Biosynthesis of flavonoids and effects of stress
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The accumulation of red or purple flavonoids is a hallmark of plant stress. Mounting evidence points to diverse physiological functions for these compounds in the stress response. Advances are also being made toward understanding how plants control the types and amounts of flavonoids that are produced in response to different cues.

Introduction
Flavonoids are ubiquitous plant secondary products that are best known as the characteristic red, blue, and purple anthocyanin pigments of plant tissues [1]. These compounds serve essential functions in plant reproduction by recruiting pollinators and seed dispersers. They are also responsible for the beautiful display of fall color in many plant species, which has recently been suggested to protect leaf cells from photo-oxidative damage, thereby enhancing the efficiency of nutrient retrieval during senescence [2]. In fact, flavonoids are a remarkably diverse group of secondary products (Figure 1) with a vast array of biological functions, including apparent roles in stress protection. The flavonols may be among the most important flavonoids in this regard; they are the most ancient and widespread of the flavonoids, synthesized even in mosses and ferns, and have a wide range of potent physiological activities [3]. Progress continues to be made in understanding the roles of flavonoids in stress protection, as well as in defining the mechanisms that control the amounts and varieties of flavonoids that are produced in plants in response to diverse environmental cues [4].

Flavonoids and stress protection
The ultra-violet (UV)-absorbing characteristics of flavonoids have long been considered to be evidence for the role of flavonoids in UV protection. Indeed, flavonoids are often present in the epidermal cell layers of leaves and in tissues that are susceptible to UV light, such as pollen and the apical meristem. The first direct evidence in support of a role for flavanoids in UV protection came from experiments with Arabidopsis mutants, which showed that lesions in chalcone synthase (CHS) or chalcone isomerase (CHI) resulted in UV-hypersensitive phenotypes [5]. Interestingly, the CHI mutants were the most sensitive to UV light and showed a corresponding decrease in sinapate esters. This evidence suggests that other phenolic compounds may be at least as important as flavonoids in UV protection, a hypothesis that has been supported by subsequent studies [6,7]. However, work by Ryan et al. [8,9] on petunia and Arabidopsis has provided new evidence that UV light induces the synthesis of flavonols with higher hydroxylation levels. Because hydroxylation does not affect the UV-absorbing properties of these compounds but does affect their antioxidant capacity, it was suggested that flavonoids may play as yet uncharacterized roles in the UV stress response. A role for flavonoids in UV protection is further supported by Bieza and Lois' [10] isolation of an Arabidopsis mutant that is tolerant of extremely high UV-B levels. This line shows constitutively high levels of a number of phenolics, including flavonoids, and upregulation of the CHS gene. Clearly, much still remains to be learned in this area, including how the synthesis of specific flavonoids and other phenolics is regulated in response to UV light, how flavonoids compare to other phenolics in contributing to UV stress protection, and whether there are roles for flavonoids in UV protection beyond the absorption of UV radiation.

There is also new evidence that flavonoids play a role in resistance to aluminum toxicity in maize [11]. Roots of maize plants that were exposed to aluminum exuded high levels of phenolics, and an aluminum-resistant variety exuded a 15-fold higher level of flavonoids when pretreated with silicon than when no such pre-treatment was applied. These observations are certainly consistent with the metal-binding activity of many flavonoids. Although some researchers have argued that flavonoids are unlikely to be effective in binding metals in an acid environment because of competition from H+ ions, Kidd et al. [11] make the interesting point that a pentahydroxyflavone, morin, is routinely used to stain for aluminum in the root apoplast. Further work is needed to corroborate their findings and to extend them to other parts of the plant, including the cell interior, and to other plant species.

After an interval of almost ten years, new evidence has been uncovered linking flavonoids with control of the polar transport of the plant growth regulator auxin. This hormone may function in the stress response by helping to control stomatal opening [12] and by allocating resources under poor growth conditions [13]. Flavonoids have limited structural similarity to auxin, but do resemble the synthetic auxin transport inhibitor naphthylphthalamic acid (NPA; Figure 2), which is believed to bind a regulatory protein

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Abbreviations
CHI chalcone isomerase
CHS chalcone synthase
FLS flavonol synthase
IAA indole acetic acid
NPA naphthylphthalamic acid
tt transparent testa
UV ultra-violet

Flavonoids and stress protection
that is associated with the auxin efflux carrier [14]. In 1988, Jacobs and Rubery [15] published evidence that flavonoids, particularly apigenin and the flavonol quercetin, could compete with NPA to perturb auxin transport. Although two additional reports provided some supporting evidence [16,17], this topic remained controversial. Studies of Arabidopsis flavonoid mutants have now provided new support for a role for flavonoids in auxin transport [14]. The transparent testa (tt) mutants, which are deficient in CHS activity, have been shown to have elevated auxin transport and altered growth phenotypes that are consistent with this [18•]. These mutants also accumulate more indole acetic acid (IAA) in the upper root than do wildtype controls, and IAA appears to leak from the root tip of these mutants [19]. Flavonoids have been shown to accumulate in the cotyledonary node, the hypocotyl–root transition zone, and the root tip of young Arabidopsis seedlings [20]. Moreover, the flavonoid biosynthetic machinery has been localized to the apical end of cortex cells in the root elongation zone of these plants [21].
Future studies will determine whether changes in the synthesis or deposition of specific flavonoids within the cell may act to change the rate or direction of auxin transport.

Studies of these types raise the long-standing question of how the subcellular distribution of flavonoid compounds is determined. Researchers are continuing to gain insights into how flavonoids are partitioned into the vacuole, most recently through analysis of the tt12 mutant of Arabidopsis [22•]. The TT12 gene encodes a protein with similarity to members of the MATE (multidrug and toxin extrusion) family of transporters, which appear to control the vacuolar sequestration of flavonoids in the seed-coat endothelium. In maize, the bronze2 locus has been found to encode a glutathione transporter that is a key player in vacuolar uptake of flavonoids [23]. These findings suggest that different plant species may use different mechanisms to distribute flavonoids among subcellular compartments or that multiple mechanisms are used in individual species. This is an area of flavonoid biology that is still poorly understood and that is of significant current interest.

Transcriptional regulation of the flavonoid pathway

One important avenue to understanding the role of flavonoids in the stress response is to understand how the expression of the biosynthetic pathway is regulated. This system represents one of the oldest examples of coordinated gene and enzyme regulation in response to environmental and developmental factors [24]. A great deal has been learned from studies in a variety of plant species, primarily about transcriptional regulation, although evidence for other types of control also exists. Recent advances in this area include the isolation by genetic approaches of transcription factors that are identified by the petunia anthocyanin1 (an1) [25] and Arabidopsis tt8 regulatory mutants [26]. Both transcription factors are new members of the basic helix-loop-helix (bHLH) family, having similarity to R1 in maize and DELILA (DEL) in snapdragon, and are required for the activation of the dihydroflavonol reductase gene as well as other genes. The Arabidopsis TT2 gene has now also been cloned by a genetic approach and found to encode an R2R3 myb domain protein that appears to function as a key regulator of proanthocyanidin accumulation in developing seed [27•]. This gene could prove to be a powerful tool for engineering proanthocyanidin synthesis in seed crops [28]. An apparent complex of novel regulatory proteins that may interact with CHS, as well as with other flavonoid genes, was recently identified in parsley using South-western and two-hybrid screening [29]. Analysis of strawberry R2R3 myb homologs by their overexpression in transgenic tobacco plants has uncovered what appears to be a negative regulator of flavonoid gene expression [30]. Gareth Jenkins’ group has come at this from the opposite direction. They studied the expression of the CHS gene to develop a model for the interaction of the various light signalling pathways, in which phytochrome B balances flux through the cryptochrome1
Enzymology of flavonoid biosynthesis

Many, although not all, of the enzymes of flavonoid biosynthesis are encoded by small gene families. The functional significance of this redundancy has been the subject of substantial interest over the years. Kimura et al. [32] reported recently that licorice (Glycyrrhiza echinata) contains two CHI isozymes that can use both 6'-hydroxychalcone and 6'-deoxychalcone, and therefore are likely to be involved in the legume-specific isoflavonoid pathway (Figure 1). One of these genes is induced by elicitor treatment, and is therefore proposed to function in the stress response related to wounding. A third enzyme can use only 6'-hydroxychalcone and presumably functions in the general flavonoid pathway. It will be interesting to discover whether other legumes use similarly duplicated genes to provide for the stress-inducible synthesis of isoflavonoids. In non-legume plants, which do not synthesize isoflavonoid phytoalexins, CHI generally appears to be encoded by a single gene; although petunia does contain a second gene with homology to CHI, the product does not appear to be involved in the flavonoid pathway [33]. Work is also underway to examine duplication in other genes of flavonoid biosynthesis, such as flavonol synthase (FLS), the only flavonoid enzyme in Arabidopsis that is encoded by a single gene family ([34]; B Winkel-Shirley, unpublished data). Flavonols are among the most important flavonoids with respect to biological activity, and the gene duplication of FLS in Arabidopsis may provide a mechanism for controlling the types and amounts of flavonols produced in different tissues and in response to different environmental cues.

This past year has seen the first reported use of a flavonoid enzyme to enhance the nutritional value of a vegetable crop by increasing the content of flavonols, which have demonstrated health-promoting activities in animals [35]. The peel of fruit from tomato plants expressing the petunia chi-a gene, under control of the double-enhanced 35S promoter, contained significantly higher levels of naringenin chalcone and quercetin 3-trisaccharide, a flavonol glycoside [36]. Surprisingly, flavonoid accumulation was not altered in the fruit flesh or leaves of these transformants. Nonetheless, tomato paste produced from the transgenic fruits had a 21-fold higher flavonol concentration than paste from comparable non-transformed tomatoes, presumably because of leaching of flavonols from the peel into the paste.

Rational approaches to the metabolic engineering of flavonoid biosynthesis will certainly depend on a detailed understanding of the enzymology of the flavonoid pathway. A wealth of new information continues to emerge regarding the reaction mechanisms of CHS and CHI, an area that has been significantly bolstered by the determination of the three-dimensional structures of these enzymes by Joe Noel’s group within the past few years [37,38]. Most recently, this group has combined site-specific mutagenesis with analysis of X-ray crystal structures to correlate the size of the active-site cavity with the length of the polyketide chain produced by CHS and related enzymes, providing insights into how the synthesis of diverse polyketides may be engineered [38]. The CHI reaction has also been examined relative to the spontaneous cyclization reaction of di- and tri-hydroxychalcones, showing that catalysis involves binding of the ionized chalcone in a conformation that accelerates ring closure [39]. In addition, some new information has emerged on the organization of flavonoid enzymes within complexes comprised of one or more enzymes. For example, a study by Rick Dixon’s group has shown that metabolic channeling in the isoflavonoid pathway controls the regiospecificity of the alfalfa isoflavone O-methyltransferase reaction [40].

Conclusions

The accumulation of anthocyanin pigments in vegetative tissues is a hallmark of plant stress, yet the role that flavonoids play in the stress response is still poorly understood. In many cases, these compounds may provide antioxidant activity as part of a general stress response, which may also explain their health-promoting qualities in animals. However, there is also evidence that flavonoids may function in plants to screen harmful radiation, bind phytotoxins, and help to regulate the stress response by controlling auxin transport. Understanding the molecular basis of flavonoid function in ameliorating stress, as well as the regulatory and biochemical mechanisms that control the types and amounts of flavonoids synthesized under different conditions, continues to be a high priority for research with an eye to engineering enhanced stress tolerance in crop plants. These efforts are benefiting from the integration of emerging technologies with the extensive genetic and biochemical tools that have been developed for this system over the years.

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


Using chlorophyll fluorescence measurements on red and yellow senescing leaves and in blue and red light, the authors implicate anthocyanins in protecting leaf cells from photo-oxidative damage and suggest that this enhances nutrient retrieval during senescence.


Cloning and characterization of the TT22 gene identifies an apparent mechanism for the transport of flavonoids into vacuoles in Arabidopsis. This mechanism differs from that described in maize.


The authors provide important new information on how different branch-points in flavonoid biosynthesis may be regulated at the transcriptional level. Cloning of the TT2 gene could provide a means for engineering proanthocyanin production in crops for human and animal consumption.


The authors provide the first evidence that there are isozymes dedicated to the production of different types of flavonoids in response to different environmental cues.


40. Liu C J, Dixon RA: Elicitor-induced association of isoflavone O-methyltransferase with endomembranes prevents the formation and 7-O-methylation of daizein during isoflavonoid phytoalexin biosynthesis. Plant Cell 2001, 13:2643-2658. The authors provide evidence from biochemical and localization experiments that channeling may be essential for determining the regiospecificity of a key enzyme in isoflavonoid biosynthesis in vivo.