A Null Mutation in the First Enzyme of Flavonoid Biosynthesis Does Not Affect Male Fertility in Arabidopsis

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Flavonoids are a major class of secondary metabolites that serves a multitude of functions in higher plants, including a recently discovered role in male fertility. Surprisingly, Arabidopsis plants deficient in flavonoid biosynthesis appear to be fully fertile. Using RNA gel blot analysis and polymerase chain reaction-based assays, we have shown that a mutation at the 3' splice acceptor site in the Arabidopsis chalcone synthase gene completely disrupts synthesis of the active form of the enzyme. We also confirmed that this enzyme, which catalyzes the first step of flavonoid biosynthesis, is encoded by a single-copy gene. HPLC analysis of whole flowers and stamens was used to show that plants homozygous for the splice site mutation are completely devoid of flavonoids. This work provides compelling evidence that despite the high levels of these compounds in the pollen of most plant species, flavonoids are not universally required for fertility. The role of flavonoids in plant reproduction may therefore offer an example of convergent functional evolution in secondary metabolism.

INTRODUCTION

The plant kingdom is characterized by unique metabolic systems that convert the products of primary metabolism into a myriad of so-called secondary products. Members of all three major classes of secondary metabolites—alkaloids, terpenoids, and phenylpropanoids—perform vital functions in higher plants (reviewed in Rhodes, 1994). Many of these products play similar roles in all plant species, for example, as plant growth substances or pigments. Others appear to have been recruited or modified during the course of plant evolution to serve specific functions, for example, in defense or symbiosis.

Flavonoids, a subclass of the phenylpropanoids, are a well-characterized group of plant products that are found in most if not all plant species. Products synthesized in the early steps of the pathway, shown in Figure 1, are found in bryophytes and ferns, whereas more complex classes of flavonoids are produced by gymnosperms and angiosperms (Stafford, 1991). In the angiosperms, flavonoids are known to function in protecting plants from predators and infectious agents, in shielding plants from UV-B radiation, as signal molecules in plant-bacteria symbioses, such as nitrogen fixation, and as pigments that attract pollinators and seed dispersers (reviewed in Chapple et al., 1994; Koes et al., 1994).

Evidence for yet another function was recently uncovered in studies of genes encoding chalcone synthase (CHS), which catalyzes the first committed step in the flavonoid biosynthetic pathway (Figure 1). In maize, a mutation at one of the two CHS genes results in white pollen that is sterile in self-crosses (Coe et al., 1981). A similar phenotype is induced in petunia plants in which CHS activity is abolished either through cosuppression or by antisense expression in anthers (Taylor and Jorgensen, 1992; van der Meer et al., 1992; Ylstra et al., 1994). This infertile phenotype is complemented by the addition of flavonols (Figure 1), a specific subclass of flavonoids, to either the pollen, anthers, or stigmas of mutants (Mo et al., 1992; Ylstra et al., 1994). Flavonols also restore the ability of mutant pollen to germinate in vitro. Other classes of flavonoids, such as flavones and dihydroflavonols, do not exhibit this activity. These parallels between such evolutionarily distant plant species suggest that flavonols might play a ubiquitous role in male fertility among angiosperms.

Numerous flavonoid-deficient mutants have been identified for Arabidopsis, including several mutant alleles of the CHS locus, transparent testa 4 (tt4) (Shirley et al., 1995). Surprisingly, all of these plants appear to be fully self-fertile, suggesting either that the mutations are leaky or that flavonoids are not required for male fertility in this species. To distinguish between these possibilities, the tt4(2YY6) allele was characterized at the molecular level. This mutation, which consists of a G-to-A transition at the 3' splice acceptor site (Shirley et al., 1995), was found to disrupt splicing of all CHS transcripts derived from the tt4 locus in both seedlings and flowers. Additional experiments confirmed that CHS is encoded by a single gene in Arabidopsis. Flavonols were not detected in mutant flowers by either epifluorescence microscopy or HPLC. Plants homozygous for the tt4(2YY6) allele are thus completely devoid of flavonoids. This work demonstrates that these compounds are not universally required for male fertility in higher plants. The

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results of this study suggest either that flavonols were recruited into this function more than once during the course of plant evolution or that this function was lost in some plant lineages.

RESULTS

Phenotype of the tt4(2YY6) Allele

The 2YY6 allele of tt4 was identified on the basis of its yellow-seeded phenotype in an ethyl methanesulfonate–treated Arabidopsis (ecotype Columbia) population (J. Greenberg, personal communication). Plants carrying this allele are also devoid of detectable levels of anthocyanins (purple pigments) in vegetative tissues at all stages of development. However, the fertility of these plants does not appear to be affected by this mutation, despite reports that flavonols are essential for pollen germination in maize and petunia. Compared with wild-type plants, tt4(2YY6) homozygotes have a normal seed set with no evidence of aborted seeds within the siliques (data not shown). This raises the possibility that the mutation does not abolish flavonoid synthesis in reproductive tissues.

As a preliminary approach to determining whether flavonoids are synthesized in male floral organs of tt4(2YY6) plants, anthers shedding mature pollen were examined under UV light. As shown in Figure 2, pollen from mutants fluoresced bright blue, in stark contrast to the dark pollen grains of the wild-type plants. This is similar to the phenotype reported previously for anthers and seeds from other flavonoid-deficient Arabidopsis lines (Shirley et al., 1995) and indicates that pollen grains of tt4(2YY6) plants contain little or no UV-absorbing flavonoids. This deficiency is consistent with a defect in the first enzyme of flavonoid biosynthesis.

Analysis of CHS mRNA Levels in Seedlings and Floral Tissues

Sequence analysis has shown that the tt4(2YY6) allele contains a G-to-A transition at the 3' splice acceptor site of the single intron in the CHS gene (Shirley et al., 1995). As a first step in exploring how this mutation could account for the flavonoid-deficient phenotype of tt4(2YY6), total RNA was isolated from 3-day-old seedlings, a stage at which flavonoid genes are normally expressed at high levels (Kubasek et al., 1992).

Figure 2. Phenotypes of Wild-Type and tt4(2YY6) Anthers under UV Light.

Anthers containing mature pollen grains were examined by epifluorescence microscopy using a UV-1B filter, uncovering substantial differences in the appearance of pollen grains and anther surface in the two genotypes. wt, wild type. Bar = 50 μm.
Flavonoid-Independent Fertility in Arabidopsis

Figure 3. Flavonoid Gene Expression in Wild-Type and tt4(2YY6) Plants.
Blots containing 10 μg per lane of total RNA extracted from 3-day-old seedlings or flowers were hybridized with probes for CHS, CHI, and DFR. C, wild type (ecotype Columbia).

and from tissues representing the full range of floral development. Steady state levels of mRNA for CHS and for genes encoding chalcone isomerase (CHI), which catalyzes the second step of flavonoid biosynthesis, and dihydroflavonol reductase (DFR), which catalyzes the conversion of dihydroflavonols to flavan-3,4-diols (Figure 1), were compared by RNA gel blot analysis.

As shown in Figure 3, CHS mRNA levels were significantly reduced in tt4(2YY6) seedlings as compared with wild-type seedlings of the same age. In flowers, CHS mRNA levels were undetectable in the mutant, perhaps reflecting the overall lower levels of these transcripts in flowers as compared with seedlings, even in wild-type plants. Interestingly, two hybridizing bands were observed for CHS in wild-type and mutant seedlings and in wild-type flowers, possibly indicating the presence of spliced and unspliced populations of CHS mRNA (which differ in length by 85 bp) or transcripts with distinct polyadenylation sites. It is unlikely that these bands represent the products of two different genes because the 2YY6 mutation reduced or eliminated both bands in this experiment, and the same probe hybridized only with the known CHS gene in high-stringency genomic DNA blots (data not shown).

In seedlings, CHI and DFR mRNA levels were essentially identical in tissues from the wild type and mutants. This is similar to what was previously reported for the W85 allele of tt4, which produces wild-type levels of CHS mRNA (Shirley et al., 1995). However, in flowers, CHI mRNA was easily detectable in the wild type but was essentially undetectable in tt4(2YY6). This suggests that the absence of CHS transcripts in tt4(2YY6) in some way also affects CHI mRNA accumulation in these tissues. No effect was seen on DFR mRNA levels, which were low but essentially equivalent in both wild-type and tt4(2YY6) flowers. It is somewhat surprising that DFR is expressed in these tissues at all because wild-type Arabidopsis flowers do not contain colored anthocyanins. It is possible that the DFR mRNA detected in these samples represents expression in only a subset of floral tissues, such as sepals, or that other late flavonoid genes needed for anthocyanin production are not expressed in Arabidopsis petals.

Analysis of CHS Splicing in tt4(2YY6)

The G-to-A transition at the CHS 3′ intron acceptor site in tt4(2YY6) suggests that the flavonoid-deficient phenotype of these plants may be due to inefficient or aberrant splicing of CHS pre-mRNA. To explore this possibility, regions flanking the intron in CHS transcripts from mutant and wild-type seedlings were amplified by reverse transcriptase–polymerase chain reaction (RT-PCR) and resolved on a high-resolution agarose gel. As shown in Figures 4A and 4B, RNA from wild-type seedlings contained primarily products corresponding to the size of spliced CHS mRNA (769 bp), although a small amount of product corresponding to unspliced transcripts (855 bp) was also present. In contrast, significant amounts of product corresponding to both the spliced and unspliced forms of CHS mRNA were synthesized using RNA from tt4(2YY6) seedlings. The spliced form appeared to be similar or identical in size to that present in wild-type plants. This result indicates that the 2YY6 allele reduces the efficiency of CHS pre-mRNA splicing but does not abolish it.

The apparent absence of flavonoids in tt4(2YY6) plants suggests that in addition to reducing the efficiency of splicing, the G-to-A transition could also affect the accuracy of CHS pre-mRNA splicing. To investigate this possibility, RT-PCR products

Figure 4. RT-PCR Analysis of CHS mRNA Splicing in Wild-Type and tt4(2YY6) Plants.
(A) Diagram of the transcribed region of the CHS gene. The black region upstream of exon 1 represents the untranslated leader sequence. The arrows labeled 1 and 2 indicate the annealing positions of the sense and antisense primers used to perform RT-PCR. The single intron present in unspliced transcripts is represented by a line connecting exons 1 and 2.
(B) RT-PCR analysis of CHS transcripts in 3-day-old seedlings. Products amplified from total RNA by using CHS-specific primers were fractionated in a high-resolution agarose–cellulose gel and stained with ethidium bromide. The positions of products derived from spliced (769 bp) and unspliced (855 bp) CHS mRNA are indicated by arrows. Size markers are given at right in base pairs. C, wild type.
splicing at some low frequency, these events would still result in a null phenotype for chalcone synthase activity and flavonoid synthesis.

In contrast, a leaky phenotype would result if some fraction of CHS mRNA in tt4(2YY6) was spliced at the correct 3' position. A survey of introns in the Arabidopsis data base shows that, although 97% have a guanosine at the 3' splice site, 2% have an adenosine at this position (Shirley et al., 1995). Deviations from the AG consensus could simply reflect errors in sequencing or in the prediction of intron/exon boundaries. However, these findings also suggest that nucleotides other than guanosine are recognized as 3' acceptor sites in some cases. Therefore, although only improperly spliced CHS mRNA was...

Figure 5. Model for Splicing of CHS Transcripts in Wild-Type and tt4(2YY6) Plants Based on Sequence Analysis of RT-PCR Products. Exon sequences are in capital letters; intron sequences are in lowercase letters. Splicing occurs at the positions indicated by the arrows, with the resulting reading frames and deduced amino acid sequences shown underneath. The AG consensus at the 3' splice site in wild-type and tt4(2YY6) plants is underlined. (A) CHS mRNA splicing in wild-type plants. The cysteine codon generated at the splice junction is indicated. (B) CHS mRNA splicing in tt4(2YY6) plants. Splicing at the AG consensus produced by the G-to-A transition in the mutant deletes the first nucleotide of exon 2. This splice junction produces a serine codon.

corresponding to spliced CHS mRNA from wild-type and tt4(2YY6) seedlings were gel purified and subcloned into a plasmid vector. The results of sequence analysis of six clones from wild-type seedlings and 18 clones from tt4(2YY6) seedlings are summarized in Figure 5. All six clones derived from wild-type plants reflected splicing at the position previously predicted based on comparison with CHS cDNAs from other plant species (Figure 5A; Feinbaum and Ausubel, 1988). However, all 18 clones from tt4(2YY6) seedlings showed that splicing had occurred at the correct 5' splice site but was shifted by one nucleotide at the 3' position to the next AG consensus site (Figure 5B). Unspliced CHS mRNA contains an in-frame stop codon that terminates translation after 68 amino acids; the shift in the reading frame caused by aberrant splicing introduces a stop codon 12 amino acids downstream of the intron, terminating translation after a total of 76 residues. Both proteins would lack the C-terminal 332 amino acids of the normal protein, including the enzyme's active site.

The possibility remains that a very small proportion of CHS transcripts are spliced either at the correct position or at cryptic splice sites. These transcripts may not have been detected by either gel staining or sequence analysis of RT-PCR products due to their low abundance. Eight AG dinucleotides that occur before the CHS active site could serve as cryptic 3' splice acceptor sites. However, none of these sites provides the correct reading frame for the CHS protein. Therefore, although it is possible that the 2YY6 mutation causes cryptic downstream...

Figure 6. Screen for the Presence of Correctly Spliced CHS mRNA in tt4(2YY6) Seedlings and Flowers. (A) Expanded view of primer annealing sites at the CHS mRNA splice junction. CHS cDNA was synthesized from total RNA by using primer 2. Primers 3 and 4 anneal perfectly to cDNA produced from correctly spliced CHS mRNA (black boxes) but contain 3' terminal mismatches when annealed to cDNA from aberrantly spliced transcripts (open boxes). The primers also anneal perfectly to unspliced CHS mRNA (not shown). S, Sall; H, Hindlll; X, Xbal. (B) Fractionation of RT-PCR products amplified from wild-type (C) or mutant (tt4) plants in high-resolution agarose-cellulose gels. Positions of products produced by the two primer pairs, 2 and 3 and 1 and 4, from unspliced and correctly spliced templates are indicated by arrows. A 100-bp ladder, given at right in base pairs, and the products obtained when the two primer pairs were used with a genomic CHS clone (G) were included as size standards.
detected among the 18 RT-PCR clones from mutant seedlings, a small amount of correctly spliced CHS mRNA may exist in seedlings and in other tissues, including flowers.

To address these possibilities, a sensitive PCR-based assay was developed that takes advantage of the inability of Taq polymerase to extend primers containing 3' terminal mismatches with the template. Two primers (3 and 4) were designed that anneal perfectly to correctly spliced CHS cDNA, as shown in Figure 6A. These primers also anneal to unspliced CHS mRNA; however, both primers contain a 3' terminal mismatch (due to the deleted guanosine) when annealed to aberrantly spliced CHS cDNA from tt4(2YY6). Total RNA was isolated from wild-type and tt4(2YY6) seedlings and mixed floral tissues. This RNA was used to generate CHS cDNA by using primer 2, which anneals in the middle of the second exon (Figure 6A). The cDNA was then used as a template in PCR reactions with either primers 2 and 3 or primers 1 and 4. As a size standard for the unspliced form, parallel reactions were performed using a genomic clone for the Arabidopsis CHS gene (Feinbaum and Ausubel, 1988). The resulting products were analyzed on a high-resolution agarose-cellulose gel. As shown in Figure 6B, both unspliced and spliced forms of CHS mRNA were detected in tissues from wild-type plants, but only unspliced mRNA was detected in either vegetative or floral tissues of tt4(2YY6) plants. This indicates that all CHS mRNA in the mutants either remains unspliced or is spliced aberrantly and that no functional CHS enzyme is produced in these plants.

CHS Protein Levels in Wild-Type and tt4(2YY6) Plants

As further evidence for the absence of correctly spliced CHS transcripts in tt4(2YY6) plants, the levels of CHS protein were examined by immunoblot analysis. Crude protein extracts were prepared from 4-day-old seedlings, in which flavonoid enzymes have been found at high levels (C.C. Cain and B.W. Shirley, unpublished data), and from flowers. As shown in Figure 7, high levels of CHS protein were present in wild-type seedlings; significantly lower levels of CHS protein were present in flowers, consistent with the low CHS mRNA levels observed in these tissues (Figure 3). In contrast, no CHS protein was detected in tt4(2YY6) seedlings or flowers in the molecular weight range of the wild-type protein (Figure 7) or in the size range predicted for the product of aberrantly spliced CHS mRNA (data not shown). This result is consistent with the finding that tt4(2YY6) plants contain no correctly spliced CHS mRNA and provides further evidence that this mutation completely interferes with the synthesis of functional CHS enzyme.

CHS Gene Copy Number in Arabidopsis

The experiments described above provide compelling evidence that tt4(2YY6) is a null allele for CHS. Plants containing this allele do not synthesize detectable levels of CHS mRNA or pro-tein in seedlings or flowers, and flavonoids appear to be absent from all tissues, including anthers (Figure 2). However, the levels required to complement a male infertile phenotype may be extremely low and could be provided by a second, weakly expressed CHS gene. Feinbaum and Ausubel (1988) previously reported that CHS is encoded by a single gene in Arabidopsis, based on low-stringency DNA gel blot analysis. However, the probe used in these experiments consisted of a 3.9-kb genomic DNA fragment containing significant amounts of potentially nonconserved flanking sequence. To eliminate the possibility that these sequences interfered with efficient hybridization to other CHS genes, filters containing Arabidopsis genomic DNA digested with four different restriction enzymes were hybridized at low stringency with a probe containing only the 1.2-kb coding region of the cloned CHS gene. As shown in Figures 8A and 8B, strong hybridization was observed with bands corresponding to the known Arabidopsis CHS gene. However, additional faintly hybridizing bands were also observed, indicating that other sequences homologous to CHS exist in the Arabidopsis genome. These sequences appear to be confined to one locus, because they are represented by single bands in the HindIII and XbaI digestions. Furthermore, two hybridizing bands were observed in the BglII and EcoRI samples, indicating that this second locus contains recognition sites for these enzymes within the region spanned by the probe.

A PCR-based assay was developed to determine whether this locus encodes a second CHS enzyme. Four degenerate primers were designed based on amino acid sequences that are identical in 80 CHS proteins from 34 different plant species, including two gymnosperms. These oligonucleotides should therefore amplify all CHS sequences present in the Arabidopsis genome. Figure 9A illustrates the annealing positions of these primers in the known CHS gene present at the TT4 locus. This gene contains EcoRI and HindIII restriction sites between primers 5 and 6; an XbaI restriction site is present between primers 7 and 8. Digestion of genomic DNA with these enzymes should specifically prevent amplification of sequences at the TT4 locus. In contrast, the second locus
These smaller products apparently resulted from internal priming at the low temperatures used in this assay, because similar products were produced from the CHS genomic clone (Figure 9B, lane P). Digestion of the genomic DNA template with EcoRI or HindIII abolished amplification of all CHS-homologous sequences when primers 5 and 6 were used. Likewise, digestion with XbaI abolished the synthesis of products using primers 7 and 8. Digestion of genomic DNA with BglII had no effect on the amplification of CHS-homologous products with either set of primers. These results are consistent with the locations of these restriction sites in the TT4 locus and with the presence of a single CHS gene in Arabidopsis.

Figure 9. PCR Amplification of CHS-Homologous Sequences from Intact and Restriction Enzyme-Digested Genomic DNA.

(A) Restriction enzyme recognition sites and annealing positions of degenerate oligonucleotides at the TT4 locus. In the expanded view, the striped box represents the untranslated leader sequence, and the black box indicates the single intron. Arrows indicate the annealing positions of primers 5 through 8. B, BglII; E, EcoRI; H, HindIII; S, Sall; X, XbaI.

(B) DNA gel blot analysis of PCR products. Two different pairs of degenerate primers were used to amplify CHS-homologous sequences from genomic DNA that was intact (G) or digested with various restriction enzymes, as given in Figure 8A. Control reactions containing either a plasmid clone (P) or no template (N) were performed at the same time. Products were fractionated in a high-resolution agarose–cellulose gel and then analyzed by low-stringency gel blot analysis, as described in Figure 8. The positions of the size markers are given at left in base pairs.
The second locus does not appear to contain recognition sites for HindIII or XbaI. Therefore, if CHS-related sequences were present at this locus, products would have been amplified from the HindIII-digested template using primers 5 and 6 and the XbaI-digested template using primers 7 and 8. This experiment therefore confirms that Arabidopsis contains a single CHS gene, although a second, faintly hybridizing locus is present. It should also be noted that the six Arabidopsis expressed sequence tags that exhibit homology to CHS are identical to the known gene, providing additional evidence that CHS is encoded by a single gene in this species.

### HPLC Analysis of Flavonol Accumulation in Wild-Type and tt4(2YY6) Plants

As final confirmation of the effects of the mutation on flavonoid biosynthesis, HPLC analysis was used to examine the levels of flavonols in flowers and pollen-bearing stamens of wild-type and tt4(2YY6) plants. Flavonols were extracted in 80% methanol and analyzed directly (flavonol glucosides) or hydrolyzed to the aglycone form before characterization. Representative profiles of these samples, as well as authentic standards for the flavonols myricetin, quercetin, and kaempferol, are shown in Figure 10.

Seven major peaks were resolved in the nonhydrolyzed samples from wild-type flowers (Figure 10A), similar to what was previously reported by Mittal et al. (1995): Two additional compounds represented by minor peaks in these extracts appear to be present at much higher levels in nonhydrolyzed extracts from stamens (Figure 10E). The majority of these compounds shifted to the positions of the quercetin and kaempferol standards upon hydrolysis (Figures 10C, 10G, 10I, and 10J). A peak that appears to correspond to myricetin was also resolved in wild-type tissues, in contrast to previous analyses of flavonoids in Arabidopsis (Mittal et al., 1995; Shirley et al., 1995), as was an as-yet-unidentified peak (Figures 10C and 10G, peak 10). The profiles for the hydrolyzed and nonhydrolyzed extracts from pollen-bearing stamens differed significantly from those of whole flowers, although in both cases significant levels of flavonoids were present.

In contrast, flavonoids were not detected in tt4(2YY6) flowers or stamens (Figures 10B, 10D, 10F, and 10H). The flavonoid glucosides and aglycones present in wild-type tissues, including those corresponding to the three flavonol standards, were undetectable above baseline levels in the mutants. Flavonoids were also absent in HPLC profiles of tt4(2YY6) seedlings, despite the high levels observed in the wild type (data not shown). We therefore conclude that the tt4(2YY6) mutation abolishes the synthesis of flavonoids, including flavonols, in Arabidopsis. Lack of these compounds in flowers and particularly in stamens demonstrates that these compounds do not play a universal role in plant reproduction.

### DISCUSSION

#### A Point Mutation at the 3' Intron/Exon Boundary Affects the Processing and Accumulation of CHS Transcripts

The tt4 locus contains the Arabidopsis gene encoding CHS, which catalyzes the first committed step in flavonoid biosynthesis. An ethyl methanesulfonate-generated allele for this locus, termed 2YY6, was previously found to contain a G-to-A transition in the AG consensus of the 3' splice acceptor site (Shirley et al., 1995). Plants homozygous for this allele produce yellow seeds, lack reddish purple anthocyanins during germination and senescence, and exhibit altered fluorescence of floral tissues under UV light. This indicates that a single point mutation can abolish all flavonoid synthesis in Arabidopsis. Identical genetic lesions have been correlated with deficiencies in the expression of structural and regulatory genes in a variety of organisms. Indeed, some 15% of human genetic diseases have been attributed to G-to-A transitions at 3' splice sites (Krawczak et al., 1992). The AG dinucleotide at the 3' splice site is a highly conserved feature of animal, fungal, and plant intron/exon boundaries and is associated with the binding of U5 small nuclear ribonucleoproteins during mRNA processing (Brown, 1986; Green, 1986). These sequences thus appear to be part of a fundamental mechanism of gene expression that has a low tolerance for mutation.

Although specific phenotypes have been correlated with G-to-A transitions at 3' splice sites in several systems, the molecular effects of such mutations have not been determined in most cases. In Citrullinemia, retinoblastoma, and Fabry disease, alteration of the G residue at the -1 position has been shown to affect recognition of authentic downstream 3' acceptor sites, causing exon skipping or the use of cryptic splice sites and usually resulting in premature translation termination (Dunn et al., 1989; Su and Lin, 1990; Okumiya et al., 1995). A few examples of this type of mutation have also been identified in plants. Three alleles of the Arabidopsis floral homeotic genes AGAMOUS (AG) and APETALA1 (AP1), namely, ag-1, ag-4, and ap1-1 (Yanofsky et al., 1990; Mandel et al., 1992; Sieburth et al., 1995), and an allele of shoot meristemless, which is a homeobox-containing gene required for meristem formation during embryogenesis (Long et al., 1996), all contain G-to-A transitions at a 3' splice site. The specific effects of these mutations on splicing have only been described for ag-4. The G-to-A transition at the 3' splice site of the fifth intron causes deletion of most or all of exon 6 due to splicing at a cryptic splice site or at the next 3' splice acceptor site (Sieburth et al., 1995).

Analysis of the tt4(2YY6) allele has provided new insights into how such lesions affect gene expression. The mutation in this allele was found to have three distinct effects on the expression of CHS. First, this mutation changes the 3' splice junction in all CHS transcripts. Correctly spliced CHS mRNA...
Figure 10. HPLC Analysis of Flavonol Glucosides and Aglycones in Reproductive Tissues.
was completely absent in these plants, demonstrating that AA sequences cannot be used as 3' splice acceptor sites within the context of the CHS gene. There was also no evidence for the use of cryptic downstream splice sites in tt4(2YY6) plants. This suggests that AG dinucleotides present in the second exon are not in an appropriate context, for example, following AU islands (Lou et al., 1993), for use as alternative acceptor sites. Instead, only one aberrantly spliced CHS mRNA species was identified in seedlings and in flowers. In these tissues, the 3' splice site was shifted by one nucleotide due to a G-to-A transition at the -1 position. This transcript encodes a severely truncated open reading frame.

Second, the mutation may reduce the overall efficiency of CHS mRNA splicing. Although the RT-PCR reactions performed in this study were not quantitative, RNA from the mutant appeared to contain a substantial amount of unspliced CHS mRNA compared with that of the wild type. Sequences spanning the 3' splice site in the wild type are an exact match to the plant consensus (CAG/G) (Brown, 1986), whereas those in the mutant (AAG/C) deviate significantly. In the context of upstream AU-rich elements (Lou et al., 1993), these sequences mediate binding of U5 small nuclear ribonucleoproteins and determine the 3' intron/exon boundary. Deviation from the consensus would be expected to interfere with one or both of these events. Because there are no other introns present in the Arabidopsis CHS gene, the mutant 3' splice site may offer the best match to the consensus. However, splicing at this site is likely to occur at a reduced efficiency with respect to wild-type transcripts. This would account for the higher proportion of unspliced CHS mRNA observed in the tt4(2YY6) plants.

A third effect of the tt4(2YY6) mutation is an overall reduction in the abundance of CHS mRNA. This effect appears to be more pronounced in flowers than in seedlings. The CHS promoter has not been sequenced, and transcription rates have not been assayed in the mutant; therefore, it is formally possible that tt4(2YY6) contains a second mutation in the promoter that interferes with transcription. A more likely explanation is that CHS transcripts are less stable in tt4(2YY6) than in wild-type plants. One possibility is that unspliced CHS mRNA is unstable. In fact, targeted degradation of unspliced mRNA has been described in yeast and mammalian systems (Malim and Cullen, 1993; Li et al., 1995). A decrease in the rate of processing of pre-mRNA to more stable spliced transcripts could thus account for the overall reduction in mRNA levels observed in tt4(2YY6). On the other hand, accelerated decay of transcripts containing premature translation terminators due to frameshift or nonsense mutations has been described for prokaryotes and eukaryotes, and several genes involved in the degradation process have been identified (for example, Lee and Culbertson, 1995). Both unspliced and improperly spliced CHS transcripts contain premature stop codons, leaving 1067 or 956 untranslated nucleotides, respectively, before the normal translation termination site. These untranslabeled sequences could render the transcripts vulnerable to degradation via this so-called nonsense-mediated mRNA decay pathway.

**Flavonoids Are Not Universally Required for Male Fertility**

We showed that the mutation at the 3' splice acceptor site in tt4(2YY6) abolishes the synthesis of functional CHS enzyme. Plants homozygous for the 2YY6 allele are therefore incapable of catalyzing the condensation of 4-coumaroyl-coenzyme A with malonyl-coenzyme A, the first committed step in the flavonoid biosynthetic pathway. Consistent with this, flavonoids appear to be completely absent from all tissues of tt4(2YY6) plants. These plants therefore provide a valuable genetic background for identifying specific physiological roles for these multifunctional secondary metabolites.

In maize and petunia, flavonoids are required for pollen germination, and the loss of CHS activity results in a male-sterile phenotype. Pollen produced by a CHS-deficient maize line appeared to be viable and metabolically active, but only a small percentage of grains germinated in vitro, and the pollen tubes reached only a fraction of the wild-type length (Taylor and Jorgensen, 1992). Pollen from petunia plants expressing a CHS antisense construct either failed to germinate in vitro (van der

**Figure 10.** (continued).

Extracts from whole flowers ([A] to [D]) and pollen-bearing stamens ([E] to [H]) were fractionated either before or after hydrolysis. Arrows indicate the positions at which the three known flavonoids should migrate in the plant extracts. 1 to 9, flavonoid glucosides; 10, unidentified flavonoid aglycone; M, myricetin; Q, quercetin; K, kaempferol.

(A) Extract from wild-type flowers.
(B) tt4(2YY6) flowers.
(C) Wild-type flowers after hydrolysis.
(D) tt4(2YY6) flowers after hydrolysis.
(E) Extract from wild-type stamens.
(F) tt4(2YY6) stamens.
(G) Wild-type stamens after hydrolysis.
(H) tt4(2YY6) stamens after hydrolysis.
(I) and (J) Authentic standards.
flavonols interferes with the ability of pollen to penetrate even
the style. Furthermore, parsley and Antirrhinum also appear
to have single-copy CHS genes (Sommer and Saedler, 1986;
Ylstra et al., 1994; Ylstra, 1995). However, in petunia, the absence of
flavonols play a structural role in the membranes of rapidly
growing pollen tubes and that these may be dispensible for
normal germination in in vitro assays was restored in both species
by the addition of flavonols but not by other classes of flavo-
oids. Fertility was also partially restored in crosses with wild-type plants or when pollination occurred in the presence of "mentor" pollen from other species, suggesting that diffusion
of flavonols from other tissues could complement the
deficiency. Fertility was completely restored when flavonoid-
deficient pollen was mixed with flavonols before crossing.

Although it is clear that pollen germination in maize and petu-
nia requires the presence of flavonols, the mechanism
underlying this phenomenon has not yet been elucidated. Dur-
ing self-pollination of antisense petunia plants, only a small
number of tubes penetrate the style and none reach the ovule
(Ylstra et al., 1994), suggesting that flavonols are essential for
early events in reproduction. However, petunia is character-
ized by binucleate pollen, "wet" stigmas that secrete a mucous
covering, and either self-compatibility or gametophytic self-
incompatibility. In contrast, both Arabidopsis and maize have
more metabolically developed trinucleate pollen, have "dry"
stigmas, and are self-fertile (Heslop-Harrison and Shivanna,
1977). Thus, a simple correlation between pollen--pistil inter-
actions at the stigma surface and a requirement for flavonols
is not apparent. There are also no obvious morphological differ-
ences in the transmitting tracts through which the pollen tubes
grow in the three species, except that the distance travelled
by the pollen tubes is much shorter in Arabidopsis (Konar and
Linskens, 1986; Heslop-Harrison et al., 1985; Elleman et al.,
1992; Kandasamy et al., 1994). It has been hypothesized that
flavonols play a structural role in the membranes of rapidly
growing pollen tubes and that these may be dispensible for
pollen germinating within a short transmitting tract (Ylstra et
al., 1994; Ylstra, 1995). However, in petunia, the absence of
flavonols interferes with the ability of pollen to penetrate even
the style. Furthermore, parsley and Antirrhinum also appear
to have single-copy CHS genes (Sommer and Saedler, 1986;
Herrmann et al., 1988), and transposon-induced mutations in
these genes have not been reported to affect fertility.

These observations suggest that flavonols are simply not
involved in the fertilization process in some plant species or
that this function can be complemented by other compounds.
It is also possible, as originally suggested by van der Meer
et al. (1992), that flavonols play an indirect role in fertility. Sev-
eral examples of cross-talk between--branch pathways of
phenylpropanoid metabolism (Loake et al., 1991; Li et al., 1993)
or the shikimate pathway (Yao et al., 1995) have been reported.
In maize and petunia, the absence of flavonols could affect
the accumulation of other compounds specifically required for
male fertility. Thus, the apparent differential requirement for
flavonols among plant species could actually reflect differences
in the integration of metabolic pathways.

Nevertheless, most plants, including Arabidopsis (Shirley
et al., 1995; Ylstra, 1995), accumulate large quantities of
flavonols in pollen grains (Wiermann and Vieth, 1983), sug-
gesting that these compounds are of fundamental importance
to the male gametophyte. One of the most interesting features
of secondary metabolites, including flavonoids, is that these
compounds are often multifunctional. It has been suggested
that flavonoids evolved first as low-abundance signaling mol-
ecules that were subsequently recruited into other functions
(Stafford, 1991). In fact, the flavonol glycosides that mediate
petunia fertility (Vogt and Taylor, 1995) were previously identi-
fied as potent inducers of the Agrobacterium vir genes (Zerback
et al., 1989). However, functions that appeared early in plant
evolution should be shared among many plant species. This
does not appear to be the case for the role of flavonoids in
reproduction. Recent studies have shown that flavonoids can
protect DNA from UV-induced cross-linking, which interferes
with replication and transcription (Kootstra, 1994; Stapleton
and Walbot, 1994). Thus, it is possible that flavonols function
principally to shield the haploid genome from UV radiation.
Such a function is also consistent with the high concentrations
of flavonols present in pollen grains.

Whether differences in the requirement for flavonols in pol-
len germination represent a case of convergent functional
evolution in maize and petunia or a loss of function in specific
plant lineages remains an open question. However, these
species-specific differences underscore the complexity of sec-
ondary metabolism and the fundamental processes mediated
by its end products. A comprehensive understanding of the
myriad functions of plant flavonoids will require studies of a
much wider representation of species. Plants carrying null mu-
tations in flavonoid genes will provide valuable tools for these
endeavors.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia and a transparent testa4 (tt4)
mutant, designated allele 2Y6, were used in these studies. Approxi-
mately 1000 (25 mg) wild-type or tt4(2Y6) seeds were surface sterilized
for 1 min in ethanol and 5 min in 50% bleach--0.05% Tween-20 and
then washed three times with sterile water (Bent et al., 1994). The seeds
were plated on Murashige and Skoog--sucrose medium (Kubasek
et al., 1992) and then stratified for 4 days in darkness at 4°C before ger-
mination under constant light at 22°C. Mature plants were grown in
soil at 22°C under a 16-hr-light/8-hr-dark cycle. Seedlings, 3-week-old
plants, or flower buds at various stages of development were harvested
into liquid nitrogen and stored at --70°C.

Microscopy

A Nikon (Tokyo, Japan) SA microscope equipped for epifluorescence
(100 W Hg lamp, UV-lB filter cube) was used to image anther prepara-
tions. A Nikon x100,3 NA Plan Fluor objective and a 3.2 projection
lens were used to photograph samples with a Nikon N6006 camera

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lens were used to photograph samples with a Nikon N6006 camera
DNA and RNA Isolation and Blot Analysis

Genomic DNA and total RNA were prepared using the methods of Watson and Thompson (1986) and Simon et al. (1992), respectively. DNA and RNA gel blots were prepared on nylon filters, as described previously (Shirley et al., 1992), or by using downward capillary transfer. Probes were synthesized from cDNA clones (Shirley et al., 1995) by polymerase chain reaction (PCR), using either T3 and T7 (chalcone isomerase [CHI] and dihydrolavonol reductase [DFR]) or specific (chalcone synthase [CHS]) primers. Digoxigenin-based probes were synthesized in 100-µL reactions with 2 µL of digoxigenin DNA labeling mix (Boehringer Mannheim) and detected according to the manufacturer's protocol with either Lumi-Phos 530 (Boehringer Mannheim) or CDP-Star (Tropix, Bedford, MA). Radioactive probes were synthesized in 50-µL reactions containing 50 µCi of α-32P-dATP (Du Pont). Probe purification, probe denaturation, and hybridization and washing of blots were conducted as described previously (Shirley et al., 1992).

Reverse Transcriptase-PCR Amplification and Sequence Analysis of CHS mRNA

CHS mRNA was amplified from total RNA by using CHS-specific primers 1 and 2 by reverse transcriptase (RT)–PCR (Shirley and Hwang, 1995). The sequences of these primers are shown in Table 1. Products corresponding to spliced CHS mRNA from fgr(2YY6) and wild-type plants were isolated from a 1% low-melting agarose (International Biotechnologies Inc., New Haven, CT)/TAE (0.04 M Tris-acetate, 0.001 M EDTA) gel, purified on Wizard PCR Prep minicolumns (Promega), digested with HindIII and PstI, and then subcloned into pHBluescript KS+. Plasmid DNA for sequence analysis was isolated using a modification of the alkaline lysis miniprep (Birnboim and Doly, 1979) in which the chromosomal precipitate was pelleted at 50,000 rpm in an ultracentrifuge (model TLA-100; Beckman, Palo Alto, CA). Sequencing was performed as described previously (Shirley et al., 1992).

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotides Used as PCR Primers</th>
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<tr>
<td><strong>Oligonucleotide Sequence</strong></td>
</tr>
<tr>
<td>1. CCAATACACCTAACTTGTG</td>
</tr>
<tr>
<td>2. TCCGACCCCAACAAATG</td>
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<tr>
<td>3. GAAGTTCAAGCGCATGTC</td>
</tr>
<tr>
<td>4. GAGTGTGCAGCTGGTTGC</td>
</tr>
<tr>
<td>5. TA(C/T)(G/C)NGA(C/T)T(A/T)(C/T)TA(C/T)T(T/C)/</td>
</tr>
<tr>
<td>7. CCGGAATTGGGAGCAGNCAGTN(C/T)TGA(A/G)(G)G</td>
</tr>
<tr>
<td>8. CCCGAGATTGTCAGCNGGCC(A/G)AANAC</td>
</tr>
<tr>
<td><em>Sequences are given 5' to 3'. A/T/G/C.</em></td>
</tr>
</tbody>
</table>

Screen for Correctly Spliced CHS mRNA

CHS cDNA was synthesized from 10 µg of total RNA by reverse transcription by using primer 2 (Table 1). This cDNA was used as the template in a PCR screen for the presence of correctly spliced CHS mRNA. Reactions containing 0.5 µM each of primers 1 and 4 or primers 2 and 3 (Table 1) were performed using an annealing temperature of 54°C. Control reactions containing a single primer were performed under identical conditions.

PCR Amplification of Sequences Homologous with CHS

PCR was performed using 5 ng of intact or restriction enzyme-digested genomic DNA and 5 µg of each degenerate primer in 100-µL reaction volumes. An annealing temperature of 46°C was used for primers 5 and 6 (Table 1); 50°C was used for primers 7 and 8 (Table 1). Products were fractionated in high-resolution agarose/cellulose (2% Synergel; Diversified Biotech, Boston, MA)/Tris-borate-EDTA gels. A 100-bp ladder (GIBCO) was used as a size standard.

Protein Extraction and Immunoblot Analysis

Proteins were extracted as described previously (Shirley and Meagher, 1990), except that 1 mL of buffer was used for each 1 g of flower tissue and for each 2 g of seedling tissue. Samples were fractionated on 10% SDS–polyacrylamide gels. Equal volumes of these samples contained approximately equal amounts of protein, as determined by Coomassie Brilliant Blue R 250 staining of the gels. Immunoblots were prepared on nitrocellulose membranes (Bio-Rad) and were processed using 5% Blotto (Johnson et al., 1984) in PBST, a 1:500 dilution of anti-CHS antiserum, and a 1:10,000 dilution of rabbit anti–chicken peroxidase conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA). Detection was performed using the ECL chemiluminescence system (Amersham), according to the manufacturer's recommendations.

HPLC Analysis of Flavonoids

HPLC analysis was performed by using a modification of the method of Graham (1991). Chromatography was performed using a chromatography station equipped with a dual-pump system (model 510; Waters, Bedford, MA), universal injector (model U6K; Waters), automated gradient controller (model 680; Waters), photodiode array detector (model 484; Waters), and a data module (model 745B; Waters). Samples were fractionated at 25°C by using a C18 reverse phase column (Nova-Pak 60 Å, 4 µm, 3.9 x 75 mm) at a flow rate of 2.5 mL/min, starting with HPLC-grade water (pH 3.0 adjusted with glacial acetic acid), with an increasing concentration of HPLC-grade acetonitrile (a linear gradient of 0 to 10% for 2 min, a linear gradient of 10 to 50% for 25 min, then a 2-min linear step to 100% acetonitrile, which was maintained for 2 min), and then a 2-min linear step back to 0% acetonitrile. Elution was monitored at 255 nm, based on absorbance maxima of authentic standards (Sigma), although similar profiles were obtained at 236 and 330 nm. Attenuation settings were 1024 for floral extracts, 64 for stamen extracts, and 512 for the standard.

Extracts were prepared by suspending 30 flowers (at a similar developmental stage to those from which stamens were removed) or stamens (bearing mature pollen) from 10 flowers in 250 or 100 µL,
respectively, of 80% (v/v) methanol. Samples were ground to a fine suspension, and the extracts were clarified by centrifugation at 14,000g for 5 min. Fifty microliters of the floral samples or 23 µL of the stamen samples was injected. Flavonol aglycones were prepared by mixing 100 or 50 µL of the floral or stamen extracts, respectively, with an equal volume of 2 N HCl. The samples were heated at 70°C for 40 min, and then 100 or 30 µL of 100% methanol was added to the hydrolyzed floral or stamen extract, respectively, to prevent precipitation of flavonol aglycones. Samples were then centrifuged at 14,000g for 5 min, and 150 µL of the hydrolyzed floral extract or 70 µL of the hydrolyzed stamen extract was injected. Retention times were compared with those of authentic standards for myricetin, quercetin, and kaempferol (Sigma) prepared at a concentration of 0.1 mg of each compound per mL in 80% (v/v) methanol, of which 40 µL was injected.

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REFERENCES


A null mutation in the first enzyme of flavonoid biosynthesis does not affect male fertility in Arabidopsis.
I E Burbulis, M Iacobucci and B W Shirley
Plant Cell 1996;8;1013-1025
DOI 10.1105/tpc.8.6.1013

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