Humans have been manipulating the genetic makeup of agricultural plants (and animals) for millennia. Recently, the pace of change has increased markedly as researchers apply molecular tools to modify a wide range of agronomic characteristics and to develop market transgenic crops that generate specific novel products (ap Rees, 1995). Among many others, these include vaccines and other pharmaceuticals, plastics, and proteins that may render certain plants effective tools for environmental decontamination (John and Keller, 1996; Raskin, 1996; Amtzen, 1997; Hezari and Croteau, 1997).

Related techniques can be adapted to manipulate endogenous biochemical pathways. Here, the objective is to generate transgenic crops in which the range, scope, or nature of a plant’s existing natural products is modified to provide beneficial commercial, agronomic, and/or post-harvest processing characteristics (Kinney, 1998). Such plants may, for example, accumulate unusual edible or industrial oils (Broun and Somerville, 1997; Zou et al., 1997), lignins with different subunit compositions that interfere less extensively with paper production (Campbell and Sederoff, 1996), starches with different milling qualities, and so on.

In theory, any biochemical pathway can be subjected to this “metabolic engineering.” In practice, however, efforts to modify the biosynthesis of a variety of plant primary and secondary metabolites have been confounded by the very metabolic flexibility that researchers are trying to exploit. Although these studies may uncover novel information about a pathway and its regulation that will inform subsequent attempts to manipulate it, such difficulties must be overcome if a genetically engineered crop is to become commercially viable.

There are four major variables that appear to affect the outcome of metabolic engineering experiments. First, it is not always possible to predict the effects that directed perturbations (i.e., changing the level or activity of a single biosynthetic enzyme) may have on the entire pathway—unidentified endogenous feedback and/or feedforward controls may constrain metabolic pathways in manners that are poorly defined. Second, manipulating primary metabolic pathways can have pleiotropic (and often detrimental) effects on plant growth and development. Third, no matter how well a transgenic plant is characterized in an experimental setting, crop plants in the field will be subjected to environmental perturbations that may adversely affect accumulation of the engineered product. Fourth, because many applications of genetic engineering demand that the engineered products get to the right place in the plant at the right time, it is critical to ensure that these products are appropriately targeted within and/or between cells in the transgenic plant.

These variables can be addressed by adapting metabolic engineering strategies in a number of ways. Using cultured plant cells as metabolic factories instead of whole plants can circumvent problems associated with inappropriate targeting and with the vagaries in metabolite accumulation that can occur in field-grown material. Growing cell cultures in defined media may also bypass a number of other potential problems, such as tissue-specific or developmental regulation of some (or all) of the genes in a pathway.

Clearly, the more that is known about the metabolic pathway in question, the better the chances that metabolic engineering will succeed. However, detailed understanding of the biochemical changes that take place at each step and of the enzymes that catalyze the reactions may not always be sufficient. It is also critical to determine how the activities of these enzymes are regulated, either at the level of gene expression or by their substrates and products through feedback and/or feedforward control.

In fact, regulatory genes are becoming valuable tools for metabolic engineering. Combined genetic and biochemical studies over the past 20 years suggest that different transcription factors operating in a single pathway may affect the expression of distinct (but sometimes overlapping) suites of “downstream” genes. These distinct suites of genes may encode enzymes that participate in individual branches of a complex pathway (see e.g., Grotewold et al., 1994; Sablowski et al., 1994; Tamagnone et al., 1998). Thus, by manipulating the expression of a single transcription factor, it is theoretically possible to affect the expression of several coordinately regulated biosynthetic enzymes. Furthermore, work in a number of systems suggests that many transcriptional regulators are capable of faithfully recognizing their homologous target genes in heterologous species. For example, Tamagnone et al. (1998) have recently shown that two transcriptional regulators that repress lignin biosynthesis in Antirrhinum also affect lignin synthesis in transgenic tobacco.

Lignin is a secondary metabolite, and metabolic engineering strategies aimed at manipulating such compounds have generally been less complicated than those in which primary metabolic pathways are targeted for modification (Kinney, 1998). Many secondary metabolites are not essential for plant viability, and so it is possible that their biosynthesis is less tightly constrained.
than is that of critical primary metabolites such as starch and lipids.

Despite the fact that they are dispensable, many secondary metabolites play important roles in plant biology. For example, specific secondary metabolites have been reported to function in defending plants from pathogens, as insecticidal compounds, and as UV protectants (Dixon and Paiva, 1995; Byrne et al., 1996; Jansen et al. 1998). Secondary metabolites are also the source of many components in the pharmaceutical arsenal against human pathogens and diseases (Kutchan, 1995). Others are food colorings, and still others play important roles in plant biology. For these reasons, secondary metabolic pathways have been subjected to intense genetic and biochemical investigation over the years. In fact, the biosynthetic pathways of many plant secondary metabolites are generally well characterized and, in some cases, a number of genes that could be used as targets for metabolic engineering strategies have been cloned.

One well-characterized pathway directs the synthesis of anthocyanins. Experiments in maize, Antirrhinum, petunia, and Arabidopsis have uncovered not only structural genes that encode enzymes in this pathway but also a number of regulatory genes. The anthocyanins are flavonoids, one of the two major classes of plant secondary metabolites that are derived from the shikimate pathway, the primary products of which include phenylalanine and tyrosine (Hermann, 1995; Shirley, 1996). There are several chemically distinct families of flavonoids in cereals, ranging from the anthocyanins and other 3-hydroxy flavonoids to the related 3-deoxy flavonoids (Shirley, 1996). Many flavonoids and phenylpropanoids (the other major class of shikimate pathway-derived secondary metabolites) are of commercial importance, either in their own right or by virtue of the agronomic benefits they confer on plants in which they accumulate (Shirley, 1996; Jansen et al., 1998).

In maize plants, enzymes carrying out steps along each of these major branches appear to be under the separate control of distinct transcriptional regulators. Upregulation of C1 and R leads to anthocyanin accumulation, whereas P is required for 3-deoxy flavonoid biosynthesis. Research with mutants has shown that P and C1/R operate independently in maize floral organs, and it has been shown that P actually binds to the promoter of a1, one of the flavonoid biosynthetic genes that it regulates (Grotewold et al., 1994; Sainz et al., 1997).

**On pages 721–740 of this issue, Grotewold et al.** bring together flavonoid biosynthesis, transgenic cell lines, and metabolic engineering to demonstrate that the ectopic expression of transcription factors is a viable method for engineering secondary metabolism in cultured plant cells. Their work also significantly extends our understanding of and appreciation for the flexibility and complexity of flavonoid biosynthesis in maize.

Grotewold et al. begin by investigating changes in flavonoid and phenylpropanoid biosynthesis that are provoked by overexpressing P or C1 and R in cultured Black Mexican Sweet (BMS) maize cells. The corresponding genes (c1, r, and p1) are not ordinarily expressed in these cells, which gives the authors a clean metabolic slate upon which to identify some of their downstream target genes.

They do this by analyzing the flavonoids and related compounds that accumulate in the transgenic lines and by investigating the effects of ectopic C1/R or P expression on the accumulation of transcripts that encode flavonoid biosynthetic enzymes. By marrying their two data sets with what is known of flavonoid biosynthesis, Grotewold et al. come up with some solid conclusions regarding the downstream targets of P and C1/R as well as some surprising information on the potential role of P in phenylpropanoid metabolism and secondary metabolite targeting.

Grotewold et al. demonstrate that many of the effects of overexpressing P and C1/R on flavonoid biosynthesis in BMS cells reflect previous analyses of genetically analogous tissues in maize plants. For example, the authors show that ectopic expression of C1/R leads to the accumulation of anthocyanins and a number of other related 3-hydroxy flavonoids. Similarly, P overexpression correlates with the accumulation of 3-deoxy flavonoids such as luteoforol and C-glycosyl flavones.

These data are significant for two reasons. First, a role for P in inducing the synthesis of 3-deoxy flavonoids had not been demonstrated previously in tissues in which P is not normally expressed. Second, although Grotewold et al. show that C-glycosyl flavones accumulate in the BMS cells overexpressing P, it is the closely related rhamnosyl derivatives that ordinarily accumulate in maize tissues. This distinction is important because rhamnosylated flavones such as maysin, which accumulates in maize silks, are thought to possess insecticidal properties (Byrne et al., 1996). The authors suggest that the rhamnosylated derivatives do not accumulate in transgenic BMS cells simply because they do not express a rhamnosyl transferase that is required for the final biosynthetic step. If this is true, then it is reasonable to assume that P may induce the accumulation of insecticidal compounds in maize reproductive tissues, which, in turn, could confer a selective advantage on plants expressing p1.

That this advantage may be quantitative is suggested by Grotewold et al.’s data showing that in P-expressing lines, the transcript levels of two flavonoid biosynthetic enzymes, a1 and c2, correlate with the level of P expression. These data support the authors’ contention and previous suggestions (e.g., Byrne et al., 1996) that p1 may
operate as a quantitative trait locus by virtue of the presumed insecticidal activity of the rhamnosylated 3-deoxy flavone derivatives whose synthesis it regulates. Thus, allelic variation of p1 may derive from the level of P expression and/or from the distinct P tissue specificities that are known to correlate with alterations in the C-terminal domain of P (Chopra et al., 1996).

Not all of the data reported by Grotewold et al. reflect parallels between flavonoid biosynthesis in BMS cells and maize plants, and the authors point out some potentially interesting differences. For example, they show that the phenylpropanoids ferulic acid and chlorogenic acid accumulate in cell lines that overexpress P. This observation is surprising because P has not been reported to affect the levels of these compounds in plants. Moreover, neither chlorogenic acid nor ferulic acid is a known precursor of the 3-deoxy flavonoids whose synthesis is regulated by P.

In support of their contention that the accumulation of ferulic acid in BMS cells has uncovered a novel regulatory role for P, Grotewold et al. point out that the chemical structure of ferulic acid is analogous to that of the C-glycosyl flavone isocoparin. Given the rather broad substrate preferences of some of the enzymes on the flavonoid biosynthetic pathway, they suggest that P may regulate a catechol O-methyl transferase that can utilize either a flavonoid or a phenylpropanoid as substrate. However, it is also conceivable that ferulic acid accumulates as a nonspecific consequence of P-induced changes in flux through the flavonoid and phenylpropanoid pathways. Regardless of the underlying mechanism, the possibility that P expression may affect phenylpropanoid biosynthesis is supported by recent work showing that chlorogenic acid, an esterified derivative of ferulic acid, accumulates in immature maize silk tissue (Byrne et al., 1996).

In addition to their efforts to explain the molecular basis for the changes in flavonoid and phenylpropanoid metabolism that are induced by expression of C1/R and/or P in BMS cells, Grotewold et al. also take some important steps toward understanding how secondary metabolites are trafficked in plant cells. Indeed, they find that the changes associated specifically with C1/R or P expression at the molecular level reflect C1/R- and P-specific differences in cell biology.

The authors show that in C1/R-expressing cells, anthocyanins accumulate first in vesicles called anthocyanoplasts, which are derived from the smooth endoplasmic reticulum. In time, the anthocyanoplasts coalesce to form a single large vacuole. By contrast, Grotewold et al. detect two distinct types of green fluorescent organelles in cells overexpressing P, neither of which appears to be closely related to anthocyanoplasts. Each type of organelle targets P-induced secondary metabolites to a distinct cellular location—either to the cell wall (the destination of most of the fluorescent material in P-expressing BMS cells) or to the vacuole.

Grotewold et al. suggest that these data reflect the inherent complexity that is likely to be a hallmark of secondary metabolite targeting in plants. Because very little is currently known about this topic, subsequent investigations of the P-induced fluorescent compounds and the organelles in which they accumulate are bound to be particularly informative. Moreover, continuing cell biological investigations of flavonoid targeting in BMS cells may complement some existing data on anthocyanin targeting. For example, one of the maize genes that has been defined as playing a role in anthocyanin synthesis, bronze-2, was recently shown to encode a glutathione S-transferase that both conjugates anthocyanin with glutathione and plays a role in targeting the conjugates to vacuoles (Mars et al., 1995).

The work reported by Grotewold et al. represents an informative and productive evaluation of the feasibility of metabolic engineering in plant cells. The majority of their data suggest that the flavonoid pathway is regulated similarly in BMS cells and in maize plants, with some of the more subtle differences likely being accounted for by deficiencies in the complement of biosynthetic genes that are expressed in the cell line. This suggests that conclusions drawn from experiments with cell lines can generally be extrapolated to whole plants.

In the future, Grotewold et al. can further refine their analyses of flavonoid biosynthesis by focusing their efforts on a series of constructs that allow C1/R and/or P expression to be induced in BMS cells upon estradiol application. In conjunction with 13C-labeling studies, this estradiol-inducible system should provide a more dynamic view of flavonoid biosynthesis in BMS cells by facilitating the identification of intermediates along the distinct branches of flavonoid biosynthesis that are controlled by C1/R or P.

Grotewold et al. may also be able to use the transformed BMS cell lines to further investigate the biological functions of the compounds that accumulate as a result of the overexpression of P and/or C1/R. For example, preliminary indications that some of these cell lines may exhibit increased UV tolerance should be pursued vigorously.

Finally, the authors’ apparent identification of two distinct trafficking pathways, each of which appears to correlate with the expression of a distinct regulatory gene (or gene pair), is most illuminating. Do these transcription factors directly regulate trafficking, or are the two trafficking pathways associated with the intrinsic qualities of the specific flavonoids whose biosynthesis is triggered by the ectopic expression of each one? Because it will allow real-time imaging of flavonoid transport, the estradiol-inducible system described by Grotewold et al. should also facilitate efforts to address this question.

Clearly, Grotewold et al.’s data represent a promising start in the engineering
of secondary metabolite biosynthesis in homogeneous plant cell cultures. However, whether the benefits of manipulating metabolic pathways in cultured cell lines will outweigh the expense of their production and maintenance, and whether it will become economically preferable to use cell cultures as biological factories instead of plants in the field, remain to be seen. My guess is that, for certain products, they will.

Crispin B. Taylor

REFERENCES


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Crispin B. Taylor
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