The inheritance of flower color in pea (Pisum sativum) has been studied for more than a century, but many of the genes corresponding to these classical loci remain unidentified. Anthocyanins are the main flower pigments in pea. These are generated via the flavonoid biosynthetic pathway, which has been studied in detail and is well conserved among higher plants. A previous proposal that the Claroïdes (B) gene of pea controls hydroxylation at the 5′ position of the B ring of flavonoid precursors of the anthocyanins suggested to us that the gene encoding flavonoid 3′,5′-hydroxylase (F3′5′H), the enzyme that hydroxylates the 5′ position of the B ring, was a good candidate for B. In order to test this hypothesis, we examined mutants generated by fast neutron bombardment. We found allelic pink-flowered b mutant lines that carried a variety of lesions in an F3′5′H gene, including complete gene deletions. The b mutants lacked glycosylated delphinidin and petunidin, the major pigments present in the progenitor purple-flowered wild-type pea. These results, combined with the finding that the F3′5′H gene cosegregates with b in a genetic mapping population, strongly support our hypothesis that the B gene of pea corresponds to a F3′5′H gene. The molecular characterization of genes involved in pigmentation in pea provides valuable anchor markers for comparative legume genomics and will help to identify differences in anthocyanin biosynthesis that lead to variation in pigmentation among legume species.

Flavonoids are a large class of polyphenolic secondary metabolites that are involved in pigmentation, defense, fertility, and signaling in plants (Grotewold, 2006). Their basic skeleton consists of two six-carbon aromatic rings, A and B, connected by ring C, a three-carbon oxygenated heterocycle. Flavonoids are divided into different subclasses according to the oxidation state of the C ring, and compounds within each subclass are characterized by modifications such as hydroxylation, methylation, glycosylation, and acylation. Anthocyanins, for example, the major water-soluble pigments in flowers, have a fully unsaturated C ring and are usually glycosylated at position 3. Two important determinants of flower color are the cytochrome P450 enzymes flavonoid 3′-hydroxylase (F3′H; EC 1.14.13.21) and flavonoid 3′,5′-hydroxylase (F3′5′H; EC 1.14.13.88). These hydroxylate the B ring of the anthocyanin precursor molecules naringenin and dihydrokaempferol, generating substrates for the production of cyanidin-3-glucoside and delphinidin-3-glucoside, which can be seen in a variety of pigmented flowers (Grotewold, 2006).

The study of genetic loci regulating floral pigmentation has a long history, beginning with crosses made between white- and purple-flowered varieties of garden pea (Pisum sativum; Knight, 1799; Mendel, 1866). Later crosses made between white-flowered P. sativum and rose-pink-flowered Pisum arvense defined two factors conferring flower color as A and B, respectively (Tschermak, 1911). The white flowers of pea anthocyanin-inhibition (a) mutants lack anthocyanins and flavones (Statham et al., 1972), in accordance with the role of A as a fundamental factor for pigmentation (Tschermak, 1911; De Haan, 1930). Another locus in pea, a2, similarly confers a white-flowered phenotype lacking anthocyanins and other flavonoid compounds (Marx et al., 1989). It was shown that A and A2 regulate the expression of genes encoding flavonoid biosynthetic enzymes (Harker et al., 1990; Uimari and Strommer, 1998), and recently they were identified as a basic helix-loop-helix (bHLH) transcription factor and a WD40 repeat protein, respectively (Hellens et al., 2010). They are likely to be components of the Myb-bHLH-WD40 transcription factor complex that regulates flavonoid biosynthesis in all plant species studied so far (Koes et al., 2005; Ramsay and
Glover, 2005). The gene encoding the Myb component of this complex in pea, as well as genes at other loci involved in pigment production, such as Claro roseus (B), Roseus (Ce), and Fuscompurpureus (Cr; Statham et al., 1972), remain to be identified.

The major anthocyanins found in wild-type pea lines that contribute to their purple flower color are delphinidin-3-O-glucoside and malvidin-3-O-glucoside (Statham et al., 1972). Rose-pink b mutants (Blixt, 1972) produce a different range of anthocyanins (pelargonidin-, cyanidin-, and peonidin-3-O-glucoside), suggesting that the B gene controls hydroxylation of the anthocyanin B ring (Statham et al., 1972) and encodes a hydroxylase. Pink-flowered mutants identified in species that are typically purple flowered, such as Petunia × hybrida (Snowden and Napoli, 1998; Matsubara et al., 2005) and Gentiana scabra (Nakatsuka et al., 2006), were found to have resulted from the insertion of transposable elements into the gene encoding F3H. If anthocyanin biosynthesis in pea were to conform to the enzymatic steps elucidated in other plant species (Grotewold, 2006), then the activity missing in b mutants would be predicted to correspond to that of a F3’5’H.

In soybean (Glycine max), however, the wp locus, which conditions a change in flower color from purple to pink (Stephens and Nickell, 1992), was reported to encode a flavanone 3-hydroxylase (F3H; EC 1.14.11.9; Zabala and Vodkin, 2005). Furthermore, an insertion/deletion mutation in a gene encoding a F3’5’H was associated with the white-flowered phenotype of the soybean w1 mutant (Zabala and Vodkin, 2007). These results suggested that anthocyanin biosynthesis in legumes, or at least in soybean, may differ from that in other plant species studied, where F3’5’H mutations result in pink flowers (Snowden and Napoli, 1998; Matsubara et al., 2005; Nakatsuka et al., 2006) and F3H mutations result in white flowers (Martin et al., 1991; Britsch et al., 1992). More recently, a Glycine soja accession carrying a w1-lp allele was described as having pale pink petal petals and a flower color designated as light purple (Takahashi et al., 2010). Our analysis here of the b mutant of pea, which is also a legume, addresses the complexity of these findings in soybean.

Transposon-tagged mutations have facilitated the isolation of genes involved in anthocyanin biosynthesis in numerous plant species, and transposon tagging is a useful technology for gene identification that remains particularly relevant for species without sequenced genomes, such as pea. Endogenous retrotransposons and DNA transposons have been identified in pea, but the transposition rate of those studied to date has been too low to be exploited for gene tagging (Shirsat, 1988; Vershinin et al., 2003; Macas et al., 2007). The identification of active DNA transposons usually occurs when sectors are found on pigmented flowers or seeds. Because most cultivated pea crop varieties have white flowers, any chance identification of sectored flowers in the field is extremely limited. A secondary purpose of this study was to carry out a screen for sectors on purple-flowered peas with the aim of identifying an active transposon.

We generated pink-flowered fast neutron (FN) deletion mutants and used these to identify the gene corresponding to B. Among the pigmentation mutants we obtained were several new b alleles, including pink-sectored mutants, which we characterized further. Stable pink b mutants were shown to carry a variety of lesions in an F3’5’H gene, including complete gene deletions. Analysis of one of these deletion lines showed that it lacked delphinidin and petunidin, the major anthocyanins of the progenitor wild-type pea variety. These results, combined with the finding that the F3’5’H gene cosegregates with b in a genetic mapping population, strongly support our hypothesis that the pea gene b corresponds to a F3’5’H.

RESULTS

Generation of New b Mutant Alleles

We used FN mutagenesis to generate pigmentation mutants in line JI 2822, which is wild type at the flower color loci A, A2, Albicans (Am), B, Ce, and Cr. The fully open petals of JI 2822 flowers are nonuniformly pigmented (Fig. 1A); the adaxial standard petal is pale purple, the two wing petals are dark purple, and the two fused abaxial keel petals are very lightly pigmented. The standard and wing petals fade to a blue purple. The JI 2822 flower is described here as purple to conform with previous naming conventions (De Haan, 1930).

M2 and M3 progeny from the mutagenized population were screened for flower color variants that differed from the wild type. Six FN lines were identified with pale pink standards, rose-pink wing petals, and lightly pigmented keel petals (Fig. 1B). Backcrosses to JI 2822 showed that four of these lines, FN 1076/6, FN 2160/1, FN 2255/1, and FN 2438/2, carried stable recessive mutations that determined the pink flower trait. These lines yielded rose-pink F1 progeny when crossed to the b mutant type line, JI 118, confirming that they carried allelic mutations. Two further lines, FN 2271/3/pink and FN 3398/2164, were stable rose-pink and allelic to b; however, sibling individuals carried flowers with pink sectors on a purple background (Fig. 1C), suggesting they were unstable at the b locus.

The b mutation is also known to confer paler stem axil pigmentation than the wild type and paler pod color in genotypes carrying the purple-podded Pur allele (De Haan, 1930; Statham et al., 1972). All six FN b alleles likewise differed from JI 2822 in having paler axillary rings. No effect on pod color was observed in the FN alleles, because JI 2822 is a green-podded genotype (pur). The FN b mutants are described here as rose pink to incorporate previous conventions (Tschermak, 1911; De Haan, 1930) yet distinguish them from cerise-pink ce and crimson-pink cr mutants.
The b Mutant Lacks Delphinidin and Petunidin

Methanol-HCl extracts of anthocyanins from the wing petals of line JI 2822 and a stable pink M3 plant, FN 2271/3/pink, were analyzed using liquid chromatography (LC) coupled with mass spectrometry (MS). Chromatograms with two major peaks showed that JI 2822 contained two major anthocyanins (Fig. 2A; 611 and 625 atomic mass units [amu]). MS data averaged across the peaks indicated that these were anthocyanins isomeric to delphinidin and petunidin glycosylated with deoxyhexose and hexose sugars (Supplemental Fig. S1). Fragmentation of the sugars as mass losses of 146 and 162 amu were consistent with Rha and Glc, respectively. Fragmentation consistent with the loss of both monosaccharide moieties individually was observed, which suggested that the anthocyanidins delphinidin (303 amu) and petunidin (317 amu) were monoglycosylated at two different positions (Supplemental Fig. S1). These results agree with earlier studies that identified delphinidin-3-rhamnoside-5-glucoside and petunidin-3-rhamnoside-5-glucoside among the anthocyanins present in wild-type pea (Statham et al., 1972).

The peaks indicating glycosylated delphinidin and petunidin were absent from FN 2271/3/pink samples (Fig. 2B). A range of ions consistent with glycosylated cyanidin and peonidin were present in FN 2271/3/pink and absent from JI 2822 (Fig. 2, C and D). These were isomeric to cyanidin glycosylated with deoxyhexose and hexose sugars (595 amu), peonidin glycosylated with deoxyhexose and hexose sugars (609 amu), and cyanidin glycosylated with a pentose and two hexose sugars (743 amu; Fig. 2C). Fragmentation of the sugars attached to cyanidin (287 amu) as mass losses of 162, 294, and 456 amu was consistent with a pentose moiety buried beneath a Glc moiety (Supplemental Fig. S1). No single loss of 132 amu, expected of an exposed pentose, was observed. These results confirmed earlier studies that identified cyanidin-3-sambubioside-5-glucoside among the anthocyanins present in b mutants (Statham et al., 1972). Fragmentation of the sugars attached to cyanidin and peonidin (301 amu) as mass losses of 146 and 162 amu was consistent with cyanidin-3-rhamnoside-5-glucoside and peonidin-3-rhamnoside-5-glucoside, also previously identified in b mutants (Statham et al., 1972).

The conversion of cyanidin and peonidin to delphinidin and petunidin requires hydroxylation at the 5’ position of the B ring of the precursor flavonoids. Because the products of this conversion were not observed in b mutants, it was presumed that the B gene controls the hydroxylation of the anthocyanin B ring (Statham et al., 1972). Our studies confirmed this conclusion and suggested to us that the gene encoding F3’5’H was a good candidate for B.

Isolation of a Pea F3’5’H Gene from a Purple-Flowered Wild-Type Plant

We performed PCR on cDNA derived from JI 2822 wing petals using primers based on aligned Medicago truncatula and soybean F3’5’H sequences. This yielded a product encoding a partial open reading frame (ORF) with extensive sequence similarity to F3’5’H. We used primers based on this new pea sequence together with primers based on the Medicago sequence for adaption-lication PCR (Sertitini et al., 1999), which enabled us to isolate genomic DNA sequences and a larger cDNA product including a TAG stop codon. Amplification and sequencing of a single PCR product, using primers at the 5’ and 3’ ends of the surmised contig, confirmed that a 1,548-bp cDNA encoded a cytochrome P450 monooxygenase 515 amino acids long.

A BLASTP search of Medicago genome pseudomolecules (version 3.5) using the chromosome visualization tool CViT (http://www.medicagohapmap.org) identified CU651565.9 on bacterial artificial chromosome (BAC) CU651565, a F3’5’H 515 amino acids in length, as the most similar sequence, with 89% identity. The predicted pea protein sequence is 79%, 78%, and 75% identical to predicted full-length F3’5’H sequences from lotus (Lotus japonicus; LjT34E9.40), soybean (AAM51564, ABQ96218, and BAJ14024), and butterfly pea (Clitoria ternatea; BAF49293), respectively. The soybean sequences are classified as CYP75A17 cytochrome P450s (Nelson, 2009). The Arabidopsis (Arabidopsis thaliana) sequence most closely related to the pea F3’5’H (48% identity) is the cytochrome P450 monooxygenase CYP75B1, encoded by TRANSPARENT TESTA7 (At5g07990; GenBank accession no. NP196416). This 513-amino acid protein has been demonstrated to have F3’H activity (Schoenbohm et al., 2000), and it lies within a separate clade when compared with other plant F3’5’H sequences (Fig. 3).
A 3,231-bp genomic DNA sequence was obtained from PCR products amplified from JI 2822 DNA using primers spanning the cDNA sequence and adapter-ligation PCR products corresponding to the promoter and 3' untranslated region (GenBank accession no. GU596479). The position of a single 530-bp intron, 915 bp downstream of the ATG start codon, was determined by alignment of the genomic DNA and cDNA sequences. A single intron is predicted in *Medicago* CU651565_9 at the same position, but in other legumes, such as soybean (Zabala and Vodkin, 2007) and lotus (LJT34E09_40), two introns are reported or annotated. In both species, the position of the predicted second intron is coincident with the position of the pea intron. The first introns are predicted in different positions, 331 and 348 bp downstream of their ATG, for lotus and soybean, respectively.

**Genetic Mapping of F3’5’H Reveals Cosegregation with b**

A cleaved-amplified polymorphic sequence (CAPS) marker for F3’5’H that distinguished the JI 15 and JI 73 alleles was generated by *TaqI* cleavage of the PCR products amplified from genomic DNA. Cosegregation of the CAPS marker with *b* was tested directly in a JI 15 × JI 73 recombinant inbred population of 169 individuals, because JI 73 carries the recessive *b* allele. JI 73 also carries *k*, the homeotic conversion of wing petals to keel petals, and *d*, the absence of pigmentation in foliage axils, whereas JI 15 carries *ce*, an independent crimson-pink flower trait. The *b*, *ce* double mutant is almost white, so single and double mutants can be distinguished easily, except in a *k* mutant background, where only the pale standard petal gives a clue to flower color. The genotypes

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**Figure 2.** LC-MS analysis of anthocyanins present in the wild type and *b* mutant lines. A, Extracted ion chromatograms showing the summed intensities of ions with masses corresponding to delphinidin and petunidin, each glycosylated with Rha and Glc, present in JI 2822. These masses are m/z = 611 (delphinin) and m/z = 625 (petunin). B, Masses corresponding to delphinin and petunin absent from FN 2271/3/pink. A and B are plotted to the same scale. C, Extracted ion chromatograms showing the summed intensities of three alternative anthocyanin ions, with masses based on glycosylated cyanidin (m/z = 743, m/z = 595) and peonidin (m/z = 609), present in line FN 2271/3/pink. D, Masses corresponding to cyanin and peonin absent from JI 2822. C and D are plotted to the same scale. Chromatographic peaks are annotated with m/z of the mass responsible for the peak.

**Figure 3.** Phylogenetic analysis of cytochrome P450 sequences. The optimal neighbor-joining tree derived from the multiple sequence alignment in Supplemental Figure S2 is drawn to scale, with the sum of branch lengths = 4.7. The Jones-Taylor-Thornton amino acid substitution model was used in phylogeny construction, and the scale bar indicates the number of amino acid substitutions per site. Percentage support for 1,000 bootstrap replicates is shown at the branch points. Labeled lines show GenBank accession numbers as follows: LJT34E09_40, *L. japonicus*; BAJ14024, soybean; BAF49293, *C. ternatea*; ADW66160, *P. sativum*; CU651565_9, *M. truncatula*; ABH06585, *Vitis vinifera*; BAE86871, *G. scabra*; P48418, *Petunia × hybrida*; CU651565_21, *M. truncatula*; NP_001064333, *Oryza sativa*; NP196416, *Arabidopsis*; ABH06586, *V. vinifera*; BAB83261, soybean; NP182079, *Arabidopsis*; NP775426, *Rattus norvegicus*. 

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at \( b \) and \( ce \) are particularly difficult to distinguish in a \( k, d \) background, where axillary pigmentation is also absent. For these reasons, the cosegregation analysis was restricted to a subset of 160 of the 169 recombinant inbred lines. The \( b \) phenotype cosegregated exactly with the JI 73 \( F3'5'H \) CAPS marker lacking a \( TaqI \) restriction enzyme site (\( bB = 71.89; \chi^2 = 2.0 \), not significant), consistent with our hypothesis that this \( F3'5'H \) identifies a single gene that corresponds to \( B \).

Identification of Lesions in \( F3'5'H \) Alleles from Pink-Flowered \( b \) Mutants

In order to provide further evidence of a correspondence between the pea gene encoding \( F3'5'H \) and \( B \), we sequenced alleles from known mutants. The \( b \) mutant type line, JI 118, carries a single nucleotide polymorphism 332 bp downstream of the ATG. This G/A transition would result in a single amino acid change, G111E (Supplemental Figs. S2 and S3). Line JI 73, the \( b \) mapping parent used above, carries a 23-bp deletion in the ORF, 291 bp from the ATG start. This deletion would introduce a change in the reading frame at position 98, resulting in the inclusion of 29 residues unrelated to the wild type followed by a premature stop codon (Supplemental Fig. S3). PCR analysis using primers that spanned the \( F3'5'H \) gene showed that lines FN 2160/1, FN 2255/1, and FN 2438/2 as well as the stable pink line FN 2271/3/pink all carry complete gene deletions (Supplemental Fig. S4). FN 1076/6 contains a genomic rearrangement that is consistent with a reciprocal break and join between the \( F3'5'H \) gene and a predicted \( Ogre \) retroelement (Neumann et al., 2003). The 5' segment of the \( Ogre \) element lies 1,330 bp downstream of the \( F3'5'H \) start codon, whereas the 3' segment lies upstream of position 1,330 at the 3' end of the \( F3'5'H \) gene (Supplemental Fig. S4).

Characterization of an Unstable Pink-Sected \( b \) Mutant

Unstable \( b \) mutants occurred in the M3 families FN 2271/3/flecked (Fig. 1C) and FN 3398/2164. It was found that sectored pink M3 siblings gave rise to sectored or stable pink M4 progeny, whereas stable pink M3 plants gave rise to stable pink M4 progeny only. Wild-type purple M3 siblings gave rise to either stable wild type, or a mix of stable wild type and stable pink, or a mix of stable wild type, stable pink, and sectored pink M4 progeny. Sectored pink M4 progeny gave rise to sectored or stable pink M5 plants in the following generation. In order to study this instability further, PCR analysis was carried out on individual flowers and progeny plants of line FN 2271/3/flecked/8.

Primers 3'pinkS1 and 3'pinkS2comp amplified 693 bp of genomic DNA and reported on exon 2. Both pairs of primers were used in conjunction with control primers designed to a pea \( Argonaute \) gene, which verified that PCR amplification had occurred, even in the absence of a \( F3'5'H \) PCR product. Genomic DNA and cDNA were prepared from the purple petals of a JI 2822 wild-type flower and from the petals of an entirely pink flower on a FN 2271/3/flecked/8 plant that carried purple/pink-sectored flowers at other nodes. PCR using primers 3'pinkS2 and 3'exTR showed the presence of the \( F3'5'H \) gene in JI 2822 and pink flower FN 2271/3/flecked/8 genomic DNA samples; however, cDNA amplification occurred in line JI 2822 only, suggesting that the \( F3'5'H \) gene was present but not expressed in the entirely pink FN 2271/3/flecked/8 flower (Fig. 4). Stable pink-flowered M4 progeny were grown from seed set on that entirely pink FN 2271/3/flecked/8 flower. When these were analyzed by PCR, exon 1 and exon 2 of \( F3'5'H \) failed to amplify from genomic DNA, suggesting that the gene was deleted in these progeny, as was observed previously in the stable pink-flowered line FN 2271/3/pink.

DISCUSSION

The early part of anthocyanin biosynthesis from chalcone to anthocyanidin is well conserved in higher plants and has been studied in detail (Grotewold, 2006). One of the key enzymes responsible for blue-purple coloration in flower petals is \( F3'5'H \), which catalyzes hydroxylation at the 3' and 5' positions of the B ring of naringenin and dihydrokaempferol, yielding flavanone and dihydroflavonol precursors of the chromophore delphinidin (Grotewold, 2006; Yoshida et al., 2009). Flowers that lack this enzyme, reported on exon 2. Both pairs of primers were used in conjunction with control primers designed to a pea \( Argonaute \) gene, which verified that PCR amplification had occurred, even in the absence of a \( F3'5'H \) PCR product. Genomic DNA and cDNA were prepared from the purple petals of a JI 2822 wild-type flower and from the petals of an entirely pink flower on a FN 2271/3/flecked/8 plant that carried purple/pink-sectored flowers at other nodes. PCR using primers 3'pinkS2 and 3'exTR showed the presence of the \( F3'5'H \) gene in JI 2822 and pink flower FN 2271/3/flecked/8 genomic DNA samples; however, cDNA amplification occurred in line JI 2822 only, suggesting that the \( F3'5'H \) gene was present but not expressed in the entirely pink FN 2271/3/flecked/8 flower (Fig. 4). Stable pink-flowered M4 progeny were grown from seed set on that entirely pink FN 2271/3/flecked/8 flower. When these were analyzed by PCR, exon 1 and exon 2 of \( F3'5'H \) failed to amplify from genomic DNA, suggesting that the gene was deleted in these progeny, as was observed previously in the stable pink-flowered line FN 2271/3/pink.

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such as rose (*Rosa hybrid*) and carnation (*Dianthus caryophyllus*), contain only cyanidin and/or pelargonidin chromophores, so their natural coloration is restricted to yellow, pink, and red but not purple or blue. Flower color also can be affected by pH, the presence of copigments, and whether the anthocyanin chromophores are polyacetylated or held in metal complexes (Yoshida et al., 2009). For example, hydrangea (*Hydrangea macrophylla*) sepal colors can be red, mauve, purple, violet, or blue, yet only one anthocyanin, delphinidin 3-glucoside, is present. It has been proposed that the anthocyanin and copigments in hydrangea sepal colors are held in a metal complex and that color depends on the concentrations of these components and the pH conditions (Kondo et al., 2005). In wild-type pea, the F35'H gene is intact and F35'H activity produces delphinidin-based anthocyanins, which confer a purple flower color. In this paper, we have presented genetic and biochemical evidence to show that b mutants lack a functional F35'H gene that results in a rose-pink flower color due to the presence of cyanidin- and peonidin-based anthocyanins. The presence of these latter 3'-hydroxylated compounds in b mutants suggests that a F3'H exists in pea, contrary to previous conclusions (Statham et al., 1972).

**Lesions Present in F35'H Alleles**

Plant P450 monooxygenases have not been characterized structurally because they are extremely insoluble when purified; however, membrane-associated mammalian P450s have been studied by homology to the crystal structure of a soluble bacterial P450 (Ferrer et al., 2008). P450s have only three absolutely conserved residues: a Cys that serves as a ligand to the heme iron, and an EXXR motif that is thought to stabilize the core around the heme (Werrick-Reichhart and Feyereisen, 2000). The Cys lies within the P450 consensus sequence FXXXRXXCG in the heme-binding loop, corresponding to FGAGRRICAG in the pea F35'H (Supplemental Fig. S2). Another consensus sequence, A/GGXD/ETT/S, corresponds to a proton transfer groove, and this corresponds to AGTDFS in the pea F35'H (Supplemental Fig. S2). The G111E mutation in the b type line, JI 118, does not occur in these conserved motifs, but the change in size and charge at this residue presumably affects protein function. Alignment of the pea F35'H sequence with homologous plant proteins (National Center for Biotechnology Information BLASTP) shows that substitutions occur at the G111 residue; however, none of the substitutes are charged residues, supporting our proposal that G111E is a detrimental change.

Line JI 73 carries a b allele with a spontaneous 26-bp deletion that is predicted to encode a truncated version of the F35'H protein. At the 3' end of the 26-bp deleted sequence, there is a 10-bp motif, ATTTCTCAAA, that is repeated at the 5' end of the deletion break point (Supplemental Fig. S3). This repeat pattern suggests that this stable b allele may have arisen from a spontaneous deletion event as a result of recombination and unequal crossing over. The same 26-bp deletion was found in lines JI 17, JI 132, and JI 2160 in the John Innes *Pisum* germplasm collection.

A genomic rearrangement consistent with a translocation event involving a retroelement was evident in line FN 1076/6. Here, sequencing showed that a break occurred in the F35'H gene, between nucleotides 1,329 and 1,330 downstream of the ATG, but we do not know whether the two fragmented portions of the F35'H gene remain on the same chromosome (Supplemental Fig. S4). The 5' end of the genomic disjunction was 95% identical to nucleotides 77,728 to 78,111 of a Ty3-gypsy *Ogre*-like retroelement (Neumann et al., 2003) identified in pea BAC clone JICPSV-29719, whereas the sequence at the 3' end of the disjunction was 95% identical to nucleotides 77,213 to 77,726 of the same retroelement. This indicates that a break occurred in the *Ogre* element between nucleotides 77,726 and 77,728 and that nucleotide 77,727 was missing from this copy of *Ogre* or was lost during the rearrangement. The presence of this retroelement does not necessarily implicate it in the mechanism of translocation but more likely reflects the abundance of the *Ogre* retroelement family. Data from 454 sequencing of cv Carrera estimated that copies of *Ogre* represent up to 33% of the pea genome (Macas and Neumann, 2007).

We gathered evidence of independent, recurring, spontaneous deletion events derived from unstable b alleles carried by lines FN 2271/3/flecked and FN 3398/2164. These sectored flowers carried an F35'H gene, presumably in nonepidermal tissue where it is not expressed, but repeatedly gave rise to stable pink deletion alleles in their progeny (Fig. 4). One possible explanation of these unstable b alleles is that FN 2271 M1 seed carried both a deletion of the b gene and a rearrangement of the chromosome carrying the wild-type B allele. This rearranged chromosome would be prone to the generation of acentric fragments that would fail to segregate properly at mitosis, generating sectors with a haploinsufficiency for many loci, including b. Individuals with the unstable phenotype would give rise to pink homozygous deletion progeny (with a wild-type karyotype). They would also generate progeny that are homozygous or heterozygous for the unstable chromosome, but the transmission of this unstable chromosome may be inefficient, or those that are transmitted efficiently may be selected for stability. In this scheme, the pink-flowered FN 2271 mutants derive from a simple deletion segregating in the population and the instability is not specifically associated with the b locus.

Alternatively, the unstable alleles at the b locus in the FN 2271 lineage may be prone to deletion, perhaps because of the action of a nearby transposon activated in the FN mutagenesis. Deletion of the b gene at one allele would be masked by the presence of the other, wild-type B allele, but the presence of such a deletion would reveal subsequent deletions of the B allele, which would be seen as pink sectors. In this scheme, deletion of b is not
generated directly by mutagenesis and the instability is associated specifically with the b gene. Pink flowers of this type could be indicators of a captured insertion element, but in no case did we find a stable pink mutant with the F3’5’H gene detectably present, even when these derived from seed set from an entirely pink flower on an unstable plant where the gene, but not the transcript, had been detected by PCR.

**F3’5’H Homologs in Legumes**

Cytochrome P450s are one of the largest enzyme families in plants. A search of annotated Medicago pseudomolecules (http://www.medicagohapmap.org) reveals 142 F3’5’H homologs (BLASTP, P > 1e-40), with approximately one-third of these located on chromosome 5. Gene clusters are found in many other organisms, and in Medicago, BACs containing five or more homologous ORFs occurred on chromosomes 2 (AC130800), 3 (AC145061), 5 (FP102223 and AC137079), and 6 (AC157489), although some of these may be pseudogenes. The soybean genome contains 712 cytochrome P450s, of which 380 are denoted pseudogenes (Nelson, 2009). Medicago BAC CU651565 carrying CU651565_9, the most similar intact ORF to pea F3’5’H, is unanchored in version 3.5 of the Medicago genome pseudomolecules; therefore, we were unable to gain any further evidence of orthology by analyzing collinearity with b gene-flanking markers. In the previous version of annotated Medicago pseudomolecules (version 3.0), BAC CU651565 was located on chromosome 3, which is syntenic with pea linkage group III, where b maps.

Another predicted Medicago F3’5’H gene, CU651565_21 (Fig. 3), lies only 52 kb from CU651565_9. The coding sequence of CU651565_21 corresponds to a protein 522 amino acids in length, which is anomalous compared with the lengths of related F3’5’H sequences (Supplemental Fig. S2). Multiple sequence alignment (Supplemental Fig. S2) suggests that CU651565_21 may in fact correspond to a 506-amino-acid protein that would be 63% identical to CU651565_9 and 62% identical to the pea F3’5’H. An alternative intron-splicing model derived from ORFs annotated in Medicago pseudomolecule version 3.0 is presented (Supplemental Fig. S5).

It is not clear whether the closest related lotus and soybean sequences are orthologous to the pea F3’5’H, because they have two introns; therefore, they are structurally dissimilar to the pea and Medicago genes. The *Petunia × hibrida* F3’5’H also has two introns, whereas the *G. scabra* F3’5’H has one, indicating that intron number is a variable feature of these genes. Diversity of exon-intron structure has been noted among genes encoding P450 enzymes, with multiple gains and losses in their evolutionary history (Werck-Reichhart and Feyereisen, 2000).

The amino acid sequence of CU651565_9, 89% identical to pea F3’5’H, is the closest match; however, the yellow (rather than purple/blue) pigmented flowers of *M. truncatula* suggest that there are differences in anthocyanin biosynthesis between these two species. All of the conserved P450 motifs are intact in CU651565_9, but a comparison with homologous sequences from other plant species shows differences that may be significant. For example, residue Phe-350, which is Leu or Val in aligned homologs (Supplemental Fig. S2), may disrupt F3’5’H function in *M. truncatula*. In support of this possibility, overexpression of the Myb transcription factor LAP1 in *M. truncatula* induced anthocyanin pigments, which were identified as glycosylated cyanidins and pelargonidins but not delphinidins (Peel et al., 2009). The absence of glycosylated delphinidins in these transgenic plants suggests a defect in F3’5’H activity, especially because glycosylated delphinidins were observed in white clover (*Trifolium repens*) overexpressing LAP1 (Peel et al., 2009).

Three soybean sequences (AAM51564, ABQ96218, and BAJ14024) are all 78% identical to pea F3’5’H; however, they are themselves nonidentical. ABQ96218 (Zabala and Vodkin, 2007) and AAM51564 (from cv Chin-Ren-Woo-Dou) are 99% identical and 509 amino acids long, respectively. They encode a CYP2 subfamily cytochrome P450, also classified as a CYP75A17 cytochrome P450 (Nelson, 2009), at locus Glyma13g04210 on linkage group F of soybean (http://soybase.org). ABQ96218, originating from cv Lee 68 and cloned from the Williams isolate L79-908, carries a G305D amino acid substitution (Zabala and Vodkin, 2007) in the conserved P450 proton-transfer groove motif that would likely render this allele nonfunctional (Supplemental Fig. S2). BAJ14024 (Takahashi et al., 2010) is a predicted F3’5’H from soybean cv Clark, 509 amino acids long, with invariant conserved motifs and 99% identical to both ABQ96218 and AAM51564.

**Flower Pigmentation in Pea and Soybean**

Soybean is believed to have been domesticated from purple-flowered *G. soja* (Takahashi et al., 2010). Studies of the standard (banner) petals of purple-flowered soybean cultivars show that these have a different sugar moiety at the 3 position of the C ring of their anthocyanidins compared with pea: the primary anthocyanins detected in soybean cv Clark (*W1W1 w3w3 W4W4 WmWm TT TdT d* and cv Harosoy (*W1W1 w3w3 W4W4 WmWm tt TdT d* were malvidin, delphinidin, and petunidin 3,5-di-O-glucoside and delphinidin 3-O-glucoside (*Iwashina et al., 2008*), whereas delphinidin and petunidin 3-rhamnoside-5-glucoside were the major anthocyanins found in the wing petals of pea line JI 2822 in this study, consistent with previous studies on line L 60 of pea (Statham et al., 1972). As the intensity of coloration in pea petals indicates (Fig. 1), the concentration of total anthocyanins in standard petals is less than in wing petals of pea at all stages of flower development (Statham and Crowden, 1974), whereas soybean flowers often have wing petals...
that are less intensely pigmented than their standard petals.

The Wp gene of soybean lies on linkage group D1b, corresponding to chromosome 2 (http://soybase.org). The wp allele is reported to contain a 5,722-bp CACTA transposable element in intron 2 of a F3H gene, F3H1, with down-regulated expression (Zabala and Vodkin, 2005). A null mutation would result in a lack of the substrates dihydrodymricetin, dihydrokaempferol, and dihydroyquercetin required for conversion into anthocyanins (Grotewold, 2006; Iwashina et al., 2008); therefore, a null mutant would be expected to have white flowers and, indeed, white-flowered mutants have been observed in other plant species (Martin et al., 1991; Britsch et al., 1992). Analysis of a wp genotype obtained by back-crossing to soybean cv Loda showed that the wp line had a low flavonoid content: 9% of the total flavonol glycosides, no detectable kaempferol 3-O-glucoside, and 28% of dihydroflavonols compared with cv Clark (Iwashina et al., 2008). The presence of dihydroflavonols indicates that F3H activity occurs in the wp mutant, suggesting that it is not a null allele. Alternatively, if the CACTA element insertion does render F3H1 null, a second F3H gene, F3H2, may be functional (Zabala and Vodkin, 2005).

Although the presence of anthocyanins in the wp mutant can be explained by the considerations above, the pale pink coloration (instead of pale purple) remained unexplained. Many factors such as copigments and vacuolar pH could influence soybean flower color, but the presence of an additional defective pigmenta-
tion gene, such as the ABQ96218 allele of F3’5’H, for example, would also cause pink flower color. A comparison of flower color and flavonoid content in available Wp and wp near-isogenic lines (Iwashina et al., 2008) and cosegregation analysis of F3H1 and wp would help to confirm which structural genes were defective.

The soybean w1 gene on chromosome 13 confers white flower color; accordingly, no HPLC peaks corresponding to anthocyanins were observed in a Clark-w1 near-isogenic line (L63-2373, w1a1, w3a3, W4W4, WmWm, TT, TdTd; Iwashina et al., 2007). However, it is not clear why a w1 encoding a defective F3’5’H gene would condition white flower color in soybean, when the pea b mutant and other F3’5’H mutants derived from purple-flowered wild-type plants (Snowden and Napoli, 1998; Matsubara et al., 2005; Nakatsuka et al., 2006) have pink flowers. Genetic linkage analysis of an F2 population segregating for w1 showed that 12 white-flowered individuals out of 39 F2 progeny carried an F3’5’H allele containing a tandem repeat insertion that would result in premature termination of the protein (Zabala and Vodkin, 2007). This linkage evidence is consistent with w1 being less than 1.1 centimorgan (Kosambi, 1944; Allard, 1956) from the tandem repeat-containing F3’5’H gene but with a high SE: the F3’5’H homozygotes in the purple flower class were not shown to be W1 homozygotes by progeny testing, and the population size is small. Thus, it is not clear that a mutated F3’5’H gene conditions white flower color in soybean.

One possibility is that w1 is a separate nonfunctional pigmentation locus, distinct from, but tightly linked to, the F3’5’H gene. This w1 locus is predicted to be functional in a G. soja line carrying the w1-lp allele, which has pale pink banner petals (Takahashi et al., 2010), and nonfunctional in Clark-w1. A cross between these two lines produced purple-flowered F2 progeny at a frequency of 0.9% (Takahashi et al., 2010), which is consistent with recombination between a distinct w1 gene and the F3’5’H gene. Soybean orthologs of genes encoding components of the Myb-bHLH-WD40 transcription factor complex that regulates anthocyanin biosynthesis (Koes et al., 2005; Ramsay and Glover, 2005), such as a and a2 (Hellens et al., 2010), have not yet been identified. These are good candidates for the proposed F3’5’H-adjacent w1 gene.

Pigmentation loci in pea, which have been studied in crosses for more than 100 years (Mendel, 1866; Tschermak, 1911), represent historic anchor markers that will aid comparative genomics between legume species as more physical maps are generated from sequenced genomes. Further biochemical studies, combined with genetic and genomic analyses, will help to elucidate the differences in anthocyanin biosynthesis that lead to variation in pigmentation among legume crop species such as soybean as well as important legume forage species such as alfalfa (Medicago sativa) and clover.

**MATERIALS AND METHODS**

**Plant Material**

The garden pea (Pisum sativum) type line for b, JI 118, also known as WBH 22 (Blüth, 1972), multiple marker line JI 73 (genotype b, also known as WBH 1231), multiple marker line JI 15 (genotype a, also known as WBH 1485), F13 recombinant inbred mapping population JI 15 × JI 73, and all F1N mutations were obtained from the John Innes Pisum Germplasm collection. Plants were grown in 16-h daylength in John Innes No. 1 compost with 30% extra grit. DNA was prepared from leaves according to Vershinin et al. (2003), and RNA was prepared from flowers according to Hofer et al. (2009).

**Mutagenesis**

A total of 1,400 seeds of line JI 2822 were subjected to 20 Gray FN irradiation from a 226Ra source at Oak Ridge National Laboratory. Irradiated M1 plants were self fertilized, and M2 families of up to four plants were screened for variant flower color phenotypes. Rose-pink mutants were backcrossed to JI 2822 to generate lines FN 1076/6, FN 2160/1, FN 2255/1, FN 2438/2, FN 2271/3/pink, and FN 3398/2164. These stable pink lines were obtained from the John Innes Pisum Germplasm collection. Plants were grown in 16-h daylength in John Innes No. 1 compost with 30% extra grit. DNA was prepared from leaves according to Vershinin et al. (2003), and RNA was prepared from flowers according to Hofer et al. (2009).

**LC-MS**

Purple (JI 2822) and pink (FN 2271/3/pink) wing petal tissue was harvested from 10 fully open flowers, ground in liquid N₂, and stored in methanol at −20°C. Sample aliquots of 10 μL containing 300 μg of tissue in methanol and 0.1 M HCl were analyzed by LC-MS using a Surveyor HPLC apparatus attached to a DecaXPlus ion-trap mass spectrometer (Thermo Fisher). Anthocyanins were separated on a
Isolation of Pea F3’S’H cDNA and Genomic DNA

Total RNA was extracted from JI 2822 wing petals using the Qiagen RNeasy Plant Mini kit. DNA was removed from RNA samples by digestion with DNA-free DNase1 (Ambion) in buffers according to the manufacturer’s protocol. Two micrograms of RNA was reverse transcribed with SuperScript reverse transcriptase (Invitrogen) from an oligo(T) primer in a 20-μL reaction. Amplification of a F3’S’H cDNA fragment from pea was achieved using 1 μL of 1:120 diluted first-strand cDNA in 20-μL PCRs containing 0.25 μM primers mtF35HF1 and mtF35HR2 (Supplemental Table S1) for 35 cycles with an annealing temperature of 62°C. Products were separated by electrophoresis on a 1% agarose gel in 1× Tris-borate/EDTA buffer. A 794-bp sequence obtained from this fragment was used to design additional primers for the amplification of 3,231-bp genomic DNA using successive rounds of adaptor ligation PCR (Sperling et al., 1999). The genomic DNA sequence was used to design primers pinkmtF1 and pinkextR for the amplification of a 1,595-bp cDNA clone, minus the ATG start codon and extending 50 bp beyond the TAG stop codon. This was cloned into a Topo4 vector (Invitrogen).

Mutation Analysis

Genomic DNA from JI 2822 and FN mutant lines was analyzed using pairs of primers that spanned the F3’S’H gene sequence in order to determine the size of deletion alleles (Supplemental Table S1). Primers PsAGO1 and PsAGO2, flanking introns 19, 20, and 21 of a pea Argonau1 cDNA clone (accession no. EF108450), were included in the reactions as internal controls. For the analysis of unstable lines, wing petal cDNA and genomic DNA from JI 2822, plant FN 2271/3/Becked/8, and its progeny were analyzed. Touchdown PCR was performed using 250 nM primers 3’pinkS2 and 3’extR, 250 μM deoxyribonucleotide triphosphates, and 1 unit of Taq polymerase in a 10-μL volume of PCR buffer. Primers PsAGO1 and PsAGO2 were included in the reactions as internal controls. Components were denatured at 95°C for 180 s, before being subjected to one cycle of 94°C for 45 s, 62°C for 45 s, and 72°C for 90 s, followed by 10 further cycles with the annealing temperature 1°C lower at each cycle. Twenty-nine further cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s were terminated at 72°C for 300 s. Reactions were held at 10°C for 300 s prior to analysis by agarose gel electrophoresis (Supplemental Fig. S4; Supplemental Table S1).

Genetic Mapping

A CAPS marker for F3’S’H was generated by TaqI cleavage of the 363- and 340-bp PCR products amplified from 100 ng of genomic DNA from parental lines JI 15 and JI 73, respectively, using primers pinkmtF1 and pinkextR for the amplification of a 1,595-bp cDNA clone, minus the AGT start codon and extending 50 bp beyond the TAG stop codon. This was cloned into a Topo4 vector (Invitrogen). Sequencing was performed using the BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems) at the John Innes Centre Genome Laboratory. Genomic DNA sequence was obtained from line JI 2822 using the primers listed in Supplemental Table S1. A 3,232-bp overlapping DNA sequence contig was generated using the program BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html). Overlapping DNA sequence contigs from b mutant lines JI 118, JI 73, and FN 1076/6 and cDNA sequences from lines JI 2822, JI 118, JI 73, and FN 1076/6 were obtained in the same way.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: JI 2822 F3’S’H cDNA sequence, GU596478; JI 2822 F3’S’H genomic DNA sequence, GU596479.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Ion fragmentation analysis of anthocyanins present in the wild type and b mutant lines.

Supplemental Figure S2. F3’S’H sequence analysis.

Supplemental Figure S3. Sequence characterization of mutant b alleles.

Supplemental Figure S4. Characterization of mutant b alleles by PCR.

Supplemental Figure S5. Proposed splicing model for Medicago gene CU615652.21.

Supplemental Table S1. Primers used for PCR and sequencing.

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