It Takes a Garden. How Work on Diverse Plant Species Has Contributed to an Understanding of Flavonoid Metabolism[1]

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Flavonoid biosynthesis is one of the most extensively studied areas of plant secondary metabolism. Modern-day reports date back to 1664 and Robert Boyle's description of the effects of acids and bases on plant pigments. Today, through the study of flavonoid metabolism in diverse plant systems, a great deal is known about the genes involved in this pathway and the variety of biological functions in which flavonoids participate. What is abundantly clear from the sum of this work is that the central flavonoid pathway has been highly conserved during the course of plant evolution, but that there has been considerable divergence in the functional roles of its end products and, perhaps related to this, the mechanisms by which expression of the pathway is controlled. For this reason, the use of a variety of species in these studies has contributed much to developing a general view of flavonoid metabolism and function across the plant kingdom, in addition to providing a variety of experimental tools to facilitate the characterization of this complex system.

ENZYME AND GENE CHARACTERIZATION

The biochemistry of flavonoid metabolism has been elucidated over the course of many years through the careful identification and characterization of numerous enzymes. This work has taken advantage of tissues from which flavonoid enzymes could be easily isolated in large quantities. Examples include irradiated parsley (*Petroselinum hortense*) cells for chalcone synthase (Kreuzaler et al., 1979), soybean (*Glycine max*) seeds, and bean (*Phaesoleus vulgarus*) cell suspension cultures for chalcone isomerase (Moustafa and Wong, 1967; Dixon et al., 1982), and matthiola (*Matthiola incana*), petunia (*Petunia hybrida*), and carnation (*Dianthus caryophyllus*) flowers for flavanone 3-hydroxylase, flavonol synthase, flavonoid 3′-hydroxylase, and dihydroflavonol reductase (Forkmann et al., 1980; Spribille and Forkmann, 1984; Britsch and Grisebach, 1986; Stich et al., 1992). These experiments uncovered the complex network of biochemical reactions that mediate the synthesis of flavonoids in plants and laid the foundation for efforts to isolate the corresponding genes.

An overview of the history of gene cloning in the flavonoid pathway (Table I) shows that most of the genes encoding enzymes of the central pathway were first isolated based on biochemical approaches (e.g. information from enzyme characterization or the use of antibodies raised against the purified protein). Mutations resulting from the insertion of transposable elements into flavonoid genes were also useful in this regard. In fact, isolation of the *bronze 1* gene, which encodes UDP-Glc:flavonoid 3-*O*-glucosyl transferase, was the first example of gene cloning using transposon tagging (Federoff et al., 1984). Cloning of the original flavonoid structural genes was carried out, for the most part, in maize and petunia, although the first flavonoid gene, for chalcone synthase, was isolated from parsley (Kreuzaler et al., 1983). Identification of genes encoding enzymes of the isoflavonoid branch pathway, which is found primarily in legumes, has come from recent work in soybean and alfalfa (*Medicago sativa*), again largely based on biochemical approaches. Arabidopsis is a bit of a late bloomer in the effort to identify genes involved in flavonoid biosynthesis. Although Arabidopsis is not particularly amenable to biochemical approaches, mutations in all of the major structural genes have been identified in this species and are proving useful for filling in a few remaining gaps. This includes the recent identification of a gene that may be involved in the synthesis of condensed tannins (Devic et al., 1999). These are major pigments in many seeds that are also of agronomic importance in the vegetative tissues of forage crops, having the beneficial property of reducing bloat in grazing ruminants and at the same time the potential to act as antifeedants and antinutrients (Morris and Robbins, 1997). Moreover, the use of maize genes to complement Arabidopsis flavonoid mutants has recently provided evidence that flavonoid enzymes have been functionally conserved over large evolutionary distances (Dong et al., 2001). Transposon and T-DNA tagging approaches in maize, petunia, and Arabidopsis are also providing long-awaited information on the genes involved in transporting flavonoids from the site of synthesis in the cytoplasm to the vacuole

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Table I. *History of flavonoid gene isolation*

Only the original genes, identified by methods other than homology-based cloning, are included. Entries in each section are listed in chronological order.

Gene	Method	Species	Citation
Central flavonoid pathway			
Chalcone synthase	Antibody screening of hybrid-arrested/selected translation products	Parsley	Kreuzaler et al. (1983)
Dihydroflavonol reductase	Transposon tagging	Maize (*Zea mays*), snapdragon (*Antirrhinum majus*)	Martin et al. (1985) and O'Reilly et al. (1985)
Chalcone isomerase	Antibody screening of expression library	Bean	Mehdy and Lamb (1987)
UDP-Glc:flavonoid 3-*O*-glucosyltransferase	Transposon tagging	Maize	Federoff et al. (1984)
Leucoanthocyanidin dioxygenase (anthocyanidin synthase)	Transposon tagging	Maize	Menssen et al. (1990)
Flavanone 3-hydroxylase	Antibody screening of expression library	Petunia	Britsch et al. (1992)
Flavonoid 3′,5′-hydroxylase	Cytochrome P450 homology	Petunia	Holton et al. (1993a)
Flavonol synthase	Dioxygenase homology	Petunia	Holton et al. (1993b)
UDP rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase	Differential cDNA library screening	Petunia	Brugliera et al. (1994)
Flavonoid 3′-hydroxylase	Cytochrome P450 homology	Petunia	Brugliera et al. (1999)
Anthocyanin 5-*O*-glucosyltransferase	Differential display	Perilla (*Perilla frutescens*)	Yamazaki et al. (1999)
Proanthocyanidin branch pathway			
Leucoanthocyanidin reductase	T-DNA tagging	Arabidopsis	Devic et al. (1999)
Aurone branch pathway			
Aureusidin synthase	Subtractive hybridization/peptide sequencing	Snapdragon	Nakayama et al. (2000)
Flavone branch pathway			
Flavone synthase II	Differential display and cytochrome P450 homology	Gerbera (*Gerbera hybrida*) torenia (*Torenia hybrida*)	Akashi et al. (1999) and Martens and Forkmann (1999)
Isoflavonoid branch pathway			
Chalcone reductase	Elicitor-induced cDNAs that matched sequence of purified enzyme	Soybean	Welle et al. (1991)
Isoflavone reductase	Antibody screening of expression library	Alfalfa	Paiva et al. (1991)
Vestitone reductase	PCR based on peptide sequence	Alfalfa	Guo and Paiva (1995)
Isoflavone-*O*-methyltransferase	PCR based on peptide sequence	Alfalfa	He et al. (1998)
Isoflavone 2′-hydroxylase	Functional analysis of cytochrome P450s	Licorice (*Glycyrrhiza echinata*)	Akashi et al. (1998)
2-Hydroxyisoflavanone synthase	Functional analysis of cytochrome P450s	Soybean, licorice	Akashi et al. (1999), Steele et al. (1999), and Jung et al. (2000)
Flavonoid 6-hydroxylase	Differential display	Soybean	Latunde-Dada et al. (2001)
Vacuolar transport of flavonoids			
Bronze-2 (glutathione *S*-transferase)	Transposon tagging	Maize	McLaughlin and Walbot (1987)
TRANSPARENT TESTA 12 (MATE transporter)	T-DNA tagging	Arabidopsis	Debeaujon et al. (2001)
Regulatory factors			
C1 (myb)	Transposon tagging	Maize	Cone et al. (1986) and Paz-Ares et al. (1986)
Lc (basic Helix Loop Helix [bHLH])	Transposon tagging	Maize	Ludwig et al. (1989)
P (myb)	Transposon tagging	Maize	Lechelt et al. (1989)
CPRF1, CPRF2	Southwestern screening of expression library	Parsley	Weisshaar et al. (1991)
delilah (bHLH)	Transposon tagging	Snapdragon	Goodrich et al. (1992)
anthocyanin11 (WD40)	Transposon tagging	Petunia	de Vetten et al. (1997)
TRANSPARENT TESTA GLABRA2 (WRKY)	Transposon tagging	Arabidopsis	Johnson and Smyth (1998)
CPRF4	Southwestern screening of expression library	Parsley	Kircher et al. (1998)
anthocyanin2 (myb)	Transposon tagging	Petunia	Quattrocchio et al. (1999)
TRANSPARENT TESTA GLABRA1 (WD40)	Positional cloning	Arabidopsis	Walker et al. (1999)
anthocyanin1 (bHLH)	Transposon tagging	Petunia	Spelt et al. (2000)
TRANSPARENT TESTA 8 (bHLH)	T-DNA tagging	Arabidopsis	Nesi et al. (2000)
CPRF5, CPRF6, CPRF7	Two-hybrid screening	Parsley	Rügner et al. (2001)

(Marrs et al., 1995; Alfenito et al., 1998; Debeaujon et al., 2001). These studies underscore the limitations of homology-based approaches because petunia and maize glutathione *S*-transferases with only 12% amino acid identity functionally complement each other in vacuolar transport of flavonoids, whereas an Arabidopsis cDNA with 50% identity to the petunia protein cannot.

The identification of genes encoding regulatory factors has relied almost exclusively on transposon and, more recently, T-DNA, tagging (Table I). This is largely due to the fact that regulatory proteins do not accumulate to high levels and therefore are not amenable to biochemical analysis; there are also limitations with regard to homology cloning across species because the conserved sequences in these proteins, such as bHLH and myb domains, are shared widely among transcription factors. Therefore, transposon tagging provided a rapid entrée to the isolation of flavonoid regulatory factors in maize, petunia, and snapdragon. Additional novel regulatory factors are now being isolated from Arabidopsis by positional cloning and T-DNA tagging. A different approach, involving the isolation of transcription factors using South-western and two-hybrid screening, has also led to the identification of an apparent complex of flavonoid regulatory proteins in parsley (Weisshaar et al., 1991; Rügner et al., 2001). The information from these efforts, together with the characterization of regulatory factors in heterologous species using transgenic plants (Lloyd et al., 1992; Quattrocchio et al., 1998; Uimari and Strommer, 1998; Bradley et al., 1999), is pointing to some similarities, but also important differences, in the mechanisms by which the flavonoid pathway is regulated in different plant species. Therefore, the availability of information from diverse systems is a crucial aspect of efforts to understand how this pathway is controlled, particularly in light of the widely different physiological requirements for flavonoids that exist among plant species.

PHYSIOLOGICAL FUNCTION

The diverse resources offered by different plant species, such as tissues that provide high yields of flavonoid enzymes and mutant lines tagged with transposable elements or T-DNA insertions, have clearly facilitated the rapid cloning of flavonoid genes. However, the use of different species has perhaps been even more crucial to efforts to define the biological functions of flavonoids in plants. Although some of these functions are common to all plants, others are more limited and appear to have evolved differently, or even independently, in different lineages. For example, isoflavonoids, which are important defense compounds and also function as signaling molecules in nitrogen fixation, are found only in legumes and a few non-legume plants. Characterization of this branch pathway in alfalfa and soybean is providing tools for metabolic engineering of isoflavonoid synthesis in other plant species (for review, see Dixon and Steele, 1999). Similarly, sorghum (*Sorghum bicolor*), maize, and gloxinia (*Sinningia cardinalis*) are among the few species known to synthesize 3-deoxyanthocyanins, which are involved both in defense (Snyder and Nicholson, 1990) and in pigmentation (Grotewold et al., 1994), and some information on the biochemistry of this branch pathway has emerged. Analysis of maize and petunia lines carrying mutations in the first enzyme of flavonoid biosynthesis uncovered a role for flavonoids in male fertility. However, a null mutant affecting the same enzyme in Arabidopsis was fully fertile, demonstrating that flavonoids are not universally required during pollen tube formation (for review, see Shirley, 1996). Likewise, flavonoids have been shown to help define host-range specificity for microbes such as *Rhizobium* spp. and *Agrobacterium* spp. (Rolfe, 1988; Zerback et al., 1989). Flavonoids also contribute to plant host recognition by parasitic plants like *Triphysaria versicolor* and *Cuscuta subinclusa*, but apparently are not required for successful parasitism of Arabidopsis with *Orobanche aegyptiaca* (Kelly, 1990; Albrecht et al., 1999; Westwood, 2000). It is clear that these biological functions are quite specialized and have required the use of particular plant species for experimental characterization.

Flavonoids also play a number of apparently universal roles in plants, which presumably arose early in, and perhaps even drove, evolution of the pathway. This is where model systems like Arabidopsis become particularly useful by providing genetic and molecular resources that may not be available in other plants. Arabidopsis also offers some simplicity with regard to the flavonoid pathway in that all but one of the enzymes of the central pathway are encoded by single genes, unlike the situation in many other plants. Therefore, mutations in this pathway disrupt expression in all tissues and under all environmental conditions. For example, Arabidopsis flavonoid mutants were used to demonstrate for the first time an unequivocal role for flavonoids in protection of plants from UV radiation (Li et al., 1993). These mutants have also provided insights into the contribution of flavonoids in the seed coat to maintaining seed dormancy (Debeaujon et al., 2000). Moreover, the long-controversial theory that flavonoids function as inhibitors of auxin transport (Jacobs and Rubery, 1988) is receiving support from studies in Arabidopsis (Jacobs and Rubery, 1988; Brown et al., 2001). In each case, Arabidopsis helps lay the foundation for expansion and application of the work in other plant species.

THE PATHWAY AS AN EXPERIMENTAL TOOL

In addition to being the subject of efforts to characterize plant metabolism, the flavonoid pathway has

contributed directly and indirectly to the discovery of several fundamental biological principles over the past 150 years. Two particularly well-known examples are Gregor Mendel's use of flower and seed coat color, among other characters of peas, to develop his theories of heredity, and Barbara McClintock's study of pigmentation patterns of maize kernels that led to the discovery and elucidation of mobile elements. More recently, analysis of pigmentation in maize kernels and vegetative tissues identified the epigenetic phenomenon known as paramutation, in which allele interactions result in heritable changes in gene expression (Chandler et al., 2000). Similarly, the effects of flavonoid transgene expression on petunia flower pigmentation uncovered the phenomenon of cosuppression (Que and Jorgensen, 1998; Metzlaff et al., 2000). The flavonoid pathway has also been a subject of interest with regard to the study of evolution, particularly in morning glory (*Ipomoea purpurea*), which offers unique genetic resources and a long history of analysis (Iida et al., 1999; Rausher et al., 1999; Durbin et al., 2000). These studies support the idea that the enzymes of flavonoid biosynthesis were recruited from primary metabolism and that gene duplication has allowed the adaptation of these enzymes for specialized functions. In addition, the flavonoid pathway, and the general phenylpropanoid pathway from which it branches, are serving as experimental models for understanding the intracellular organization of metabolism, with recent work in alfalfa and Arabidopsis providing new information on channeling of intermediates and the assembly of multienzyme complexes (for review, see Winkel-Shirley, 2001). Again, each of these efforts has benefited from the unique features of a particular plant species with respect to flavonoid metabolism.

METABOLIC ENGINEERING

The availability of well-defined structural and regulatory genes from a variety of species has fueled interest in engineering flavonoid metabolism, both for the floriculture industry and for nutritional enhancement of plants (for review, see Dixon and Steele, 1999; Forkmann and Martens, 2001). Significant progress has been made toward engineering modified flower and plant coloration by the exchange of flavonoid genes between species. However, this has been more challenging than might have been expected due to the complexity of factors, such as proper vacuolar pH and the need for accessory proteins such as cytochrome b5s, that contribute to flavonoid-associated pigmentation in plants. At the same time, genes are becoming available that permit the production of beneficial isoflavonoids in nonlegume species (Akashi et al., 1998, 1999; Steele et al., 1999; Jung et al., 2000; Yu et al., 2000) and a petunia chalcone isomerase gene was recently used to increase the levels of flavonols, which also have nutritional benefits, in tomato (*Lycopersicon esculentum*) fruit (Muir et al., 2001). There is also an interest in modifying the production of condensed tannins (proanthocyanidins) in forage crops such as *Lotus corniculatus* (Morris and Robbins, 1997), an effort that may be facilitated by the isolation of additional biosynthetic genes, such as the putative leucoanthocyanidin reductase recently cloned in Arabidopsis (Tanaka et al., 1997; Devic et al., 1999).

FUTURE DIRECTIONS

Could all of this knowledge have come from the study of a single model plant? And will one model system provide answers to the many questions that remain? Certainly not. Model plants such as Arabidopsis and rice (*Oryza sativa*) will undoubtedly continue to facilitate efforts to address specific questions regarding the regulation and function of the flavonoid pathway. Arabidopsis is already well developed in this regard and is providing new information on general, as well as some specialized, functions of flavonoids, as described above. Genomics tools such as microarrays are also generating new information on coordinate expression of genes, including those of the flavonoid pathway, in Arabidopsis as well as in maize (Bruce et al., 2000; Harmer et al., 2000). On the other hand, very little has yet been published on the flavonoid pathway in rice, although one flavonoid mutant that disrupts pigmentation in the leaf and pericarp has now been described (Reddy et al., 1995). It will be interesting to see how this emerging experimental system will fit into the overall effort to understand flavonoid metabolism, perhaps by integrating genomics data from rice with the extensive genetic resources available for flavonoid biosynthesis in barley (Jende-Strid, 1991).

There will also always be applications that are best studied in particular plant species. For example, Lers et al. (1998) have identified an isoflavone reductase-like gene in grapefruit that appears to be correlated with UV-induced resistance to *Penicillium digitatum*. Likewise, insights into mechanisms by which plant regulate vacuolar pH may soon come from mutants that alter flower color by disrupting a vacuolar Na^+H^+ exchanger in morning glory (Yamaguchi et al., 2001) and a putative regulatory gene in petunia (Griesbach, 1998). Of course, there is still the question of how plants modify the basic flavonoid skeleton to generate the thousands of variants that are found in nature. It is quite clear that only through the combined efforts in a diverse array of plant systems will we arrive at the level of understanding needed to effectively engineer flavonoid metabolism for the agronomic, horticultural, and nutritional enhancement of plants. At the same time, the knowledge derived from studying flavonoid biosynthesis in diverse plant species will continue to expand our understanding of cellular metabolism and molecular evolution, as well as other fundamental biological phenomena.

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