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Molecular Analysis of the *anthocyanin2* Gene of Petunia and Its Role in the Evolution of Flower Color

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The shape and color of flowers are important for plant reproduction because they attract pollinators such as insects and birds. Therefore, it is thought that alterations in these traits may result in the attraction of different pollinators, genetic isolation, and ultimately, (sympatric) speciation. *Petunia integrifolia* and *P. axillaris* bear flowers with different shapes and colors that appear to be visited by different insects. The *anthocyanin2* (*an2*) locus, a regulator of the anthocyanin biosynthetic pathway, is the main determinant of color differences. Here, we report an analysis of molecular events at the *an2* locus that occur during *Petunia* spp evolution. We isolated *an2* by transposon tagging and found that it encodes a MYB domain protein, indicating that it is a transcription factor. Analysis of *P. axillaris* subspecies with white flowers showed that they contain *an2*$^-$ alleles with two alternative frameshifts at one site, apparently caused by the insertion and subsequent excision of a transposon. A third *an2*$^-$ allele has a nonsense mutation elsewhere, indicating that it arose independently. The distribution of polymorphisms in *an2*$^-$ alleles suggests that the loss of *an2* function and the consequent changes in floral color were not the primary cause for genetic separation of *P. integrifolia* and *P. axillaris.* Rather, they were events that occurred late in the speciation process, possibly to reinforce genetic isolation and complete speciation.

INTRODUCTION

Flowers are the structures containing the male and female sex organs of angiosperms. Flowers of diverse species display a wide range of different morphologies and pollination strategies. For instance, flowers of wind-pollinated species usually possess small and inconspicuous petals or no petals at all, whereas flowers of insect-pollinated plants usually possess large, brightly colored, and patterned petals that serve as visual signals and a landing site for visiting insects.

Recent experiments suggest that the wide variety of plant and flower morphologies may have depended on the evolution of a relatively small number of genes. First, mutations at single loci, usually isolated by breeders or researchers, are sufficient to cause fundamental alterations in inflorescence architecture (Doebley et al., 1997; Souer et al., 1998). Similarly, the different shapes, colors, and color patterns of naturally occurring *Mimulus* (monkeyflower) spp are due to alterations at only a few (major) loci (Bradshaw et al., 1995). Second, even very different inflorescence and flower architectures appear to be determined by genes that often encode conserved proteins but that differ in their expression patterns (reviewed in Doebley and Lukens, 1998).

The isolation of key regulatory loci and analysis of the molecular alterations that have taken place in them will provide new insights into the evolution and diversification of flower morphology. The biosynthesis of anthocyanin flower pigments is particularly suited for such studies, because it is a well-defined biochemical pathway that is being studied simultaneously in distinct species with different flower morphologies and pollination strategies, such as maize (wind pollinated with flowers that lack petals), Arabidopsis (self-pollinating with white flowers), Antirrhinum (insect pollinated with colored flowers), and *Petunia* spp (both white and colored flowering species that are visited by different insects) (reviewed in Martin and Gerats, 1993; Holton and Cornish, 1995; Mol et al., 1998).

It is generally believed that the structural genes encoding the enzymes of the anthocyanin pathway are similar in all angiosperm species (Holton and Cornish, 1995). Recently, however, it was shown that one of the last steps in the pathway, glutathionation, is catalyzed by glutathione *S*-transferases of different evolutionary origin in maize and *Petunia* spp (Marrs et al., 1995; Alfenito et al., 1998). Moreover, the

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expression of the structural genes appears to be regulated differently in distinct species (reviewed in Mol et al., 1998; Weisshaar and Jenkins, 1998). Mutational analyses showed that in maize, transcription of the entire set of structural anthocyanin biosynthesis genes is controlled as a single unit by two families of regulatory genes. The so-called *c1*/*pl* and the *r*/*b* gene families comprise multiple paralogous genes that encode functionally similar proteins that include a MYB domain and a basic helix-loop-helix (bHLH) domain, respectively, and that have distinct expression patterns (Ludwig and Wessler, 1990; Cone et al., 1993).

In Antirrhinum and *Petunia* spp flowers, however, the pathway appears to be regulated in at least two distinct units (Martin et al., 1991; Quattrocchio et al., 1993). Mutations at the *anthocyanin1* (*an1*), *an2*, *an4*, and *an11* loci of *P. hybrida* cause inactivation of structural genes acting late in the pathway in specific parts of the flower (Beld et al., 1989; Quattrocchio et al., 1993; Huits et al., 1994a). The same holds true for mutations at *delila* (*del*) and *rosea* of Antirrhinum (Martin et al., 1991). Remarkably, the early structural genes remain transcriptionally active in these *Petunia* spp and Antirrhinum mutants, suggesting that they are activated by another set of regulatory genes (Moyano et al., 1996; Quattrocchio et al., 1998).

These findings can be explained in at least two ways: (1) the regulators of flower pigmentation in Antirrhinum and *Petunia* spp are not related to *c1* and *r* from maize; or (2) *c1*- and *r*-related genes do control flower pigmentation, and regulatory differences are due to divergent evolution of the target structural genes. Although several regulatory anthocyanin genes have now been isolated from Antirrhinum and *Petunia* spp, the data are too incomplete to exclude either of these two possibilities.

Molecular analysis of the *an11* locus of *Petunia* spp showed that it encodes a WD-40 repeat protein that is highly conserved in nature, even in animals and yeast that cannot synthesize anthocyanins (de Vetten et al., 1997). However, no information is available on the function of these *an11* homologs in their cognate hosts. The *an1* locus includes a gene that encodes a bHLH protein but this gene cannot be considered to be an ortholog of the maize *r* gene (C. Spelt and R. Koes, unpublished data), because another petunia gene, *jaf13*, has greater similarity to *r* (Quattrocchio et al., 1998). Although *jaf13* can replace the function of *r* in some functional assays, it cannot complement r^- mutants (Quattrocchio et al., 1998). Similarly, the *del* gene of Antirrhinum, encoding a bHLH protein with homology to R (Goodrich et al., 1992), failed to complement r^- mutants (Mooney et al., 1995). Therefore, it is not clear whether *jaf13*, *del*, and *r* are truly orthologous. However, our knowledge of the factors that control anthocyanin biosynthesis is far from complete because, for instance, information on the products encoded by *an2* and *an4* of *Petunia* spp and *rosea* of Antirrhinum is lacking. These loci are particularly interesting because they appear to have played a role in the diversification of flower color and patterns in nature.

P. integrifolia and *P. axillaris* subspecies occur naturally in South America in partially overlapping areas. Yet, both species remain genetically separated, apparently because their flowers are visited by different insects (Wijsman, 1982, 1983). *P. integrifolia* flowers have a purple corolla with a short wide tube and are pollinated by bees (Figure 1A), whereas *P. axillaris* flowers have a white corolla with a long narrow tube (Figure 1B), which is typical of moth-pollinated flowers (Wijsman, 1982). Manual cross-pollination, however, readily produces fertile progeny. In fact, the garden petunia (*P. hybrida*) is thought to be derived from such interspecific crosses (Wijsman, 1983; Sink, 1984; Koes et al., 1987).

Genetic analyses showed that differences at five loci, *an2*, *an4*, *hydroxylation-at-five* (*hf1*), *flavonols* (*fl*), and *pollen* (*po*), are responsible for the different floral organ colors of various subspecies of *P. integrifolia* (genotype $An2^+An4^+Hf1^+fl^-Po^+$) and *P. axillaris* (genotype $an2^-an4^-hf1\text{-}1fl^-po^-$ or $an2^-an4^-hf1\text{-}1Fl^+po^-$) (Wijsman, 1983). The $an4^-$ and po^- markers of *P. axillaris* are responsible for the yellow anthers, but because these markers do not affect coloration of the corolla, they are not discussed here (cf. van Tunen et al., 1991; Quattrocchio et al., 1993). The markers $an2^-$, *hf1-1*, and Fl^+ contribute in different degrees to the white color of the *P. axillaris* flower corolla. The $an2^-$ genotype strongly reduces but does not completely abolish the transcription of structural anthocyanin genes and coloration, when compared with $An2^+$. On their own, neither Fl^+ nor the partially

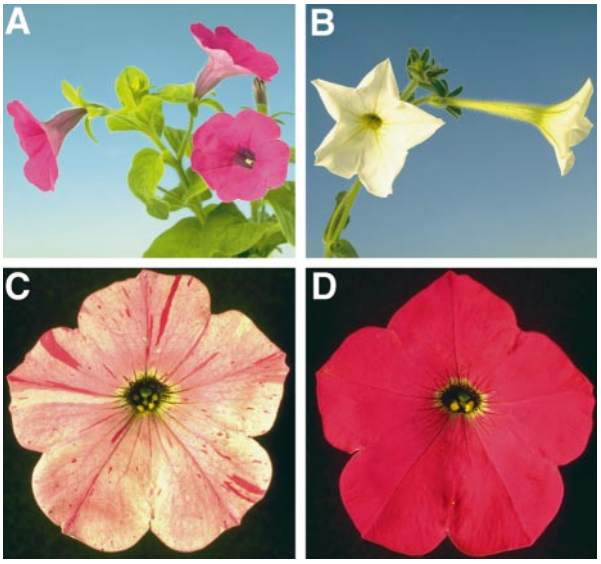

Figure 1. Flower Phenotypes of *Petunia* spp.

(A) Flowers of *P. integrifolia* subsp *violacea* line S9.
(B) Flowers of *P. axillaris* subsp *axillaris* line S1.
(C) *P. hybrida* flower harboring the unstable *an2-W82* allele.
(D) *P. hybrida* flower harboring a revertant allele derived from *an2-W82*.

active *hf1-1* allele contributes strongly to the white flower color. *Fl*$^+$ makes the flower more bluish (copigmentation) without reducing the accumulation of delphinidin-type anthocyanins. Mutation of *Hf1* results in the formation of reddish cyanidin-type anthocyanins instead of delphinidin-type anthocyanins. Only the combination of *Fl*$^+$ and *hf1-1* (or *hf1*$^-$) reduces the synthesis of anthocyanins; for unknown reasons, flavonol synthesis competes with cyanidin synthesis and not so much with delphinidin synthesis (Wiering and De Vlaming, 1984). During the evolution of petal color, the role of the *an2* locus appears to have been more prominent than the combination of *hf1* and *Fl*$^+$. This is because (1) *P. axillaris* accessions can be either *Fl*$^+$ or *fl*$^-$ (Wijsman, 1983), indicating that this difference arose late, after the genetic separation from *P. integrifolia*; (2) *An2*$^+$*fl*$^-$*hf1-1* flowers can still accumulate a considerable amount of anthocyanins (see, e.g., Figures 6H and 6I in Quattrocchio et al., 1998), whereas (3) the effect of an *an2*$^-$ allele in a *Hf1*$^+$*Fl*$^+$ background is considerably stronger (see Figures 6J and 6K in Quattrocchio et al., 1998).

To gain further insight into the molecular mechanisms that control flower pigmentation and into the evolution of those mechanisms, we conducted a molecular analysis of the *an2* locus. We isolated *an2* by transposon tagging and showed that it encodes a MYB domain protein. A comparison of the *an2* alleles in *Petunia* spp showed that the lack of *an2* function in *P. axillaris* flowers results from two independent loss-of-function mutations, one of which was apparently induced by the insertion and excision of a transposable element. Analysis of *an2* polymorphisms indicated that mutations in *an2* occurred relatively late in the speciation process, suggesting that in this instance, floral color change was not the primary speciation event but rather a mechanism to reinforce genetic separation.

RESULTS

Molecular Analysis of the *an2* Locus

P. hybrida line W82 in the Amsterdam collection harbors an unstable *an2* allele, herein named *an2-W82*, that is presumably identical to the *an2-n* allele described earlier (Cornu, 1977). The flowers of *an2-W82*/*an2-W82* plants have pale-colored corolla limbs with sectors and spots of a different intensity (Figure 1C). Among progeny obtained by self-pollination of *an2-W82* homozygotes, we identified four plants with full-colored flowers (Figure 1D) that originated from independent sporogenic reversion events.

Because most unstable alleles in *P. hybrida* contain insertions of the 284-bp transposable element *dTph1* (van Houwelingen et al., 1998), we anticipated the presence of *dTph1* in the *an2-W82* allele. To clone a fragment of the *an2* locus, we isolated *dTph1* flanking sequences that were present in two *an2-W82* homozygous plants, but not in a homozygote for the revertant allele *An2*$^+$*-R1*, by a combination of inverse polymerase chain reactions (PCRs) and differential screening of cloned amplification products (Souer et al., 1995). One of these *dTph1* flanking sequences, *jaf41*, hybridized with a 2.2-kb fragment in *an2-W82* homozygous plants, whereas a 1.9-kb fragment was detected in plants harboring four independently derived excision alleles (Figure 2A). This result showed that *jaf41* contains part of the *an2* locus. We subsequently isolated the complete locus and cDNA clones by hybridizing *jaf41* with corresponding libraries that were made from the *An2*$^+$ *P. hybrida* line V26.

The sequence of an *an2* cDNA revealed a large open

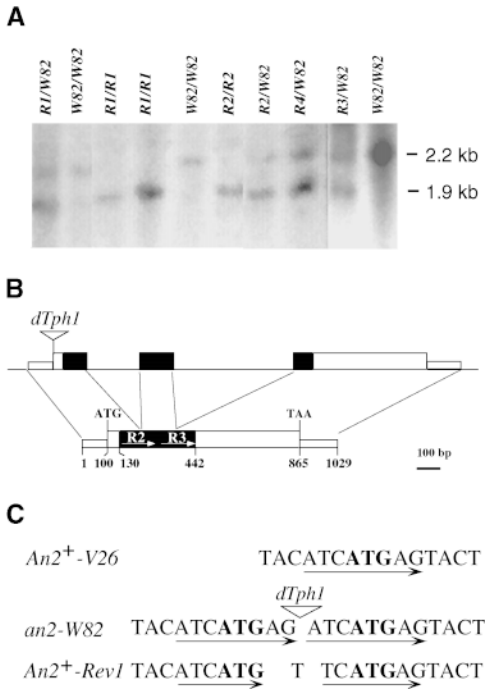

Figure 2. Molecular Analysis of *an2*.

(A) DNA gel blot of *P. hybrida* plants harboring the mutable *an2-W82* allele (*W82*) or four independently derived *An2*$^+$ revertant alleles (*R1* to *R4*) hybridized with the *dTph1* flanking sequence *jaf41*. The size of the hybridizing fragments (in kilobases) is indicated at right.

(B) Diagram showing the structure of the *an2* gene (top) and cDNA (bottom). Exon sequences are shown as a block; the protein coding sequences are blocks that are twice as high. The region encoding the MYB domain is shown in black, and the two repeats (R2 and R3) that make up this domain are indicated with arrows. The triangle shows the position of the *dTph1* insertion in the *an2-W82* allele.

(C) Sequence around the ATG translation start in wild-type *An2*$^+$, unstable *an2-W82*, and revertant *An2*$^+$*-Rev1* alleles. The ATG at the start of the *an2* ORF is marked in boldface. The arrows denote the 8-bp target site duplicated by the *dTph1* insertion (triangle).

reading frame (ORF) of 765 bp (Figure 2B) that is preceded by a small (69 bp) ORF. In *an2-W82*, the *dTph1* insertion duplicated 8 bp, including the ATG start codon of the large ORF (Figure 2C), without lowering the *an2* mRNA level (data not shown). This suggests that the mutant phenotype is caused by reduced translation initiation of the large ORF and implies that this ORF encodes AN2. The finding that naturally occurring *an2*$^-$ alleles contain mutations that disrupt the 765-bp ORF lends further support to this conclusion (see below).

Database searches revealed that the AN2 protein is similar to a range of so-called MYB domain proteins from animals and plants. Parsimony analysis showed that, in general, AN2 is more related to plant MYB proteins than to the animal c-MYB, the prototype of this superfamily (Figure 3A). The protein with the highest similarity found in the databases is that encoded by the *myb75* gene of Arabidopsis, a gene whose function is not known (Kranz et al., 1998). Because the majority of genes in this Arabidopsis family have been sequenced, this suggests that *myb75* may be the Arabidopsis homolog of *an2*. However, AN2 does not display significantly greater similarity to other MYB domain proteins implicated in flavonoid biosynthesis, such as MYB305 and MYB340 of Antirrhinum (Moyano et al., 1996), C1 and P from maize (Paz-Ares et al., 1987; Grotewold et al., 1991), or MYBPH3 from *P. hybrida* (Solano et al., 1995), than it does to MYB domain proteins involved in other processes (Figure 3B). In all cases, the similarity was restricted to the two repeats that make up the MYB domain, whereas no similarities were detectable in the C-terminal half of these proteins (Figure 3B). Comparison of the genomic and cDNA sequences showed that the *an2* gene is split by two introns (Figure 2B) in positions that are conserved in other *myb*-related genes.

Polymorphisms among *an2* Alleles

To investigate the evolutionary relationships between the various *An2*$^+$ and *an2*$^-$ alleles of different *Petunia* spp, we conducted DNA gel blot analyses. These experiments showed that *P. axillaris* subsp and *an2*$^-$ *P. hybrida* lines contain genomic *an2* fragments with nearly identical restriction maps, whereas the *P. integrifolia* subsp and *An2*$^+$ *P. hybrida* lines harbored different polymorphic fragments (data not shown). We also detected polymorphisms by using PCR amplification of an *an2* fragment that spans both introns (Figure 4A). In *an2*$^-$ lines of *P. axillaris* or *P. hybrida*, this amplification generated a 1.2-kb fragment, whereas the same fragment in *An2*$^+$ lines of *P. integrifolia* and *P. hybrida* varied in size between 1.2 and 1.4 kb (Figure 4A). Further mapping with different primer combinations showed that the polymorphism resides in the second *an2* intron (data not shown). To determine the nature of the polymorphism, we PCR amplified and sequenced the second intron of the *an2* allele of *P. axillaris* S1 and *P. integrifolia* S9 and S12. Comparison of these sequences showed that the polymorphism resulted from two insertions or deletions—one of 36 bp and a second of variable length (~50 bp in *P. integrifolia* S12 and 122 bp in *P. integrifolia* S9) (Figure 4A). A closer examination of these insertions/deletions and surrounding region did not reveal specific features (e.g., repeated sequences) that might account for a mechanism by which the polymorphism was established.

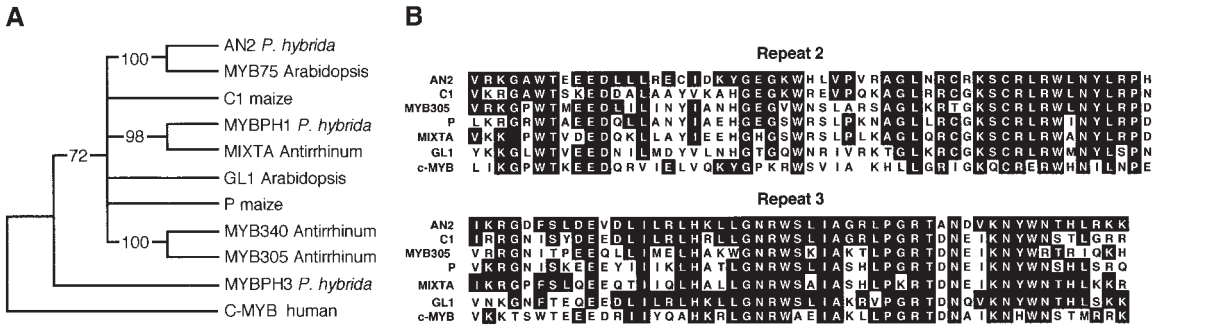

Figure 3. *an2* Encodes a MYB Domain Protein.

(A) A neighbor-joining phylogenic tree of AN2 and selected MYB domain proteins from other species. The numbers in the branches indicate the percentage of bootstrap support after 500 replicates. For construction of the tree, we used only the MYB domains; for c-MYB, we also excluded repeat 1 of its MYB domain, because this repeat is not found in the plant proteins.

(B) Alignment of the MYB domain of AN2 and proteins controlling anthocyanin (C1) and phlobaphene (P) synthesis in maize, phenylpropanoid metabolism (MYB305) in Antirrhinum and *Petunia* spp (MYBPH3), cell shape in Antirrhinum (MIXTA) and *Petunia* spp (MYBPH1), trichome development in Arabidopsis (GL1), and hematopoiesis in humans (c-MYB) (reviewed in Martin and Paz-Ares, 1997), and MYB75 of Arabidopsis, a protein with unknown function. Residues on a black background denote sequence similarity between AN2 and one or more of the other proteins.

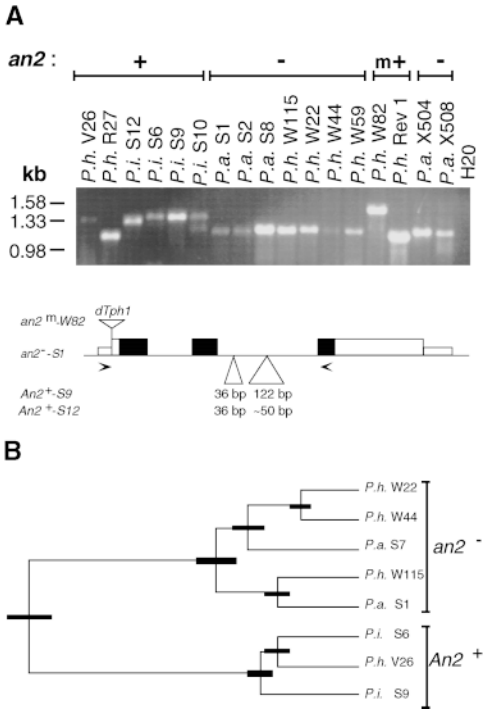

Figure 4. Phylogenetic Relationships of *an2* Alleles.

(A) Polymorphic *an2* fragments amplified from *P. integrifolia* (*P.i.*), *P. axillaris* (*P.a.*), and *P. hybrida* (*P.h.*) lines. The lane marked H20 is a control in which water without template DNA was used for amplification. The *an2* genotype of the lines used is indicated by + (*An2*$^+$), − (*an2*$^-$), and m (*an2* mutable). The positions of the two length markers are indicated at left. The diagram below shows the structure of the *an2* gene as in Figure 1A, the positions of the PCR primers (arrowheads), and the insertions/deletions (open triangles under the gene) responsible for the polymorphisms in the amplification products.

(B) A phylogenetic tree constructed by alignment of complete *an2* protein coding sequences using the UPGMA (for unweighted pair method using arithmetic averages) algorithm. The thick bars indicate the standard error in the positions of the branch points. The sequences have the GenBank accession numbers AF146702 (*P. hybrida* V26), AF146703 (*P. integrifolia* S9), AF146704 (*P. integrifolia* S6), AF146705 (*P. hybrida* W115), AF146706 (*P. hybrida* W22), AF146707 (*P. hybrida* W44), AF146708 (*P. axillaris* S1), and AF146709 (*P. axillaris* S7).

Alignments of the *an2* mRNA sequences also showed that the *an2*$^-$ and *An2*$^+$ alleles fall in two separate groups (Figures 4B and 5). The phylogenetic trees that were obtained in this way were fully consistent with the distribution of restriction fragment length polymorphisms and intron size polymorphisms described above.

Taken together, these data indicate that *An2*$^+$ and *an2*$^-$ alleles comprise two distinct groups that have been maintained in genetically separated populations, during which time the observed polymorphisms (restriction fragment length polymorphisms, the intron size polymorphisms, and sequence polymorphisms in the coding region) arose. The high degree of similarity between *an2*$^-$ alleles indicates that the *an2*$^-$ alleles found in *P. hybrida* are introgressed *an2*$^-$ alleles of *P. axillaris*.

Natural *an2*$^-$ Alleles Contain Frameshift and Nonsense Mutations

To determine the nature of the mutation that inactivated the *an2*$^-$ alleles, we determined the *an2* mRNA level in petals of different *Petunia* spp lines. Because *an2* mRNAs were of too low abundance to detect by RNA gel blot analysis, we used a quantitative reverse transcription (RT)–PCR assay. As an internal control, we coamplified products of the *chsA* gene, encoding the enzyme chalcone synthase that catalyzes the first step in flavonoid synthesis. Even though *chsA* is expressed independently of *an2*, its temporal expression pattern during petal development is very similar to that of the *an2*-controlled genes involved in anthocyanin synthesis (Quattrocchio et al., 1993, 1998). Figure 5 shows that all the *an2*$^-$ *Petunia* spp lines tested express *an2* transcripts in amounts that are only slightly lower than *An2*$^+$ lines do. Given that these lines represent different species, the relatively small variations in *an2* transcript abundance most likely result from differences in genetic background and/or decreased mRNA stability due to a blocked translation (see Discussion).

To determine the structure of the *an2* transcripts detected in *An2*$^+$ and *an2*$^-$ lines, we cloned the RT-PCR products and determined their sequence. In the *an2*$^-$ alleles of *P. axillaris* X504 and *P. hybrida* W44, W22, and W59, we found a 4-bp insertion after codon 127, just downstream of the

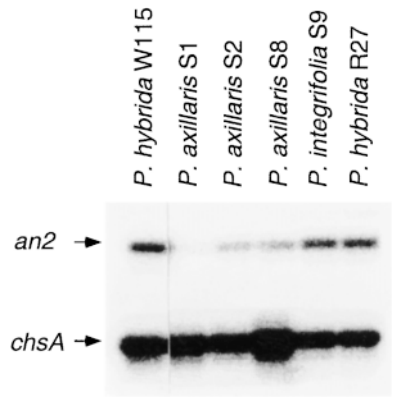

Figure 5. Analysis of *an2* Transcripts in *an2*$^-$ and *An2*$^+$ *Petunia* spp Lines.

Transcripts of the genes *an2* and, as control, *chsA* were detected by quantitative RT-PCR.

conserved MYB domain. This insertion results in a reading frame shift and a premature stop codon, thereby preventing the translation of the nonconserved C-terminal half of the AN2 protein (Figures 6A and 6B). In the *an2*$^{-}$ alleles in *P. axillaris* S1 and X508 and *P. hybrida* W115, we found a 1-bp deletion in codon 127, which also prevents translation of the C-terminal half of AN2 (Figure 6B). Partial sequencing of the *an2*$^{-}$ allele of *P. axillaris* S8 showed that this allele contained the same 1-bp deletion.

Partial sequencing of the *an2-S7* mRNA showed that the reading frame was intact at codon 127. We subsequently determined the complete mRNA sequence. This showed that *an2-S7* contains a nonsense mutation farther downstream at codon 196 (Figure 6B).

The Frameshift and Nonsense Mutations Are Responsible for *an2* Inactivation

We considered the possibility that the frameshift and nonsense mutations arose in *an2* alleles that had been already inactivated by other mutations. It is very unlikely that mutations upstream of the frameshift had inactivated *an2* prior to the frameshift, because the truncated protein encoded by *an2-W22* has the same sequence as the corresponding region in that encoded by *An2*$^{+}$*-V26* (Figure 6A). However, we could not exclude that inactivation had occurred downstream from the frameshift.

To identify evolutionary old mutations that might have inactivated the *an2*$^{-}$ alleles before the frameshift and nonsense

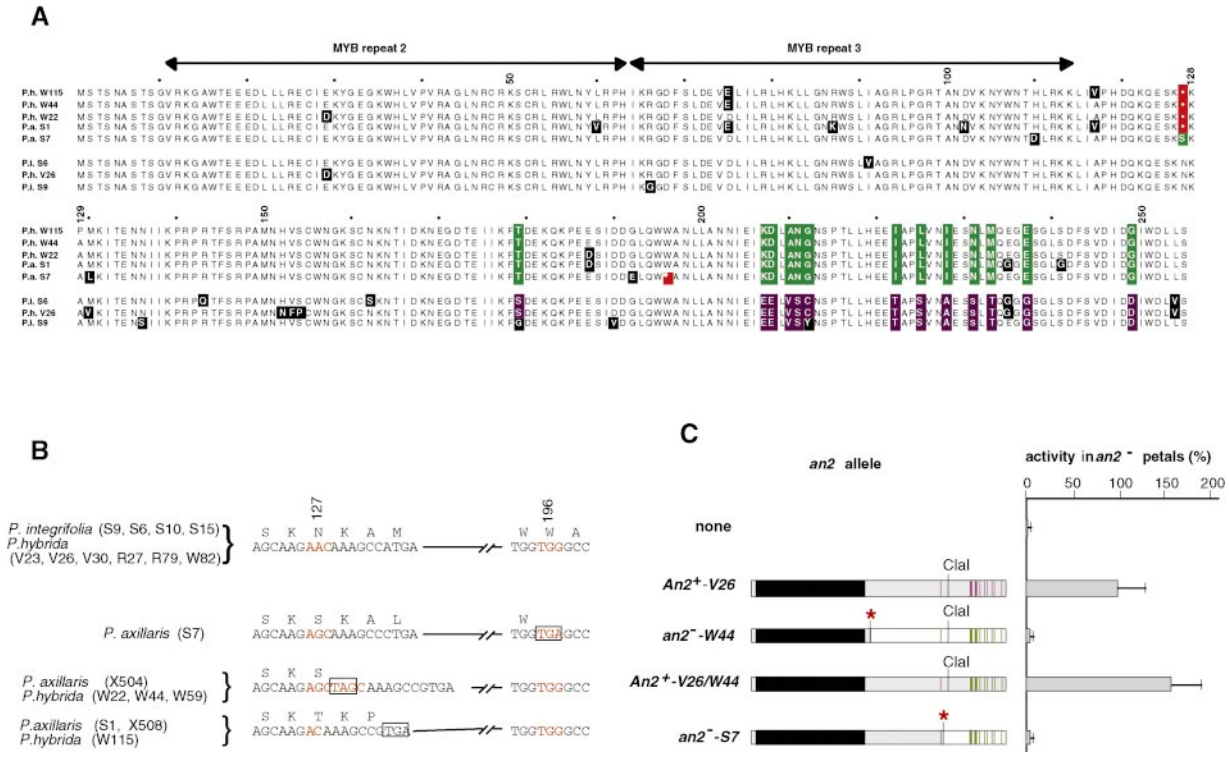

Figure 6. Analysis of the Mutations That Inactivate *an2*$^{-}$ Alleles.

(A) Alignment of translated *An2*$^{+}$ and *an2*$^{-}$ alleles. To produce the translations of the *an2*$^{-}$ alleles, the frameshift or nonsense mutations were ignored. The positions of these frameshift and nonsense mutations in *an2*$^{-}$ alleles are indicated by a dot on a red background. Polymorphisms specific for *An2*$^{+}$ and *an2*$^{-}$ alleles are indicated in purple and green, respectively. Other polymorphisms are highlighted in black. Numbering of the amino acid residues is indicated above the sequences (dots indicate every tenth residue). For species abbreviations, see the legend to Figure 4A.

(B) DNA and deduced protein sequences around the frameshift and nonsense mutations in *an2*$^{-}$ alleles. Codons 127 and 196, at which frameshift and nonsense mutations were found, are in red. The broken lines denote the sequences in between these two regions. Stop codons that terminate the AN2 protein coding sequence are boxed.

(C) Structure and activity of *An2*$^{+}$, *an2*$^{-}$, and hybrid alleles. The maps denote the mRNA structure of the different alleles. Black regions denote the MYB domain, and gray regions denote other translated sequences. White regions indicate mRNA sequences that are not translated due to the presence of a frameshift (*an2-W44*) or a nonsense mutation (*an2-S7*) at the site marked by a red asterisk. The green and purple bars indicate the position of polymorphisms, as given in **(A)**. The activity of these alleles, when expressed from the 35S promoter, was measured after microprojectile delivery into *an2-W115* petal cells by using a *dfrA–luc* reporter gene. Activities are given as the mean ±SE of five independent bombardment assays and are expressed in arbitrary units.

mutation arose, we searched for sequence polymorphisms that are consistently present in (nearly) all *an2*$^-$ alleles. Therefore, we aligned the translated AN2 sequences while ignoring the frameshift and nonsense mutations in the *an2*$^-$ alleles (Figure 6A). This revealed some sequence variations for single alleles that are scattered throughout the protein (Figure 6A). This distribution pattern indicates that these mutations arose relatively late in evolution, after the frameshift and nonsense mutations, and therefore were not responsible for gene inactivation in nature. Because the *an2*$^-$ alleles are no longer under selection pressure, one might expect that more such mutations will accumulate at a high rate.

At 13 positions, all clustered in the C terminus, the proteins of the *An2*$^+$ and *an2*$^-$ alleles differed consistently (Figure 6A), indicating that these variations occurred soon after the genetic separation of both groups of alleles. To examine whether these early alterations resulted in an inactive AN2 protein, we used a conserved ClaI restriction site to replace the last 65 codons of the wild-type *An2*$^+$*-V26* cDNA with those from *an2*$^-$*-W44* and fused the cDNAs to the constitutive 35S promoter of the cauliflower mosaic virus. To assay the activity of the various *an2* constructs, we used a complementation assay in which they were delivered by particle bombardment into *an2*$^-$ petal cells. To measure AN2 activity, we assayed the expression of a codelivered reporter gene (*luc*, encoding luciferase) driven by the *an2*-responsive promoter of the *dfrA* gene, which encodes dihydroflavonol 4-reductase, a "late" enzyme in the anthocyanin pathway. To normalize the *dfrA*-driven LUC activity for variations in transformation efficiency, we measured the activity of a codelivered reporter gene consisting of the β-glucuronidase coding sequence driven by the 35S promoter (35S–*gus*). Figure 6C shows that *an2-W115*, containing a −1-bp frameshift at codon 127, is a null allele because it fails to induce the *dfrA–luc* reporter gene. The hybrid allele *An2*$^+$*-V26*/*W44*, however, activated the *dfrA* promoter at least as efficiently as the wild-type *An2*$^+$*-V26* allele. This indicates that the polymorphisms near the former stop codon of *an2-W44* or its progenitor allele did not inactivate the protein. This apparent high tolerance for sequence variations cannot be taken as evidence that this part of the AN2 protein is without function, because truncation of the C-terminal domain, as in *an2-S7*, results in complete loss of AN2 activity (Figure 6C).

Taken together, these data strongly suggest that inactivation of *an2* in nature was caused by the frameshift and nonsense mutations, and not by the sequence variations observed in the C terminus of the protein.

DISCUSSION

To elucidate the molecular mechanisms that control flower pigmentation patterns, we studied a set of regulatory genes that control the tissue-specific transcription of the structural genes encoding enzymes of the anthocyanin pathway. Early experiments indicated that transcription of these structural genes in *P. hybrida*, Arabidopsis, and tobacco could be activated by combined expression of the *c1* and *r* genes from maize (Lloyd et al., 1992; Quattrocchio et al., 1998). However, this does not necessarily mean that *r*- and *c1*-homologous genes control pigmentation of the flower in vivo. In fact, the finding that *an11* and *an1* encode, respectively, a WD-40 protein (de Vetten et al., 1997) and a bHLH protein (C. Spelt and R. Koes, unpublished data) that had not been previously identified in other species indicated a different direction. Here, we report the isolation of *an2*, a third regulator of the anthocyanin pathway in *Petunia* spp, which acts in concert with *an1* and *an11* to activate transcription of structural anthocyanin genes in the petal limb.

an2 Encodes a MYB Domain Protein

The homology of AN2 to various MYB domain proteins suggests that AN2 is a transcription factor that activates a subset of structural anthocyanin genes. Such an activity would be consistent with the results of transient expression assays, which show that AN2 can activate promoter activity of the *dfr* gene (Figure 6C and Quattrocchio et al., 1998). Whether this activity involves binding of AN2 to the promoter of the structural genes or operates via an intermediate regulatory gene is currently under investigation.

Strikingly, AN2 does not display significantly more similarity to MYB305 and MYB340, two regulators of early structural genes in Antirrhinum (Sablowski et al., 1994; Moyano et al., 1996), or *C1* and *Pl* from maize. This finding, together with the observation that *an2*, *myb305*/*myb340*, and *c1* control different sets of target structural genes in their respective hosts (reviewed in Mol et al., 1998), may at first sight suggest that these genes are not homologs. However, subsequent expression assays and complementation experiments showed that AN2 can replace C1 and vice versa, indicating that *c1* and *an2* are homologous genes (Quattrocchio et al., 1998). The different sets of target anthocyanin biosynthesis genes controlled by *an2* and *c1* in their cognate hosts therefore appear to be due to divergent evolution of the target genes rather than of the regulatory proteins (Koes et al., 1994; Quattrocchio et al., 1998).

For C1, it was shown that its specificity resides in the DNA binding MYB domain (Sainz et al., 1997; Williams and Grotewold, 1997) and that this domain interacts with bHLH proteins encoded by the *r* gene family (Goff et al., 1992). However, also in this more limited domain, the similarity between AN2 and C1 does not appear to be significantly higher than it is between functionally unrelated MYB domain proteins (Figure 3), even though AN2 is the only MYB protein known that can complement mutations in *c1* and interact with maize R proteins or the petunia homolog JAF13 in transient expression assays (Quattrocchio et al., 1998) and in a yeast two-hybrid assay (A. Kroon and R. Koes, unpublished data). The C-terminal half of AN2 functions in yeast as a

transcriptional activation domain (A. Kroon and R. Koes, unpublished data), similar to the C-terminal domain of C1 (Goff et al., 1992). This suggests that the similarity between C1 and AN2 is higher than can be detected by sequence alignments or, in other words, that a significant number of alterations are tolerated—especially in the C-terminal domain—without loss of activity or specificity. This may account for the relatively high sequence divergence in the (former) C-terminal domains of *an2*$^-$ and *An2*$^+$ alleles (Figure 6A). Nevertheless, this domain appears essential for activity, because the truncated protein encoded by *an2-S7* is inactive (Figure 6C).

In *P. hybrida*, mutations in either of the regulators *an1* or *an11* leads to a complete loss of expression of their target anthocyanin genes in all pigmented tissues and, consequently, to completely white flowers (Quattrocchio et al., 1993). The function of *an2*, on the other hand, seems highly redundant, because in *an2*$^-$ mutants, pigmentation of seeds, the flower stem, anthers, and the corolla tube is unaltered, whereas only pigmentation of the corolla limb is reduced. In *an2*$^-$ corolla limbs, some residual expression of target genes remains detectable (Quattrocchio et al., 1993); as a consequence, the corolla limb is patchy and pale colored rather than completely white. In a *P. axillaris* background, however, the formation of the pale petal color is prevented by the *hf1-1* and *Fl*$^+$ alleles, which in combination also reduce anthocyanin formation (Wiering and De Vlaming, 1984). In transient expression assays, we could not detect activity of the *an2*$^-$ alleles, even when they were expressed from the strong 35S promoter, indicating that they are null alleles. Therefore, it is possible that the residual expression of structural genes in *an2*$^-$ corolla limbs is controlled by another paralogous locus. We recently isolated a candidate AN2 paralog by yeast two-hybrid screens (A. Kroon and R. Koes, unpublished data), a finding that may help to solve this issue.

The Role of Transposons in the Generation of *an2*$^-$ Alleles

In natural isolates of *P. axillaris*, we found three different *an2*$^-$ alleles harboring either a nonsense mutation or a +4- or a −1-bp frameshift mutation. Our data strongly suggest that these were the mutations that inactivated *an2* in nature. First, *an2*$^-$ *Petunia* spp lines still express *an2* transcripts, indicating that *cis*-acting elements in the promoters of the corresponding *an2* alleles are still working properly. The slightly lower abundance of *an2* transcripts in *an2*$^-$ lines compared with *An2*$^+$ lines may be due to the different genetic backgrounds of the *Petunia* spp lines and to reduced stability of *an2* transcripts because of premature termination of translation. The latter phenomenon was also observed for transcripts with frameshifts of *an11* (de Vetten et al., 1997), *an1* (C Spelt, F. Quattrocchio, J. Mol, and R. Koes, manuscript in preparation), and *alf* (Souer et al., 1998) and was reported by others as well (van Hoof and Green, 1996).

Because the +4- and the −1-bp frameshifts found in most *an2*$^-$ alleles are located on the same site, it is unlikely that they were generated by completely independent events. Closer inspection of the 4-bp insertion sequence shows that it has precisely the structure predicted for the insertion and subsequent excision of a transposon, strongly suggesting that the +4-bp and −1-bp *an2*$^-$ alleles were generated by two independent excisions of the same transposon (Figure 7). The petunia transposons *dTph1* (Gerats et al., 1990), *dTph2* (van Houwelingen et al., 1998), *dTph3* (Kroon et al., 1994), *dTph4* (Renckens et al., 1996; Alfenito et al., 1998), and *dTph5* (C. Spelt and R. Koes, unpublished data) all belong to the *Activator* superfamily and generate an 8-bp target site duplication upon insertion. After excision, a footprint is left behind that consists of remains of the target site duplication (⩾7 bp of each target site duplication; cf. Coen et al., 1986; Kunze et al., 1997) often separated by one or more inverted nucleotides of the target site duplication. Figure 7A shows that the −1- and +4-bp frameshift mutations can be explained, consistent with existing models for footprint formation, by two independent excisions of the same *Activator*-like transposon. However, in our analyses of footprints produced by *dTph1* elements, we never found the relatively large deletions in the target site duplication inferred in Figure 7A, suggesting that these may be rare events (van Houwelingen et al., 1999).

More recently, two families of petunia transposons—*Ps1* (Snowden and Napoli, 1998) and *dTph6* (C. Spelt and R. Koes, unpublished data)—were discovered that belong to the so-called CACTA family. *Ps1* and *dTph6* also belong to the so-called CACTA family. These transposons generate a 3-bp

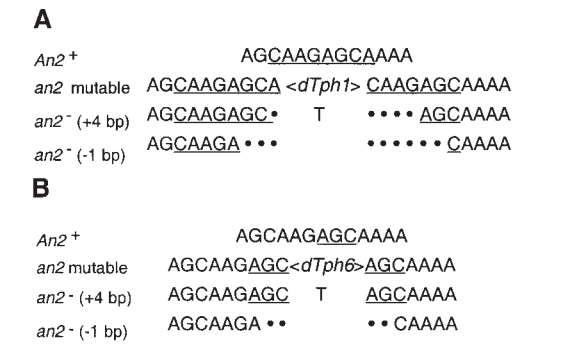

Figure 7. Model Showing How the Insertion and Excision of a Transposable Element May Have Created *an2*$^-$ Alleles with Two Different Frameshifts (−1 and +4 bp) at One Site.

(A) Model based on the insertion and excision of an *Activator*-like element, such as *dTph1*.

(B) Model based on the insertion and excision of a CACTA element, such as *dTph6*.

In **(A)** and **(B)**, the nucleotides that are duplicated by the transposon insertion are underlined; nucleotides that have been lost during subsequent transposon excision and break–repair are shown as dots.

target site duplication upon insertion and produce footprints that consist of remains of the target site duplication (≤3 bp), often separated by small inversions of the target site duplication without missing nucleotides at the symmetry axis (cf. Coen et al., 1986; Kunze et al., 1997). Figure 7B shows that the −1- and +4-bp frameshift *an2*$^-$ alleles are fully consistent with the insertion and excision of a CACTA transposon.

All of the above-mentioned transposons were identified in *P. hybrida* initially. However, DNA gel blot experiments showed that all *P. axillaris* and *P. integrifolia* accessions analyzed contained numerous copies of *dTph1* (Gerats et al., 1990; Huits et al., 1994b), *Ps1* (Snowden and Napoli, 1998), and *dTph6* (F. Quattrocchio, C. Spelt, and R. Koes, unpublished data).

Taken together, these data suggest that in nature, the −1- and +4-bp frameshift in the *an2*$^-$ alleles of *P. axillaris* were most likely generated by a transposable element, presumably a member of the CACTA family, although the involvement of an *Activator*-like element cannot be excluded.

The Evolution of Flower Color in Petunia

The genetic separation of populations followed by mutation, selection, and genetic drift is thought to be the basis for the creation and divergence of species (Coyne, 1992). Traditionally, it is believed that this requires physical isolation, for instance, by geographical factors (allopatric speciation). However, speciation can in principle also occur between individuals that grow side by side (sympatric speciation). As flower shape and color attract specific pollinating animals, alteration of one or more of these traits may cause the attraction of different pollinators and genetic isolation (Vickery, 1992). For example, *Mimulus* spp mutations at only a few loci are sufficient to alter floral traits, such as shape, color, color pattern, and nectar yield, and switch from bee to hummingbird pollination (Bradshaw et al., 1995). Although this is consistent with a sympatric speciation scenario, it remains to be determined if *Mimulus* spp arose sympatrically. One problem with such sympatric speciation scenarios is to envision how the first mutation, for example, a new color, becomes fixed in the population, because it might initially be at a disadvantage as long as the flower retains its original shape.

The sequences of the *an2* alleles provide a record of their history from which one can deduce how different flower colors arose in the genus *Petunia* in relation to speciation events. Figure 8 provides a model that summarizes and explains our findings. First, the presence of mutated *an2*$^-$ alleles in *P. axillaris* subsp indicates that the common ancestor of *P. integrifolia* and *P. axillaris* was colored flowering (*An2*$^+$) and that the white (*an2*$^-$) *P. axillaris* flowers arose by subsequent loss of *an2* function. This is in contrast to the evolution of kernel pigmentation in maize, which was established by gain-of-function mutations that altered the expression pattern of the regulatory *c1* and *r* genes of its progenitor Teosinte (Hanson et al., 1996). Second, we postulate that the ancestral petunia population split for unknown reasons (e.g., a geographical barrier or alterations in flower shape) into two genetically isolated groups that provided the foundation for today's *P. integrifolia* and *P. axillaris* subsp.

The old polymorphisms in *An2*$^+$ and *an2*$^-$ alleles are not spread randomly over the gene but rather are clustered in regions in which sequence alterations are relatively easily tolerated without loss of function, such as the intron and the 3′ end of the coding sequence (Figure 4). This indicates that the *an2* gene was after the genetic separation, at least for some time, still under selection pressure in both *Petunia* spp populations. This implies that no inactivating mutations had occurred and that the flowers were still colored (*An2*$^+$) at this stage of speciation. In the group that founded *P. axillaris*, at least two independent *an2*$^-$ alleles arose through the generation of a nonsense mutation and the insertion and excision of a transposon, respectively. Presumably, unknown circumstances (e.g., the insects visiting the flowers) favored white corolla limbs in this population, which explains why the *an2*$^-$ alleles became fixed and both the *An2*$^+$ progenitor and *An2*$^+$ revertant alleles (in which transposon excision generated an active *An2*$^+$ allele) seem to have disappeared. In this respect, it is interesting that insects (bees) are able to associate color with other characteristics such as scent and reward (Srinivasan et al., 1998).

Because in *P. hybrida*, other anthocyanin genes, such as *an1* (a regulatory gene) and *an3* (encoding the enzyme flavanone 3β-hydroxylase), are more susceptible to transposon insertions than is *an2* (van Houwelingen et al., 1998), it

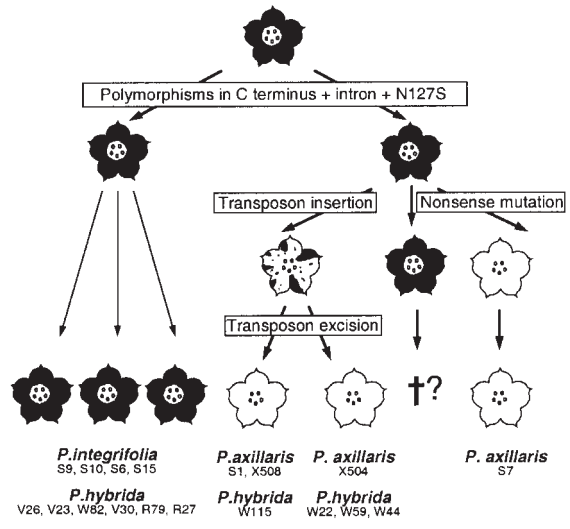

Figure 8. Model Summarizing Events at the *an2* Locus during *Petunia* spp Evolution.

The rectangles describe molecular alterations at the *an2* locus. The resulting flower phenotypes are indicated by the cartoons. The cross indicates the possible extinction of an allele.

is likely that the *an2*$^-$ mutants were only a few out of many white-flowering petunia mutants that arose in nature. We assume that only the *an2* alleles became fixed, because they only reduce anthocyanin synthesis in the petal limb, without additional effects on flavonoid accumulation. Mutation of any of the other known anthocyanin genes also reduces the synthesis of flavonoids in other tissues, such as the flower tube and the seed coat, and/or reduces the synthesis of other flavonoid compounds, such as flavonols (Quattrocchio et al., 1993; Huits et al., 1994a; van Houwelingen et al., 1998), which is apparently disadvantageous in a natural habitat.

Taken together, our data indicate that the flower color change caused by *an2*$^-$ mutations occurred after the genetic separation of both *Petunia* groups. Therefore, it seems that the *an2*$^-$ mutation(s) was not the primary cause of speciation but rather a reinforcement mechanism (Coyne, 1992) that helped to complete the genetic separation.

METHODS

Plant Materials

The inbred lines of *Petunia axillaris* subsp *axillaris* (S1 and S2) and subsp *parodii* (S7 and S8) and *P. integrifolia* subsp *inflata* (S6), subsp *violacea* (S9 and S10), and subsp *integrifolia* (S12) have been described previously (Wijsman, 1982). The *P. axillaris* accessions X504 and X508 (our numbering) were obtained around 1980 from the Botanical Garden of Liebig University (Giessen, Germany) and from R.N. Bowman (Goldsmith Seeds, Gilroy, CA), respectively. The *an2-W82* allele was kept in the Amsterdam collection in the *P. hybrida* line W82 and is presumably identical to *an2-n* (Cornu, 1977).

Nucleic Acid Analysis

DNA extraction, DNA gel blot analysis, polymerase chain reaction (PCR) amplification, and sequence analyses were performed as previously described (Souer et al., 1995; de Vetten et al., 1997). The flanking sequence of the *dTph1* element in *an2-W82* (*jaf41*) was identified by established procedures (Souer et al., 1995; de Vetten et al., 1997) and used to screen a petal cDNA library and a genomic library of *P. hybrida* V26. *an2* cDNAs from other lines were isolated by reverse transcription (RT)–PCR, and crucial regions were sequenced in at least two independently amplified products and in many cases by sequencing the corresponding region in PCR products amplified from genomic DNA. Analyses of DNA sequences were performed by using the program Geneworks 2.3 (Intelligenetics, Mountain View, CA). Maximum parsimony analysis was performed with the program PAUP 3.1.1 (Smithsonian Institution, Washington, DC).

Quantification of mRNAs by RT-PCR analysis was performed as described previously (Quattrocchio et al., 1998).

Transient Expression Assays

an2 cDNA fragments were ligated between the cauliflower mosaic virus 35S promoter and nopaline synthase polyadenylation signal and delivered into *an2-W115* mutant petal limbs by particle bombardment. AN2 activity was determined by measuring the induction of a *dfrA–luc* reporter gene and normalization for transformation efficiency using a 35S–β-glucuronidase gene, as described previously (de Vetten et al., 1997; Quattrocchio et al., 1998).

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