (sunlight), and carbon (CO₂). Man has also known how to grow, harvest, and process plant tissues since the beginning of modern agricultural practice. Plants contain an amazing diversity of genes encoding enzymes for thousands of distinct biochemical reactions and this natural genetic diversity dwarfs growing efforts in combinatorial chemistry to increase the available space of chemical entities. As a distinct advantage over synthetic chemical transformations

and in contrast with microbes, plant cells are highly compartmentalized, facilitating, for example, the separation of substrates from the products. Thus, plants provide amazing opportunities for their use as natural factories.

PLANT METABOLIC ENGINEERING

In 1991, Bailey defined metabolic engineering as “the improvement of cellular activities by manipulation of enzymatic, transport, and regulatory functions of the cell with the use of recombinant DNA technologies.”^[1] Today, the availability of the complete genome sequence for several plants, together with the development of powerful techniques for the transformation and stable or transient expression of genes in plants brings plant metabolic engineering as a strong alternative to classical chemical synthesis for the production of pharmaceuticals and other important industrial compounds. Plant metabolic engineering involves the manipulation of existing metabolic pathways by either increasing or diverting flux to desired or from undesired products, respectively, or the generation of chemical entities not normally found in the plant production system (cells or whole plants, see next section) through the introduction of genes from other organisms. Essential elements in the toolbox of the metabolic engineer are mechanisms to eliminate or overexpress gene activity.

Strategies available to eliminate the activity of specific enzymes in a pathway involve one of several possible approaches:

1. Identification of a mutant gene for the corresponding enzyme. Traditionally, plant breeding has taken advantage of natural allelic diversity to introduce mutant alleles into the genetic background of interest. Alternatively, screens have been conducted to identify mutants with desired phenotypes. Transposons have provided powerful tools, facilitating the identification of mutants in any gene of interest through what is

currently known as “reverse genetics.” More recently, targeting-induced local lesions in genome (TILLING) have permitted the identification of single-nucleotide mutations in any gene in a randomly mutagenized plant population.^[2]

4. Knocking out gene function by targeted RNA degradation. Double-stranded RNA interference (dsRNAi) today provides probably the preferred method to knock out gene function.^[3] An emerging alternative to this is the use of RNase P-mediated RNA degradation.^[4] Both of these approaches are dependent on the availability of methods for introducing and expressing RNA in the host plant.
5. Interfering with protein function using specific inhibitors or antibodies. There are many protein inhibitors of metabolic enzymes that, when overexpressed, could have the potential to inhibit specific enzymatic steps (e.g., Ref.^[5]). Non-protein inhibitors of metabolic enzymes are also extensively used, resulting in some potent plant herbicides for which resistance can be easily manipulated, or in the formation of new compounds, when nonessential pathways are inhibited.^[6,7]

The expression of genes in plants or plant cells is dependent on several factors that include:

1. A method to introduce genes into the plant. Such methods are available for a number of plants. While *Agrobacterium*-mediated transformation provides a facile method and usually results in a low number of integration events, minimizing problems of cosuppression of endogenous genes, introduction of genes by particle bombardment has none of the host plant limitations imposed by the host preference of *Agrobacterium* and has been used in a broad range of plants.^[8]
2. Promoters to direct gene expression in the appropriate spatial and temporal landscape. A large number of plant promoters capable of directing robust gene expression in almost every plant tissue have been identified.^[9,10] An emerging alternative to using natural promoters is the generation of “artificial” or “synthetic” promoters that would provide the desired expression patterns.^[11]
3. A source for the gene encoding the enzyme of interest. The completion (or near completion) of the sequencing of various plant genomes together with the availability of a large number of expressed sequence tags (ESTs) for plants where complete genome sequencing is impractical provide an enormous opportunity for the selection of genes encoding plant metabolic enzymes.

CHOICE OF PLANT SYSTEM FOR METABOLIC ENGINEERING

Whole Plants

Compared to animals, cells in culture (plant or animal), or microbes, whole plants provide a cheap and simple platform for large-scale mass production. Transgenic plants can be grown, maintained, and harvested using equipment already available from classical agricultural practices. The choice of a production plant for metabolic engineering depends on the specific metabolite to be produced and whether the necessary precursors are already present. Specialized organs (e.g., leaf hairs, glands, or trichomes) can be used both to sequester compounds and to provide for an accessible source for metabolite extraction.^[12] There is a growing concern in using crop species for the production of phytochemicals, particularly pharmaceuticals, in areas where those crops are grown for food consumption purposes. Thus, plant species that do not hybridize with major crops or with wild relatives are gaining importance for the purposes of genetic manipulation.

Plant Cells in Culture

Cell culture offers the advantage of growth in minimal space without requirement for greenhouses and allows for development of uniform conditions to optimize phytochemical production. While cell culture may not always mimic the biochemical profile of cells as in the growing plant, it is possible to manipulate culture conditions, such that the appropriate array of genes are expressed and metabolites are biosynthesized and accumulated (e.g., treatment with elicitors and other stress inducers).^[13] Cell lines may be transformed with genes encoding biosynthetic enzymes or regulatory factors and assessed for modification of metabolite profiles. Given that conditions are established for maximizing accumulation of the desired chemical constituents, plant cell culture is a useful alternative to growing differentiated plant tissues. In addition, cell culture may provide a platform to test genes that may later be utilized for the more time-consuming production of transgenic plants. For large-scale production of metabolites, whether they represent novel drugs, or unusual metabolites needed for performing nutritional studies, cell cultures are a good alternative to harvesting metabolites from rare plant species or from those refractory to transgenic manipulation.

Using Plant Genes in Microorganisms

Microorganisms (e.g., the gram-negative bacterium *Escherichia coli*) possess a biochemical profile similar

to plant plastids, such as chloroplasts. Because of the biochemical similarity between the microorganism and the plant plastid, the results obtained by genetic modification can be applied later to testing in plants. The advantage of using the microorganism initially is the savings in time as compared to production of a transgenic plant. Examples of this approach include application to manipulation of the carotenoid and isoprenoid biosynthetic pathways. This heterologous system has been used to test the function of plant transgenes encoding putative enzymes that utilize substrates produced by the bacterium.^[14,15] Alternatively, *E. coli* cells have been used to screen for genes that may positively or negatively influence pathway flux.^[16] These organisms have also proven to be expedient tools for testing potential strategies for manipulating pathway flux for pathways that operate in plant plastids.^[17]

STRATEGIES FOR INCREASING FLUX OF EXISTING PATHWAYS

Manipulating the Activity of “Rate Limiting” Steps

Classically, flux constraints have largely been attributed to the presence of one or a few “rate limiting” or bottleneck enzymes in a pathway. The flux theory put forward by Kacser and Burns, and supported by elegant experimentation, questions the existence of single rate limiting enzymatic steps in biochemical pathways.^[18,19] These studies provided the theory behind metabolic control analysis (MCA), which furnishes a tool to interpret why experiments aimed at manipulating metabolic pathways fail, largely a consequence of the distribution of the control of flux over multiple enzymes in a pathway, instead of one as the “rate limiting step” concept would suggest.^[20] Nevertheless, several examples are available in the literature in which the expression of one or a few genes results in a significant increase in flux. For example, the expression of the chalcone isomerase enzyme in tomatoes resulted in a significant increase in the accumulation of flavonols, which are important nutraceutical components of the human diet.^[21] Similarly, the overexpression of phytoene synthase in canola seeds dramatically increased flux into the carotenoid pathway.^[22] Increasing the metabolic flux of upstream pathways that feed precursors for the synthesis of the desired compounds provides an additional strategy for engineering metabolism, with a good example provided by the increase of isoprenoid precursor for the manipulation of carotenoids.^[23]

The experimental success in increasing metabolic flux by expressing one or a few key biosynthetic enzymes contrasts with the emerging notion that

enzymes in metabolic pathways are often associated in large macromolecular complexes, or metabolons.^[24] The most extreme example of a metabolon is the formation of a “metabolic channel” in which pathway intermediates are directly transferred between catalytic sites without diffusion. Clearly, if channeling is a general characteristic in metabolic pathways, the perturbation of the correct stoichiometry of the enzymes in a channel, for example, by overexpression, could have results opposite to what was expected. To what extent the formation of metabolons contributes to the distribution of the overall flux in a pathway over multiple enzymes, as suggested by MCA, remains to be established.

Metabolic Flux Analysis and Modeling

Metabolic flux is the rate at which the material is processed through a specific pathway. A general misconception is that an increase in flux through a pathway will result in the increased accumulation of the pathway products. This assumption is wrong because it fails to consider that pathway products can be the substrates for other chemical reactions. A case example is provided by the biosynthesis of amino acids. While the overall accumulation of amino acids can be increased by enhancing flux through their biosynthetic pathways, the accumulation of the free amino acids (i.e., those not incorporated into proteins) may not be affected. Thus, when investigating flux with the ultimate objective of modeling metabolic pathways, one needs to take into account the ultimate fate of the metabolites. The classical mass-balance approach requires measurements of the rates of substrate uptake, rates of metabolite secretion, and rates of biomass formation. Together with the estimation of metabolic pools and with knowledge of the metabolic networks involved, a fairly accurate model of flux through a pathway can be obtained. However, many plant metabolic pathways involve reversible reactions, multiple compartments, and parallel competing pathways that significantly complicate the mass-balance approach.^[25] These complications can in part be solved by stable-isotope labeling under steady-state conditions, resulting in what is known as stable-isotope metabolic flux analysis, which has been applied to modeling flux through a number of plant metabolic pathways.^[25]

Expression of Multiple Genes in Plants: Progress and Limitations

One way to overcome the limitations imposed by the flux theory is to overexpress multiple genes encoding enzymes of a pathway. There are significant limitations to introducing multiple genes in a transgenic plant and ensuring that they will all be expressed at the high

desired level, as exemplified by the attempts to increase the levels of monoterpenoid alkaloid biosynthesis by coexpressing tryptophan decarboxylase and strictosidine synthase.^[26] In these studies, the analysis of a large number of transgenic tobacco plants showed a dramatic variation in the levels of expression of these two genes, largely a consequence of silencing and cosuppression.^[26] While part of the problems encountered could be solved by minimizing the sequence homology between the transgenes and carefully controlling transgene copy number, significant hurdles prevent the “stacking” of genes in transgenic plants by conventional methods. Sequential sexual crossing of transgenic plants has permitted the stacking of up to three transgenes, and significant progress has been recently made in cotransformation techniques that permit the simultaneous insertion of multiple genes.^[27,28] Emerging approaches to solve the problems associated with stacking transgenes include the expression of multiple genes from a single promoter creating translational fusions of multiple enzymes, which can then be cleaved into separate enzymes by the inclusion of convenient protease cleavage sites.^[29] An alternative to the use of protease sites is the synthesis of a transcript, resembling a polycistronic mRNA, encoding multiple enzymes in a pathway. By having internal ribosome entry sites between the different subunits, translation of each enzyme can be achieved.^[30] However, neither of these promising approaches has yet been utilized for the successful engineering of a plant metabolic pathway.

Diverting Flux Using Loss of Function Approaches

While the overexpression of metabolic enzymes provides a powerful tool in metabolic engineering, it is often as important to minimize flux through a pathway that results in an undesired product. As described before, several approaches are currently available for the downregulation or knockout of a specific enzymatic step. RNA interference (RNAi) is indeed emerging as a powerful complement to more classic loss of function methods.^[31] But often, the most impressive pathway manipulation results are obtained when gain and loss-of-function approaches are combined to increase flux through one pathway while decreasing flux through a competing pathway. This combinatorial approach has proven to be very successful in modifying multiple lignin traits in aspen trees.^[32]

Targeting Entire Pathways with Transcription Factors

According to MCA, increasing the speed of a rate limiting step in a biochemical pathway is often

insufficient for a significant increase in the overall flux in the pathway. Metabolic pathways are often controlled by one or a few regulatory proteins that bind to the promoters of the genes encoding the enzymes in a pathway and activate their transcription. Thus, transcription factors provide an attractive alternative to the manipulation of single or multiple enzymes for increasing flux. Because of the conspicuous pigmentation provided by anthocyanin pigments, the flavonoid pathway provides one of the best-studied cases of control of a plant metabolic pathway.^[33] Anthocyanin accumulation is regulated in many plant species by the concerted action of transcription factors corresponding to the MYB and bHLH families.^[34] Not surprisingly, the regulators of anthocyanin biosynthesis have been among the first ones used to demonstrate the potential of transcription factors to activate entire pathways.^[35] As examples of the successful application of this approach, the maize P1 (MYB family member) and C1 and R transcription factors have been used to activate separate branches of flavonoid biosynthesis in maize cultured cells, the ORCA3 (AP2 family member) to manipulate the accumulation of alkaloids in *Catharantus roseus* cultured cells, and the maize C1 and R to increase the accumulation of flavonols in tomato.^[36–38] Transcription factors provide a powerful complement to other approaches to manipulate the accumulation of desired phytochemicals. As an example, a chimeric form of the maize C1 and R regulators of anthocyanin biosynthesis was combined with the silencing of a structural gene in the flavanol/anthocyanin pathway, flavanone 3-hydroxylase, to increase the content of phytoestrogenic isoflavones in soybean seeds.^[39]

The identification of novel plant repressor domains, such as the EAR motif, expands the use of transcription factors to not only activate entire metabolic pathways, but also to inhibit them.^[40] In addition to using natural transcription factors, an emerging approach is to artificially create transcription factors, primarily from the Zn-finger family, with known DNA-binding specificities.^[41] Thus, the use of artificial DNA-binding domains fused to either transcriptional activation or repression motifs is likely to provide a powerful future tool for altering flux through entire metabolic pathways using a single transgene.

MAKING NEW COMPOUNDS IN PLANTS

Plants as a Source of Industrial Materials

Plants continue to be the unique source of a number of very important industrial materials that include wood, cotton, cork, and rubber. However, the advent of petroleum as a source of carbon for the chemical industry

has resulted in a significant reduction in the use of plants as the feedstock for organic industrial chemicals. In 1930, 30% of the organic chemicals were derived from plants, down to 1% in 1960.^[42] Is there a future for metabolic engineering to revive the role of plants as a fundamental source of industrial materials? This question can only be answered by taking into account the economics involved in growing and harvesting the plants, extracting the desired compound, and separating it from other chemicals. Previous analyses highlighted the challenges associated with this.^[43] One of the best-described examples of how plants could be used to manipulate industrial products is the formation of polyhydroxyalkanoates (PHA) in plants. These polyesters of 3-hydroxyacids have unique biodegradable and elastomeric properties, which make them highly desirable in a number of applications, such as the medical industry, and for making environmental friendly plastics. Polyhydroxyalkanoates accumulate in numerous bacteria and fungi for carbon storage, and the genes from bacteria were successfully introduced into the plastids of *Arabidopsis*, resulting in a significant accumulation of polymer.^[27] As of today, the accumulation of PHAs of various compositions and chain lengths has been accomplished in a number of crop plants, yet significant hurdles need to be overcome to compete in price with cheaper plastics with similar properties obtained by chemical synthesis.^[44]

Another plant polymer with multiple industrial applications, which is the subject of several efforts to modify its accumulation and properties, is starch. Starch is a polymer of two glucan polymers, the mainly linear amylose and the branched amylopectin. Depending on the proportion of amylose to amylopectin, and the length and branching of the chains, starches can have very different properties. Currently, a large number of enzymes are known in maize and other starch-producing plants that influence these fundamental properties of starches.^[45]

Plants as Pharmaceutical Factories

Plant parts have a widespread use in traditional medicine (such as Indian ayurvedic medicine and Chinese herbal remedies), providing clues to bioactive principles with novel health and nutritional benefits that will contribute to resources for metabolic engineering. The small fraction of plants so far surveyed (<15% of terrestrial plants) has revealed an astonishing potential for the biosynthesis of small (<1000 Da) molecules, with over 100,000 of these phytochemicals already described.^[46] These phytochemicals accumulate largely as a consequence of biotic and abiotic interactions of plants with their environment, and are mainly derived from what has been classically known as secondary

metabolism. In addition to the important role that these compounds play as nutraceutical components of animal diet, many of these metabolites are bioactive, providing the basis for a large fraction of all the pharmaceuticals currently available in the market. Thus, a major thrust in plant metabolic engineering continues to be to exploit their huge biochemical diversity to make new compounds from existing ones or to increase the levels of compounds with biomedical or nutraceutical significance. Future genomic investigations of medicinal plants will yield novel enzymes and genes to further expand the repertoire available for metabolic engineering of either endogenous pathways or transfer to heterologous species for enhanced accumulation of novel drugs or lead compounds for further drug development, or production of novel enzymes for bioprocessing.

The exploitation of medicinal plants for metabolic engineering of undiscovered metabolites and metabolic pathways is still at its infancy and is likely to require the initial investment of molecular markers to provide plant genotype fingerprints followed by bioactivity guided fractionation to identify the target molecule(s) and further basic studies (precursor feeding, bioinformatics approaches to identify genes) to elucidate the relevant biosynthetic pathways.

Plants as Nutraceutical Factories

Modification of plants by metabolic engineering can have a profound impact on human health. For example, the grass family (Poaceae) members are the most important food crops worldwide, and include maize, wheat, barley, sorghum, pearl millet, and rice. The endosperm tissues of these crops serve as major food staples; unfortunately, they are deficient in nutritionally essential carotenoids. Endosperms of these food crops are generally low in provitamin A (1–10%) relative to nonprovitamin A carotenoids.^[47] For example, the consumption of carotenoid-poor cereal crops is associated with vitamin A deficiency affecting 250 million children in developing countries.^[48] Improved vitamin A nutrition could eliminate approximately 1.3–2.5 million annual deaths.^[49] Carotenoids also have important health benefits in developed countries. In humans and animals, various carotenoids derived from plant sources act as antioxidants, protect against diseases such as cancer, heart, and eye diseases, and are important in vision, while other carotenoids are precursors to vitamin A and to retinoid compounds involved in development.^[50–56] An alternative approach to alleviating vitamin A deficiency worldwide is to improve levels of provitamin A carotenoids in food staples such as corn, wheat, and rice by metabolic engineering. Preliminary success with metabolic engineering of the pathway in rice, tomato, tobacco, and canola points

to the potential of this approach.^[57–60] Unexpected products in these transgenic plants, however, suggest that the technology is limited by current deficiencies in understanding of endogenous gene expression. Despite the preliminary successes using laboratory-optimized experimental transgenic materials, future integration of the pathway in local varieties will also entail pyramiding of multiple traits, a challenge that can be addressed by continuing research on pathway interactions and competition for common substrates.

A similar success was recently reported in the manipulation of folates (including tetrahydrofolate) in tomato fruits. Tetrahydrofolate is an essential cofactor for several one-carbon reactions including the DNA biosynthesis cycle and the methylation cycle, and hence, folate deficiency results in anemia. Diaz de la Garza and coworkers have recently shown that increasing up to 100-fold the biosynthesis of pteridine, a precursor for folate biosynthesis, resulted in a two-fold increase in the folate content in tomato fruit.^[61] In this study, the investigators utilized a mammalian GTP cyclohydrolase I to bypass the negative feedback regulation featured in regulation of the corresponding plant enzyme.

Using Plant Compartments for Chemical Sequestration

Massive accumulation of metabolites may sometimes be problematic if transgenes are expressed constitutively throughout the plant. Several strategies can be used to overcome this problem. One approach is through the use of tissue-specific promoters, allowing for accumulation in either a specific organ or tissue. Alternatively, gene expression can be controlled in such a way that biosynthetic enzymes or metabolites are directed to specific cell compartments such as the vacuole or chloroplast. For the purpose of chloroplast accumulation, either the plastid genome itself may be engineered with the desired transgene(s) or nuclear-encoded transgenes may be outfitted with chloroplast-targeting signals. Powerful new plastid transformation methods have been developed over the past few years.^[62] The expression of metabolic enzymes in plastids provides several advantages over the nuclear expression. Among these advantages is the higher level of expression possible, given the large number of plastids that a plant cell can harbor, and the impact on biosafety, given that in most crops, plastids are under maternal plastid inheritance, minimizing the risks associated with gene flow from pollen derived from genetically modified plants.

In addition to targeting metabolic pathways to specific organelles, it is conceivable that pathway intermediates or final products could be sequestered

in specific subcellular compartments. This could have a significant impact on metabolite production by removing toxic compounds and increasing flux through the displacement of equilibrium. However, the challenge that remains is our ignorance of the possible pathways by which phytochemicals traffic within or between cells. It is well established that many phytochemicals are sequestered in the vacuole by modifications that include glutathione conjugations followed by the action of specific transporters, particularly from the ABC family.^[63–65] However, it is becoming evident that, in addition to transporters, phytochemicals can traffic between compartments using specialized vesicles, minimizing the risks of undesired chemical reactions and decreasing their toxicity.^[66,67] It is evident that once we learn more on the trafficking of phytochemicals, enormous opportunities will become available to manipulate these processes as an additional tool in the toolbox of the metabolic engineer.

EMERGING TECHNOLOGIES

Plant Diversity as a Source of New Genes

In the course of plant evolution, certain plant lineages have evolved unique biosynthetic pathways that offer opportunities to use genomic approaches to capture this biochemical diversity and provide tools for manipulating plants and other organisms that do not produce the compounds or where accumulation is blocked at a particular biosynthetic step. Even in a single species, such as maize, where many diverse lines are available, allelic variation offers opportunities to use molecular tools to direct breeding efforts using molecular probes for these particular alleles. For example, using association mapping, Buckler's group has identified specific candidate genes associated with starch accumulation and these results may be applied to metabolic engineering of starch accumulation by directing breeding efforts using molecular probes representing these novel alleles.^[68]

Gene Shuffling and Directed Enzyme Evolution

Directed alteration of enzyme activity and substrate specificity can broaden the repertoire of metabolites produced in plants and other organisms. Error-prone DNA polymerases can be used for PCR amplification to generate mutations in plant genes, which are then expressed in bacteria or in other organisms that can be transformed with high efficiency, which are then screened for accumulation of novel compounds.^[69] Gene shuffling technologies can produce a large number of enzyme variants, by shuffling fragments

from an existing library.^[70,71] While the applications of gene shuffling are multiple and include selecting for enzymes with higher stability, the recent identification of new glyphosate *N*-acetyltransferase enzymes that resulted in increased resistance of various plants to the herbicide glyphosate provides a good example of the power of the approach.^[72,73]

Need for New Technology

With the growing interest in metabolomics, a field that provides the necessary data for metabolic engineering, current methodologies are limited in giving resolution of metabolic constitution in complex organisms having multiple cell types and suborganellar compartments. Therefore, advancements in the fields of metabolomics and metabolic engineering will require new types of analytical technology that provide 3-D resolution overlaid with temporal variation. The future availability of such tools will have a far-reaching impact extending from plant metabolic engineering to medical research and diagnostics. Another area in which significant improvement is needed is in the integration of chemistry and biology, for example, to better establish how small molecules bind to proteins in enzyme–substrate or ligand–receptor relationships.

CONCLUSIONS

Despite the significant advances in the field of plant metabolic engineering, many of them described here, predicting the outcome of a metabolic engineering strategy (predictive metabolic engineering) remains a significant challenge.^[74] This highlights the urgent need to continue to study fundamental aspects of plant metabolism including trafficking of small molecules and how those molecules interfere with fundamental cellular processes.

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REFERENCES

- Bailey, J.E. Toward a science of metabolic engineering. *Science* **1991**, 252 (5013), 1668–1675.
- Till, B.J.; Colbert, T.; Tompa, R.; Enns, L.; Codomo, C.; Johnson, J.; Reynolds, S.H.; Henikoff, J.G.; Green, E.A.; Steine, M.N.; Comai, L.; Henikoff, S. High-throughput TILLING for functional genomics. In *Plant Functional Genomics: Methods & Protocols*; Grotewold, E., Ed.; Humana Press: Totowa, NJ, 2003; 205–220.
- Baulcombe, D. RNA silencing in plants. *Nature* **2004**, 431 (7006), 356–363.
- Rangarajan, S.; Raj, M.L.; Hernandez, J.M.; Grotewold, E.; Gopalan, V. RNase P as a tool for disruption of gene expression in maize cells. *Biochem. J.* **2004**, 380 (Pt 3), 611–616.
- Rausch, T.; Greiner, S. Plant protein inhibitors of invertases. *Biochim. Biophys. Acta* **2004**, 1696 (2), 253–261.
- Darmency, H. Genetics of herbicide resistance in weeds and crops. In *Herbicide Resistance in Plants*; Powles, S.B., Holtum, J.A.M., Eds.; CRC Press: Boca Raton, FL, 1994; 263–297.
- Forkmann, G.; Martens, S. Metabolic engineering and applications of flavonoids. *Curr. Opin. Biotechnol.* **2001**, 12 (2), 155–160.
- Twyman, R.M.; Christou, P. Plant transformation technology: particle bombardment. In *Handbook of Plant Biotechnology*; Christou, P., Klee, H., Eds.; John Wiley & Sons, Ltd.: Chichester, U.K., 2004; 263–289.
- De Buck, S.; Depicker, A. Gene expression and level of expression. In *Handbook of Plant Biotechnology*; Christou, P., Klee, H., Eds.; John Wiley & Sons, Ltd.: Chichester, U.K., 2004; 331–345.
- Mueller, A.E.; Wassenegger, M. Control and silencing of transgene expression. In *Handbook of Plant Biotechnology*; Christou, P., Klee, H., Eds.; Chichester, U.K., 2004; 291–330.
- Bhullar, S.; Chakravarthy, S.; Advani, S.; Datta, S.; Pental, D.; Burma, P.K. Strategies for development of functionally equivalent promoters with minimum sequence homology for transgene expression in plants: cis-elements in a novel DNA context versus domain swapping. *Plant Physiol.* **2003**, 132, 988–998.
- Wagner, G.J.; Wang, E.; Shepherd, R.W. New approaches for studying and exploiting an old protuberance, the plant trichome. *Ann. Bot. (Lond.)* **2004**, 93 (1), 3–11.
- Radman, R.; Saez, T.; Bucke, C.; Keshavarz, T. Elicitation of plants and microbial cell systems. *Biotechnol. Appl. Biochem.* **2003**, 37 (Pt 1), 91–102.
- Matthews, P.D.; Luo, R.; Wurtzel, E.T. Maize phytoene desaturase and zetacarotene desaturase catalyze a poly-Z desaturation pathway: implications for genetic engineering of carotenoid

- content among cereal crops. *J. Exp. Bot.* **2003**, *54*, 2215–2230.
15. Li, Z.H.; Matthews, P.D.; Burr, B.; Wurtzel, E.T. Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway. *Plant Mol. Biol.* **1996**, *30*, 269–279.
 16. Gallagher, C.E.; Cervantes-Cervantes, M.; Wurtzel, E.T. Surrogate biochemistry: use of *Escherichia coli* to identify plant cDNAs that impact metabolic engineering of carotenoid accumulation. *Appl. Microbiol. Biotechnol.* **2003**, *60*, 713–719.
 17. Matthews, P.D.; Wurtzel, E.T. Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase. *Appl. Microbiol. Biotechnol.* **2000**, *53* (4), 396–400.
 18. Kacser, H.; Burns, J.A. The control of flux. *Symp. Soc. Exp. Biol.* **1973**, *27*, 65–104.
 19. Niederberger, P.; Prasad, R.; Miozzari, G.; Kacser, H. A strategy for increasing an in vivo flux by genetic manipulations. *Biochem. J.* **1992**, *287*, 473–479.
 20. Morandini, P.; Salamini, F. Plant biotechnology and breeding: allied for years to come. *Trends Plant. Sci.* **2003**, *8* (2), 70–75.
 21. Muir, S.R.; Collins, G.J.; Robinson, S.; Hughes, S.; Bovy, A.; De Vos, C.H.R.; van Tunen, A.; Verhoeyen, M.E. Over expression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. *Nat. Biotechnol.* **2001**, *19*, 470–474.
 22. Ravanello, M.P.; Ke, D.; Alvarez, J.; Huang, B.; Shewmaker, C.K. Coordinate expression of multiple bacterial carotenoid genes in canola leading to altered carotenoid production. *Metab. Eng.* **2003**, *5* (4), 255–263.
 23. Botella-Pavia, P.; Besumbes, O.; Phillips, M.A.; Carretero-Paulet, L.; Boronat, A.; Rodriguez-Concepcion, M. Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors. *Plant J.* **2004**, *40* (2), 188–199.
 24. Winkel, B.S.J. Metabolic channeling in plants. *Annu. Rev. Plant Biol.* **2004**, *55*, 85–107.
 25. Schwender, J.; Ohlrogge, J.; Shachar-Hill, Y. Understanding flux in plant metabolic networks. *Curr. Opin. Plant Biol.* **2004**, *7* (3), 309–317.
 26. Leech, M.J.; May, K.; Hallard, D.; Verpoorte, R.; De Luca, V.; Christou, P. Expression of two consecutive genes of a secondary metabolic pathway in transgenic tobacco: molecular diversity influences levels of expression and product accumulation. *Plant Mol. Biol.* **1998**, *38*, 765–774.
 27. Nawrath, C.; Poirier, Y.; Somerville, C. Targeting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. *Proc. Natl. Acad. Sci. USA* **1994**, *91* (26), 12760–12764.
 28. Halpin, C.; Boerjan, W. Stacking transgenes in forest trees. *Trends Plant Sci.* **2003**, *8* (8), 363–365.
 29. Beck von Bodman, S.; Domier, L.L.; Farrand, S.K. Expression of multiple eukaryotic genes from a single promoter in *Nicotiana*. *Biotechnology* **1995**, *13*, 587–591.
 30. Martinez-Salas, E. Internal ribosome entry site biology and its use in expression vectors. *Curr. Opin. Biotechnol.* **1999**, *10* (5), 458–464.
 31. Tang, G.; Galili, G. Using RNAi to improve plant nutritional value: from mechanism to application. *Trends Biotechnol.* **2004**, *22* (9), 463–469.
 32. Li, L.; Zhou, Y.; Cheng, X.; Sun, J.; Marita, J.M.; Ralph, J.; Chiang, V.L. Combinatorial modification of multiple lignin traits in trees through multigene cotransformation. *Proc. Natl. Acad. Sci. USA* **2003**, *100* (8), 4939–4944.
 33. Mol, J.; Grotewold, E.; Koes, R. How genes paint flowers and seeds. *Trends Plant Sci.* **1998**, *3* (6), 212–217.
 34. Irani, N.G.; Hernandez, J.M.; Grotewold, E. Regulation of anthocyanin pigmentation. *Recent Adv. Phytochem.* **2003**, *38*, 59–78.
 35. Goff, S.A.; Klein, T.M.; Roth, B.A.; Fromm, M.E.; Cone, K.C.; Radicella, J.P.; Chandler, V.L. Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues. *EMBO J.* **1990**, *9*, 2517–2522.
 36. Grotewold, E.; Chamberlin, M.; Snook, M.; Siame, B.; Butler, L.; Swenson, J.; Maddock, S.; Clair, G.S.; Bowen, B. Engineering secondary metabolism in maize cells by ectopic expression of transcription factors. *Plant Cell* **1998**, *10* (5), 721–740.
 37. van der Fits, L.; Memelink, J. ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. *Science* **2000**, *0*, 1–3.
 38. Bovy, A.; de Vos, R.; Kemper, M.; Schijlen, E.; Almenar Pertejo, M.; Muir, S.; Collins, G.; Robinson, S.; Verhoeyen, M.; Hughes, S.; Santos-Buelga, C.; van Tunen, A. High-flavonol tomatoes resulting from the heterologous expression of the maize transcription factor genes LC and C1. *Plant Cell* **2002**, *14* (10), 2509–2526.
 39. Yu, O.; Shi, J.; Hession, A.O.; Maxwell, C.A.; McGonigle, B.; Odell, J.T. Metabolic engineering to increase isoflavone biosynthesis in soybean seed. *Phytochemistry* **2003**, *63* (7), 753–763.

40. Hiratsu, K.; Matsui, K.; Koyama, T.; Ohme-Takagi, M. Dominant repression of target genes by chimeric repressors that include the EAR motif, a repression domain, in *Arabidopsis*. *Plant J.* **2003**, *34*, 733–739.
41. Jantz, D.; Amann, B.T.; Gatto, G.J. Jr.; Berg, J.M. The design of functional DNA-binding proteins based on zinc finger domains. *Chem. Rev.* **2004**, *104* (2), 789–799.
42. Ohlrogge, J. Plant metabolic engineering: are we ready for phase two? *Curr. Opin. Plant Biol.* **1999**, *2* (2), 121–122.
43. Hitz, B. Commentary: economic aspects of transgenic crops which produce novel products. *Curr. Opin. Plant Biol.* **1999**, *2* (2), 135–138.
44. Snell, K.D.; Peoples, O.P. Polyhydroxyalkanoate polymers and their production in transgenic plants. *Metab. Eng.* **2002**, *4* (1), 29–40.
45. Fernie, A.R.; Willmitzer, L. Carbohydrate metabolism. In *Handbook of Plant Biotechnology*; Christou, P., Klee, H., Eds.; John Wiley & Sons, Ltd.: Chichester, U.K., 2004; 525–557.
46. Verpoorte, R. Pharmacognosy in the new millennium: leadfinding and biotechnology. *J. Pharm. Pharmacol.* **2000**, *52* (3), 253–262.
47. Graham, R. Wheat: Research at Waite Agricultural Research Institute in Australia. CGIAR Micronutrients Project 1997, Update No. 2.
48. Underwood, B.A.; Arthur, P. The contribution of vitamin A to public health. *FASEB J.* **1996**, *10* (9), 1040–1049.
49. Humphrey, J.; West, K. Jr.; Sommer, A. Vitamin A deficiency and attributable mortality among under-5-year-olds. *Bull. World Health Organ.* **1992**, *72* (2), 225–232.
50. van den Berg, H.; Faulks, R.; Granada, H.F.; Hirschberg, J.; Olmedilla, B.; Sandmann, G.; Southon, S.; Stahl, W. The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J. Sci. Food Agric.* **2000**, *80* (7), 880–912.
51. Lee, C.; McCoon, P.; LeBowitz, J. Vitamin A value of sweet corn. *J. Agric. Food Chem.* **1981**, *29* (6), 1294–1295.
52. Bendich, A.; Olson, J. Biological actions of carotenoids. *FASEB J.* **1989**, *3*, 1927–1932.
53. Giovannucci, E.; Ascherio, A.; Rimm, E.B.; Stampfer, M.J.; Colditz, G.A.; Willett, W.C. Intake of carotenoids and retinol in relation to risk of prostate cancer. *J. Natl. Cancer Inst.* **1995**, *87* (23), 1767–1776.
54. Sommerburg, O.; Keunen, J.E.; Bird, A.C.; van Kuijk, F.J. Fruits and vegetables that are sources for lutein and zeaxanthin: the macular pigment in human eyes. *Br. J. Ophthalmol.* **1998**, *82* (8), 907–910.
55. Kohlmeier, L.; Kark, J.D.; Gomez-Gracia, E.; Martin, B.C.; Steck, S.E.; Kardinaal, A.F.; Ringstad, J.; Thamm, M.; Masaev, V.; Riemersma, R.; Martin-Moreno, J.M.; Huttunen, J.K.; Kok, F.J. Lycopene and myocardial infarction risk in the EURAMIC Study. *Am. J. Epidemiol.* **1997**, *146* (8), 618–626.
56. Kiefer, C.; Hessel, S.; Lampert, J.M.; Vogt, K.; Lederer, M.O.; Breithaupt, D.E.; von Lintig, J. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J. Biol. Chem.* **2001**, *276* (17), 14110–14116.
57. 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* (5451), 303–305.
58. Shewmaker, C.K.; Sheehy, J.A.; Daley, M.; Colburn, S.; Ke, D.Y. Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects. *Plant J.* **1999**, *20* (4), 401–412.
59. Mann, V.; Harker, M.; Pecker, I.; Hirschberg, J. Metabolic engineering of astaxanthin production in tobacco flowers. *Nat. Biotechnol.* **2000**, *18* (8), 888–892.
60. Rosati, C.; Aquilani, R.; Dharmapuri, S.; Pallara, P.; Marusic, C.; Tavazza, R.; Bouvier, F.; Camara, B.; Giuliano, G. Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *Plant J.* **2000**, *24* (3), 413–419.
61. Diaz de la Garza, R.; Quinlivan, E.P.; Klaus, S.M.; Basset, G.J.; Gregory, J.F. III; Hanson, A.D. Folate biofortification in tomatoes by engineering the pteridine branch of folate synthesis. *Proc. Natl. Acad. Sci. USA* **2004**, *101* (38), 13720–13725.
62. van Bel, A.J.; Hibberd, J.; Pruffer, D.; Knoblauch, M. Novel approach in plastid transformation. *Curr. Opin. Biotechnol.* **2001**, *12* (2), 144–149.
63. Edwards, R.; Dixon, D.P.; Walbot, V. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci.* **2000**, *5* (5), 193–198.
64. Theodoulou, F.L. Plant ABC transporters. *Biochem. Biophys.* **2000**, *2000*, 79–103.
65. Rea, P.A.; Li, Z.-S.; Lu, Y.-P.; Drozdowicz, Y.M. From vacuolar GS-X pumps to multispecific ABC transporters. *Ann. Rev. Plant Phys.* **1998**, *49*, 727–760.
66. Lin, Y.; Irani, N.G.; Grotewold, E. Sub-cellular trafficking of phytochemicals using auto-fluorescent compounds in maize cells. *BMC Plant Biol.* **2003**, *3* (3), 10.
67. Grotewold, E. The challenges of moving chemicals within and out of cells: insights into the

- transport of plant natural products. *Planta* **2004**, *219*, 906–909.
68. Wilson, L.M.; Whitt, S.R.; Ibanez, A.M.; Rocheford, T.R.; Goodman, M.M.; Buckler, E.S.T. Dissection of maize kernel composition and starch production by candidate gene association. *Plant Cell* **2004**, *16* (10), 2719–2733.
69. Schmidt-Dannert, C.; Umeno, D.; Arnold, F. Molecular breeding of carotenoid biosynthetic pathways. *Nat. Biotechnol.* **2000**, *18* (7), 750–753.
70. Stemmer, W.P.C. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc. Natl. Sci. USA* **1994**, *91*, 10747–10751.
71. Jestin, J.L.; Kaminski, P.A. Directed enzyme evolution and selections for catalysis based on product formation. *J. Biotechnol.* **2004**, *113* (1–3), 85–103.
72. Eijssink, V.G.; Bjork, A.; Gaseidnes, S.; Sirevag, R.; Synstad, B.; van den Burg, B.; Vriend, G. Rational engineering of enzyme stability. *J. Biotechnol.* **2004**, *113* (1–3), 105–120.
73. Castle, L.A.; Siehl, D.L.; Gorton, R.; Patten, P.A.; Chen, Y.H.; Bertain, S.; Cho, H.J.; Duck, N.; Wong, J.; Liu, D.; Lassner, M.W. Discovery and directed evolution of a glyphosate tolerance gene. *Science* **2004**, *304* (5674), 1151–1154.
74. Sweetlove, L.J.; Last, R.L.; Fernie, A.R. Predictive metabolic engineering: a goal for systems biology. *Plant Physiol.* **2003**, *132*, 420–425.