An Intragenic Suppressor of the Arabidopsis Floral Organ Identity Mutant apetala3-1 Functions by Suppressing Defects in Splicing

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The Arabidopsis floral organ identity gene APETALA3 (AP3) specifies the identity of petals and stamens in the flower. In flowers mutant for the temperature-sensitive ap3-1 allele, the petals and stamens are partially converted to sepals and carpels, respectively. ap3-1 contains a single nucleotide change in the AP3 gene that alters both an amino acid in the AP3 protein and the 5' splice consensus site for intron 5. Surprisingly, the Ap3-1 mutant phenotype is not due to the missense mutation but instead is due to defects in splicing; specifically, exon 5 is frequently skipped by the splicing machinery at the restrictive temperature. In a screen for suppressors of ap3-1, we isolated an intragenic suppressor, ap3-11, that functions to suppress the splicing defects of ap3-1. Using a reverse transcriptase-polymerase chain reaction assay, we demonstrate that the percentage of full-length exon 5-containing AP3 RNAs correlates with the phenotype of the flowers in both ap3-1 and ap3-11. Rather surprisingly, the ap3-11 suppressor mutation is located in intron 4. One model explaining the function of ap3-11 is that the ap3-11 suppressor creates a novel branch point sequence that causes exon 5 to be more frequently recognized by the splicing machinery. The identification of such a suppressor strongly suggests that exon-scanning models of intron-exon recognition are operative in plants.

INTRODUCTION

APETALA3 (AP3) is an Arabidopsis gene that is required for proper development of the petals and stamens in the flower. Both ap3 and pistillata (pi) mutants exhibit organ identity transformations in which sepals develop in place of petals and carpels in place of stamens (Bowman et al., 1989; Jack et al., 1992). AP3 and its partner protein, PI, are also sufficient to specify petal and stamen identity in the flower (Krizek and Meyerowitz, 1996a; Riechmann and Meyerowitz, 1997a). AP3 and PI proteins form a highly specific heterodimer that binds in a sequence-specific manner to DNA (Davies et al., 1996; Riechmann et al., 1996). The AP3 and PI proteins conserve a DNA binding and dimerization domain called the MADS domain (Goto and Meyerowitz, 1994). AP3 and PI also conserve a second domain, the K domain, which has been postulated to play a role in the dimerization specificity of AP3 and PI (Ma et al., 1991; Huang et al., 1996).

Currently, nine AP3 alleles have been isolated and sequenced to determine the molecular lesion responsible for the mutant phenotype (Jack et al., 1992; T. Jack, unpublished data). Eight of these alleles exhibit an identically strong (and most likely null) AP3 mutant phenotype. Three of these phenotypically strong alleles are missense mutations (ap3-6, ap3-9, and ap3-12), three are nonsense mutations (ap3-3, ap3-4, and ap3-5), and two contain mutations in canonical splice donor and acceptor sequences (ap3-7 and ap3-10). In flowers for these strong ap3 alleles, organ identity conversions are complete; sepals develop in place of petals and stamens in place of carpels.

One ap3 allele, ap3-1, is temperature sensitive and exhibits a weaker Ap3 phenotype. When grown at 23°C, homozygous ap3-1 flowers exhibit an intermediate Ap3 phenotype, with a partial conversion of petals to sepals and stamens to carpels. At a lower temperature (e.g., 16°C), the second-whorl organs have large petal sectors and the third-whorl organs are either stamens (most often fertile) or carpelloid stamens (Bowman et al., 1989). Sequencing of the AP3 gene from homozygous ap3-1 plants identified a single nucleotide change located 2 bp from the 3' end of exon 5 (Jack et al., 1992). This change causes a nonconservative missense mutation at the C-terminal end of the conserved K domain in the AP3 protein (K153M). The K domain has been postulated to be important for protein–protein interactions, possibly mediating either AP3/PI heterodimer formation or interaction with accessory factors (Ma et al., 1991; Krizek and Meyerowitz, 1996b; Riechmann and Meyerowitz, 1997b).

Similarly, a temperature-sensitive mutation is present in the K domain of the AP3 homolog in Antirrhinum, DEFICIENS (DEF). The Def-101 allele contains a deletion of a single amino acid at the N terminus of the K domain. At the restrictive temperature, the DEF-101 protein was unable to

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form DNA binding heterodimers with wild-type GLOBOSA (GLO; the Antirrhinum PI homolog) in a DNA binding assay (Zachgo et al., 1995). Unlike Def-101, the ap3-1 mutation does not function by altering the activity of the AP3 protein; Sablowski and Meyerowitz (1998) clearly demonstrate that ap3-1 exhibits a defect in splicing, and the defect in splicing is responsible for the Ap3 mutant phenotype. More specifically, at 25°C, exon 5 (which contains the mutation) is frequently skipped by the splicing machinery, resulting in AP3 cDNAs that fuse exon 4 directly to exon 6. The fusion of exon 4 to exon 6 results in an in-frame 14-amino acid deletion in the AP3 protein, which results in a nonfunctional AP3 protein.

Here, we report the isolation of an intragenic suppressor that functions to suppress the splicing defects of ap3-1. This intragenic suppressor, ap3-11, is a very weak ap3 allele; homozygous ap3-11 ap3-1 flowers exhibit very weak conversions of petals to sepals and stamens to carpels, and the flowers are self-fertile at 29°C. In ap3-11 ap3-1 flowers, the aberrant splicing patterns observed in ap3-1 are observed much less frequently. In both ap3-1 and ap3-11 ap3-1 flowers, the percentage of correctly spliced AP3 RNAs correlates with the phenotype of the flowers; the higher the percentage of exon 5-containing RNAs produced, the less severe the AP3 mutant phenotype. Rather surprisingly, the ap3-11 suppressor mutation is located in an intron. One model explaining the function of ap3-11 is that the ap3-11 suppressor creates a novel branch point sequence that causes exon 5 to be more frequently recognized by the splicing machinery.

RESULTS

Isolation of Intragenic ap3-1 Suppressors

We performed a genetic suppressor screen utilizing the temperature-sensitive ap3-1 allele. When homozygous ap3-1 plants are grown at 16°C, the second whorl develops as sepaloid petals, and the third whorl most frequently develops as stamens that often have carpelloid features, but the resulting flowers are most often self-fertile. At 23°C, homozygous ap3-1 plants exhibit a stronger Ap3 phenotype; the stamens are converted to carpelloid stamens or staminoid carpels, and the flowers are male sterile (Figure 1B; cf. wild type in Figure 1A). At higher temperatures (i.e., 29°C), the stamen-to-carpel transformation in the third whorl is more complete. To isolate suppressors of ap3-1, we mutagenized homozygous ap3-1 seeds with ethylmethane sulfonate and grew M₁ plants at 16°C, a temperature at which homozygous ap3-1 plants are self-fertile. We screened the M₁ generation for plants that exhibited a suppressed phenotype at 23°C. The phenotype of plants carrying these suppressors is intermediate between ap3-1 grown at 16°C and the wild type (Figures 1D and 1E). The first-whorl sepals and fourth-whorl carpels of plants carrying these suppressors were indistinguishable from those of the wild type. The second whorl developed as petals that were slightly sepaloid and were not fully elongated and thus were distinguishable from wild-type petals. The third whorl of the ap3-1 suppressors developed into either carpelloid stamens or stamens that most often produced pollen at 23°C; as a result, self-fertile flowers developed in plants carrying all four ap3-1 suppressors.

Genetic Characterization of ap3-1 Suppressors

To determine whether the suppressors were intragenic or extragenic, we performed two genetic tests. First, all four suppressors, when crossed to ap3-1, exhibited an F₁ phenotype intermediate between the homozygous suppressor and ap3-1 (Figure 1F). Similarly, when the suppressors were crossed to the strong ap3-3 allele, they also exhibited an intermediate phenotype (Figure 1G), suggesting that the suppressors might be intragenic (see below for a detailed description of the phenotype of the transheterozygotes).

To confirm that the suppressors were intragenic, we crossed them with wild-type (Landsberg erecta [Ler]) plants. In the F₂ generation, plants with wild-type flowers and plants exhibiting the suppressed floral phenotype segregated, but plants with an Ap3-1 phenotype failed to segregate. For one suppressor, we examined ~3000 F₂ progeny and did not detect any plants exhibiting Ap3-1 flowers, demonstrating very tight linkage between the suppressor mutation and AP3. The simplest interpretation of these genetic tests is that these suppressor mutations are intragenic.

Molecular Defects of ap3-1 Suppressors

Because genetic evidence suggested that the ap3-1 suppressors were intragenic, we sequenced the AP3 gene from all four suppressors to identify the second-site intragenic suppressor mutation. Genomic DNA from the suppressors was isolated, and regions of AP3 containing the exons and the intron-exon boundaries were amplified by polymerase chain reaction (PCR) and directly sequenced (Figure 2A). Rather surprisingly, none of the suppressors contained a second mutation in the AP3 coding region. All four suppressors, however, exhibited a single nucleotide change of G to A in intron 4 that was 33 bp 5’ to the 3’ splice site (Figure
Figure 1. Phenotypes of Mutant Flowers.

(A) Wild type (wt).
(B) ap3-1 grown at 23°C.
(C) ap3-3.
(D) ap3-11 suppressor (isolate 98-1) grown at 23°C.
(E) Suppressor isolate 104 grown at 23°C.
(F) ap3-1/ap3-11 transheterozygote.
(G) ap3-3/ap3-11 transheterozygote.
(H) ap3-3/ap3-3 flower containing a genomic copy of the AP3 gene containing the ap3-1 mutation grown at 23°C. The AP3-1 transgene (T[AP3-1]) partially rescues the strong Ap3-3 phenotype; in this flower, petaloid sepals are present in whorl 2 and staminoid carpels in whorl 3.
(I) ap3-3/ap3-3 flower containing a genomic copy of the AP3 gene containing the ap3-1 and ap3-11 mutations. The AP3-11 transgene (T[AP3-11]) rescues the strong Ap3-3 phenotype (compare with ap3-11 flower in (D)). In this flower, petals (not fully extended) and stamens (sometimes infertile) develop in whorls 2 and 3, respectively.
2B). This mutation was present in all four suppressors but was not present in either the wild type or the ap3-1 strain. Because the suppressor mutations were generated in an ap3-1 background, all four suppressors also retained the ap3-1 mutation in addition to the intron 4 mutation. We characterized one of these intragenic suppressors (isolate 98-1) in more detail. Because both genetic and molecular evidence suggest that this intragenic suppressor is an ap3 allele, we refer to it as ap3-11.

Genomic DNA (2.5 kb) encompassing the promoter, introns, exons, and 3′ region of the AP3 gene from ap3-11 and ap3-1 was sequenced. The only difference observed between ap3-1 and ap3-11 was the mutation in intron 4; at all other positions, the sequences were identical.

Figure 2. AP3 Gene Structure.

(A) The intron-exon structure of AP3 is shown at the top, and a schematic diagram of the conserved domains of the AP3 protein is shown at the bottom. The hatched regions indicate the location of the MADS box, and the filled-in region indicates the location of the K box. Numbers above the boxes refer to AP3 exons. The ap3-1 mutation is a single base change of an A to a T located 2 bp from the 3′ end of exon 5. This mutation results both in a missense mutation in the AP3 protein (K153M) and in a mutation in the 5′ splice site for intron 5. Exon sequences are in capital letters, and intron sequences are in lowercase letters.

(B) Schematic diagram of the exon 4 through exon 6 region of AP3 showing the location of the ap3-11 suppressor mutation. ap3-11 contains a single base change of a G to an A located in intron 4 33 bp 5′ to the 3′ splice site for intron 4. Numbers above the boxes refer to AP3 exons. Black boxes indicate the location of the K box.

Phenotype of ap3-11 Homozygous and Transheterozygous Flowers

As mentioned above, the ap3-11 mutation was generated in an ap3-1 background, and we did not analyze plants that contained only the ap3-11 mutation. In the following phenotypic description, we refer to the ap3-11 ap3-1 double mutant as ap3-11. Homozygous ap3-11 flowers exhibit a phenotype that is intermediate between wild-type flowers and ap3-1 flowers grown at 16°C (Figure 1D and Table 1). The second-whorl organs in ap3-11 flowers are composed primarily of petal tissue but occasionally contain small sepaloid sectors. Compared with wild-type petals, ap3-11 petals are both narrower in the apical part of the petal and shorter, rarely exceeding the height of the tall stamens. Overall, the shape is more similar to first-whorl sepalas than to wild-type petals. Third-whorl positions in ap3-11 flowers most frequently develop into pollen-producing stamens. Some third-whorl organs in ap3-11 flowers develop with carpelloid features, such as stigmatic tissue developing on the margins of the anther. Unlike ap3-1 flowers, ap3-11 flowers are not temperature sensitive; ap3-11 flowers were scored at both 16 and 23°C, and no dramatic differences were observed in the phenotypes at these two temperatures (Table 1).

Transheterozygotes between ap3-1 and ap3-11 exhibit a phenotype intermediate between ap3-11 homozygotes and ap3-1 homozygotes (Figure 1F). We scored transheterozygous flowers at both 16 and 23°C; at 16°C, the phenotype is considerably less severe (Table 2). At 23°C, second-whorl organs in ap3-1/ap3-11 flowers are sepaloid petals that appear white like petals but are shaped like sepals and do not elongate like wild-type petals. These organs are shorter than the petals that develop in ap3-11 homozygotes but are considerably more petaloid than the second-whorl organs that develop in ap3-1 homozygotes at 23°C. The third-whorl organs develop as either carpelloid stamens or stamens. Compared with plants grown at 23°C, ap3-1/ap3-11 transheterozygous flowers grown at 16°C have larger petals that contain fewer sepaloid sectors.

We also examined the phenotype of transheterozygotes between the strong ap3-3 allele and ap3-11 (Figure 1G). ap3-3/ap3-11 flowers exhibit a phenotype intermediate between ap3-3 and ap3-11 mutants, and overall, the phenotype is more severe than that of the ap3-1/ap3-11 transheterozygotes. The second whorl of these flowers develops as petaloid sepals and the third whorl as staminoid carpels or carpelloid stamens. No fertile stamens were observed in ap3-11/ap3-3 transheterozygous flowers.

It is interesting that ap3-11 flowers exhibit a second and third whorl phenotype much closer to that of the wild type than do the 35S::AP3–Met-153 ap3-3 flowers described in Sablowski and Meyerowitz (1998). 35S::AP3–Met-153 ap3-3 plants contain an AP3 cDNA that contains the exon 5 mutation. This mutation results in the K153M amino acid change in the AP3 protein. Like ap3-11 flowers, 35S::AP3–Met-153 flowers exhibit fairly strong rescue of third-whorl organs (i.e., fertile stamens); however, unlike ap3-11, the second whorl is only
weakly rescued. In both ap3-11 and 35S::AP3–Met-153, the same AP3 protein product is produced—an AP3 protein that contains the K153M change. Despite the fact that these two genotypes produce the same AP3 protein, they rescue second and third whorl phenotypes differently, suggesting that there may be a difference in expression when comparing ap3-11 and 35S::AP3–Met-153. The fact that a construct containing AP3 introns 4 and 5 in the context of an ap3-1cDNA (35S::ap3-1 minigene) rescues petals more completely than does 35S::AP3–Met-153 (Sablowski and Meyerowitz, 1998) suggests that the problem is not due to transcription initiation driven by the cauliflower mosaic virus 35S promoter but instead to some post-transcriptional phenomenon that results in an increase in AP3 activity when introns are present in the pre-mRNA.

**ap3-11 Suppresses the Splicing Defects of ap3-1**

To investigate whether the ap3-11 mutation functions to suppress the splicing defects of the ap3-1 mutation, we performed a reverse transcriptase (RT)-PCR assay to examine the AP3 RNAs produced in ap3-1 and ap3-11 flowers. Total flower RNA was prepared from wild-type, ap3-1, and ap3-11 plants grown at 16, 23, and 29°C. First-strand cDNA was prepared from this RNA by using oligo(dT) as a primer, and RT-PCR was performed by using primers located in AP3 exons 2 and 7 (Figure 3A). With wild-type RNA isolated from flowers at all three temperatures, the RT-PCR assay yielded only a single 332-bp RT-PCR product (Figure 3B). This RNA contained all of AP3 exons 2 to 6, including exon 5. When ap3-1 RNA was used in this assay, however, two bands were produced. One is the same size as the wild-type band (332 bp), but the second band is smaller (290 bp). The smaller band was subcloned and sequenced, and the sequence reveals that it is missing exon 5: in this shorter product, exon 4 is fused directly to exon 6, resulting in a 42-bp deletion in the cDNA and a 14–amino acid in-frame deletion in the AP3 protein.

In the RT-PCR assay, differences were observed in the ratio of long product to short product, depending on the temperature at which the ap3-11 plants are grown. When RNA from

| Table 1. Third-Whorl Phenotype of ap3-1 and ap3-11 Flowers a |
|-----------------|-----------------|-----------------|
| Floral Organ    | 16°C  | 23°C  |
|                 | 1 to 20 | 21 to 30 | 1 to 20 | 21 to 30 |
| ap3-1           |       |       |       |       |
| St fertile      | 5.5   | 5.3   | 0     | 0     |
| St infertile    | 0.2   | 0.2   | 5.6   | 4.1   |
| C st            | 0     | 0     | 0.2   | 1.8   |
| C st c          | 0     | 0     | 0     | 0.8   |
| Carpels         | 0     | 0     | 0     | 0     |
| Filaments       | 0     | 0     | 0     | 0     |
| Other           | 0     | 0     | 0.1   | 0     |
| Sum of all organs | 5.7   | 5.3   | 5.9   | 6.0   |
| Number scored   | 10    | 6     | 20    | 20    |

| ap3-11          |       |       |       |       |
| St fertile      | 5.0   | 5.1   | 5.6   | 5.8   |
| St infertile    | 0     | 0     | 0     | 0     |
| C st            | 0     | 0.1   | 0     | 0     |
| C st c          | 0     | 0     | 0     | 0     |
| Carpels         | 0     | 0     | 0     | 0     |
| Filaments       | 0     | 0     | 0     | 0     |
| Other           | 0     | 0.1   | 0     | 0     |
| Sum of all organs | 5.0   | 5.2   | 5.6   | 5.8   |
| Number scored   | 20    | 8     | 20    | 3     |

a All plants were grown under identical conditions. The average number of organs per flower is given.

b Numbers refer to the position of the flower on the inflorescence, with flower 1 being the first flower to develop.

c Stamens (St) produce visible pollen grains.

d Stamens do not produce visible pollen grains.

e Carpelloid (C) stamens. Organs primarily staminoid with carpelloid features, as described in Bowman et al. (1989).

f Staminoid carpels. Organs primarily carpelloid with staminoid features, as described in Bowman et al. (1989).

g Includes various types of filaments and filamentous structures.

h Includes other types of mosaic organs.
plants grown at 23 or 29°C was assayed, the ratio of the long-to-short product was <1. By contrast, when RNA from plants grown at the permissive temperature of 16°C was assayed, the ratio of the long-to-short product was >1. The RT-PCR experiments were repeated several times, and in all experiments, more of the full-length exon 5-containing product was detected at 16°C, whereas more of the exon 5-deleted form was detected at 23 and 29°C. Figure 3B shows one representative experiment. In summary, the ratio of exon 5-containing to exon 5-deleted products correlated with the phenotype of the flowers; ap3-1 plants grown at 16°C exhibited a less severe Ap3 phenotype and produced a higher ratio of long-to-short product.

**A Genomic AP3 Fragment from ap3-11 Complements the ap3-3 Mutant**

To further support the hypothesis that the ap3-11 mutation was intragenic and located in intron 4 of AP3, we constructed transgenic plants that contained a genomic copy of the AP3 locus from the wild type, ap3-1, and ap3-11. Constructs containing genomic DNA spanning the AP3 locus isolated from the wild-type AP3, ap3-1, and ap3-11 were transformed into wild-type plants. For each construct, at least five independent transgenic lines were analyzed. In a wild-type background, none of the AP3 constructs altered the phenotype of the flowers. To test whether these constructs could rescue a strong ap3 allele, we crossed transgenic lines to the strong, and likely null, ap3-3 mutant (Figure 1C). When transgenic lines that contained wild-type AP3 were crossed into an ap3-3 background, 100% of the F2 kanamycin-resistant plants exhibited a wild-type phenotype (data not shown). When the ap3-1-containing transgene was crossed to an ap3-3 mutant, none of the F2 kanamycin-resistant plants exhibited an Ap3-3 phenotype, but some of the kanamycin-resistant plants exhibited flowers with a phenotype that was intermediate between the Ap3-3 and Ap3-1 phenotypes (Figure 1H and Table 3). Specifically, the second whorl developed as petaloid sepals, and the third whorl developed most frequently as carpelloid stamens, although staminoid carpels, infertile stamens, carpels, and filaments were also observed. At 16°C, the phenotype was less severe, but fertility of the stamens was reduced compared with ap3-1 homozygotes. Based on this result, we conclude that the ap3-1-containing transgene partially rescues the Ap3-3 phenotype.

When transgenic lines that contained the AP3 region from ap3-11 plants were crossed into an ap3-3 background, the F2 kanamycin-resistant plants exhibited either a wild-type phenotype (ap3-3/+ or +/+), or a phenotype very similar to homozygous ap3-11 plants (Figure 1I and Table 3). The degree of rescue was variable in different plants. Some plants exhibited strong rescue and a phenotype very similar to ap3-11 homozygotes. In these flowers, the second whorl developed as petals (slightly reduced in size) and the third whorl as stamens (most often producing pollen) or carpelloid stamens. Other kanamycin-resistant F2 plants exhibited weaker rescue. Specifically, flowers from the weaker class resembled ap3-1 flowers when grown at 23°C; the second whorl developed as sepaloid petals rather than petals, and the third whorl frequently developed staminoid carpels and carpelloid stamens rather than stamens; as a result, the overall fertility of the flowers was reduced. In summary,

<table>
<thead>
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<th>Floral Organ</th>
<th>16°C</th>
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<tr>
<td></td>
<td>1 to 10</td>
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<tr>
<td>St fertile</td>
<td>4.9</td>
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<td>St c</td>
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<tr>
<td>Carpels</td>
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<td>Filaments</td>
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<tr>
<td>Other</td>
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</tr>
<tr>
<td>Sum of all organs</td>
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<tr>
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*a* All plants were grown under identical conditions. The average number of organs per flower is given.

*b* Numbers refer to the position of the flower on the inflorescence, with flower 1 being the first flower to develop.

*c* Stamens (St) produce visible pollen grains.

*d* Stamens do not produce visible pollen grains.

*e* Carpelloid (C) stamens. Organs primarily staminoid with carpelloid features, as described in Bowman et al. (1989).

*f* Staminoid carpels. Organs primarily carpelloid with staminoid features, as described in Bowman et al. (1989).

*g* Includes various types of filaments and filamentous structures.

*h* Includes other types of mosaic organs.
ap3-1 Suppresses Splicing Defects

Figure 3. RT-PCR Assay Demonstrates That ap3-11 Suppresses the Splicing Defects of ap3-1.

(A) Schematic diagram showing the location of the two oligonucleotides used for RT-PCR. The oligonucleotides (indicated by arrows) are located in exons 2 and 7 of AP3. These oligonucleotides detected two products in ap3-1 and ap3-11. One product is 332 bp and contains sequences from all seven exons. The second product is 290 bp and is completely missing exon 5 sequences; in this shorter RT-PCR product, exon 4 is fused directly to exon 6.

(B) RT-PCR assay using RNA isolated from wild-type, ap3-1, and ap3-11 plants grown at 16, 23, and 29°C. Using wild-type (wt) RNA isolated at all three temperatures, only the longer 332-bp product was detected. For ap3-1 (-1) and ap3-11 (-11), both the longer and shorter (290 bp) products were detected at all three temperatures. For ap3-1, the ratio of longer to shorter product varied depending on temperature; at 23 and 29°C, more of the exon 5-deleted RNA was detected, whereas at 16°C (the restrictive temperature), more of the exon 5-containing product was detected. For ap3-11, more of the longer product was detected at all three temperatures. In all cases, the higher the percentage of full-length AP3 RNA, the weaker the Ap3 mutant phenotype. The ratios reported do not reflect the absolute ratio of RNAs produced; instead, they reflect the ratio of the intensity of larger (i.e., exon 5-containing) to smaller (exon 5-deleted) bands within a single lane.

these results demonstrate that the AP3 genomic fragment containing the ap3-11 mutation is capable of rescuing the strong ap3-3 mutant.

DISCUSSION

In this study, we describe the isolation and characterization of an intragenic suppressor of the temperature-sensitive ap3-1 allele. Several lines of genetic evidence demonstrate that the ap3-11 suppressor is a second-site intragenic suppressor. First, transheterozygous combinations of ap3-11 with other ap3 alleles result in phenotypes that are intermediate between the weak ap3-11 allele and the stronger ap3-1 and ap3-3 alleles. Second, when backcrossed to the wild type, plants with an Ap3-1 phenotype fail to segregate in the F2 generation, demonstrating very tight linkage between the ap3-11 mutation and ap3-1. Third, a genomic fragment containing the AP3 gene from ap3-11 plants can rescue the phenotype of strong ap3-3 mutants.

Molecular analysis of ap3-11 demonstrates that the suppressor mutation is located in intron 4 of AP3, suggesting that the suppression is not due to a second mutation in the AP3 protein. As is convincingly demonstrated by Sablowski and Meyerowitz (1998), the phenotype of ap3-1 plants is caused by a defect in splicing; specifically, exon 5 is frequently skipped by the splicing machinery, resulting in a 14-amino acid deletion that inactivates the AP3 protein. The temperature sensitivity of the ap3-1 mutation is not due to the production of a temperature-sensitive AP3 protein but rather is due to the temperature sensitivity of a component of the splicing machinery (Sablowski and Meyerowitz, 1998). The ap3-1 mutation is located 2 bp from the 3’ end of exon 5, and this mutation causes two separate defects. First, this mutation causes a missense mutation (K153M) in the AP3 protein. The missense mutation, however, is not responsible for the Ap3-1 phenotype; when the K153M mutation is engineered in the context of an AP3 cDNA and expressed under the control of the broadly expressed cauliflower mosaic virus 35S promoter, it partially rescues the strong ap3-3 mutant, demonstrating that an AP3 protein with the K153M mutation is still functional (Sablowski and Meyerowitz, 1998).

Second, the ap3-1 mutation results in a mutation in the 5’ splice consensus sequence for intron 5, and it is this defect that causes the Ap3-1 phenotype. The 5’ splice site for AP3 intron 5 is a poor match to the consensus 5’ splice sequence (consensus sequence CAG/GUAAGU; AP3 sequence AAG/GUCACA; Figure 4A). The most critical bases in the 5’ splice site consensus are the GU dinucleotide at the extreme 5’ end of the intron; these two bases are conserved in nearly 100% of Arabidopsis introns, including AP3 intron 5 (Brown, 1996). At other positions, however, the match to the consensus is poorer. The 5’ splice site for AP3 intron 5 matches the consensus at only one position between +3 and +6. The A nucleotide at position +3 is conserved in 68% of Arabidopsis introns, whereas the +4 to +6 positions are conserved in ~50% of introns (Brown, 1996). There is evidence that conservation at these positions is functionally important; for example, mutations at the +5 position have been shown to decrease splicing efficiency in plants (McCullough et al., 1993).

Although not essential for proper splicing, exon sequences adjacent to the intron-exon boundary have been demonstrated to be important for efficient splicing. The 5’ splice site for AP3 intron 5 matches the consensus at the –1 and –2 positions, which are conserved in 78 and 62% of Arabidopsis introns, respectively, but it does not match at –3, which is less conserved (36% of introns; Brown, 1996). The mutation in ap3-1 is a mutation away from the 5’ consensus at position –2 (AG/GU to TG/GU). Similarly, the Arabidopsis spindly-1 (spy-1) mutation contains a mutation in the last base of the exon (a G/GU to A/GU) and results in the skipping of exon 8 in SPY (Jacobsen et al., 1996).
Mechanisms of Splicing in Plants

Most of the biochemical details of the pre-mRNA splicing reaction are based on experiments performed with yeast and animals (reviewed in Staley and Guthrie, 1998). The similarity between the structure of plant and animal introns as well as the conservation of components of the splicing machinery suggest that most of the basic mechanisms of splicing are conserved between animals, plants, and fungi. For example, plant introns contain 5’ and 3’ splice consensus sites; U1, U2, U4/U6, and U5 small nuclear RNAs (snRNAs); and small nuclear ribonucleoproteins (snRNPs), such as U1A, U2B”, SmG, and PRP8. One notable difference between plants and mammals is the failure of plants to conserve the polypyrimidine tract that is located between the branch point and the 3’ splice site; however, plants contain a U-rich sequence that often is located at a position similar to the polypyrimidine tract in animals.

Plant introns, unlike animal introns, are AU rich, and the overall percentage of AU bases and the location of AU-rich stretches in the intron have been demonstrated to be an important determinant for efficient splicing in dicots (Goodall and Filipowicz, 1989; Baynton et al., 1996). Based on these data, it was postulated that perhaps consensus branch point sequences were not necessary and that AU-rich sequences in combination with consensus 5’ and 3’ splice sites might be sufficient to direct accurate and efficient splicing. Recent data, however, suggest that branch point sequences, at least in some cases, play an important role in the efficiency of splicing in plants. In one study (Simpson et al., 1996), branch point mutations were engineered into the pea legumin and wheat amylase introns, and these mutations resulted in at least a fivefold decrease in splicing efficiency but did not affect 3’ splice site selection.

The importance of consensus branch point sequences in plant splicing has been debated for many years. An early re-
port utilizing a mutagenized branch point consensus in a normally efficiently spliced synthetic intron demonstrated little effect on the efficiency or accuracy of splicing (Goodall and Filipowicz, 1989). This paper led to the hypothesis that splicing in plants might proceed by a slightly different mechanism than in mammals and yeast. The lack of an in vitro system for studying plant splicing, however, has made this hypothesis difficult to test directly.

In 80% of plant introns, sequences with a match to the consensus branch point sequence are located between 20 and 60 bases upstream of the 3' splice site, and the average position of the first consensus branch point sequence is at ~27 (Brown et al., 1996; Simpson et al., 1996). In only a few cases have branch points been mapped experimentally, so it is not known whether these branch point consensus sequences represent actual sites of lariat formation.

### Intron versus Exon Definition

The two models for how introns are recognized by the splicing machinery are referred to as the intron definition and exon definition models. The intron definition model proposes that the splicing machinery scans through the pre-mRNA and identifies sequences/signals that are present in introns. According to the intron definition model, all of the signals necessary for the splicing of a particular intron are contained in the intron; in other words, the splicing machinery need only recognize key sequences, such as the 5' splice site, 3' splice site, and branch point sequence, and splicing of that intron can proceed.

The second model, the exon definition model, proposes that the splicing machinery scans through the pre-mRNA and identifies the exons. This model initially was proposed to explain the splicing of mammalian genes, which often have multiple small exons separated by very large introns (reviewed in Berget, 1995). In mammals, the splicing machinery assembled at the branch point and 3' splice site of

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**Figure 4.** Does ap3-11 Suppress the Splicing Defects of ap3-1 by Creating a New Branch Point Sequence?

**A** Alignment of 5’ splice site sequences. The top line shows the consensus 5’ splice site sequence. Conserved sequences in both the exon (capital letters) and intron (lowercase letters) are indicated. The second line shows the wild-type AP3 sequence at the 5’ splice site for intron 5. The third line shows the location of the ap3-1 mutation two base pairs from the 3’ end of exon 5. The ap3-1 mutation is a change of an A to a T, which results in a 5’ splice site sequence that is a poorer match to the consensus.

**B** Alignment of branch point sequences. The top line shows the consensus branch point sequence based on probable Arabidopsis branch points (Brown, 1996). The favored location for a branch point sequence in the intron is between 18 and 40 bases 5’ to the 3’ splice site, but the most favorable branch point sequences are located between positions ~30 and ~40. The second line shows the most probable branch point used by the wild type (wt) and ap3-1; this sequence is a good match to the consensus and is located at position ~19. The bottom line shows the location of the ap3-11 suppressor mutation, which creates a close match to the branch point consensus at position ~33.

**C** Schematic model for how ap3-11 might be functioning. The ap3-11 mutation could result in the creation of a new branch point at position ~33. This new branch point is postulated to suppress the Ap3-1 phenotype by allowing a more favorable interaction between the splicing machinery assembled at the 5’ splice site of intron 5 and the splicing machinery assembled at the branch point and 3’ splice site of intron 4. In ap3-1 alone, a combination of a nonconsensus 5’ splice site sequence for intron 5 and a branch point for intron 4 located at a less than optimal location results in exon skipping of exon 5. We propose that this defect can be partially suppressed by the creation of a new more favorable branch point at position ~33 in intron 4, which stabilizes the interaction between the splicing machinery assembled at the 5’ splice site of intron 5 and the splicing machinery assembled at the branch point and 3’ splice site of intron 4. In ap3-1 alone, a combination of a nonconsensus 5’ splice site sequence for intron 5 and a branch point for intron 4 located at a less than optimal location results in exon skipping of exon 5.
The intragenic suppressor, ap3-11, suppresses the splicing defects of ap3-1 and partially restores AP3 function to the flower. The ap3-11 suppressor mutation is a change from a G to an A, and the mutation is located in intron 4 33 bp 5′ to the 3′ splice site. The location of the ap3-11 suppressor mutation in intron 4 is close to the location of branch point consensus sequence. We propose that this mutation creates a new branch point sequence in intron 4 (Figures 4B and 4C). According to our model, the A nucleotide that is created at −33 in ap3-11 would be the site of lariat formation in ap3-11. This new branch point presumably strengthens interactions between components of the splicing machinery assembled at introns 4 and 5, which in turn strengthens the interaction of the U1 snRNP for the 5′ splice site of intron 5. The fact that the suppressor mutation is located in the preceding intron suggests that there is an interaction between the splicing machinery assembled at adjacent introns (e.g., introns 4 and 5), and presumably, this interaction takes place across exon 5.

AP3 intron 4 contains a second close match to the branch point consensus sequence located at position −19, and this branch point likely is utilized in both the wild type and ap3-1. The putative branch point sequence created by the ap3-11 mutation at −33 may be located at a more optimal distance from the 3′ splice site than the branch point sequence located at position −19; the position of the −19 branch point may be too close to the 3′ splice site to mediate efficient splicing. Based on experiments in mammals, the spacing between the branch point and the 3′ splice site is important for efficient splicing, and reducing the spacing between the branch point and the 3′ splice site reduces the efficiency of splicing (Wierenga et al., 1984). Similar experiments have not been performed in plants, so no information is available that compares the efficiency of splicing by using branch points located at different positions. In fact, the precise location of branch points in plant introns has been experimentally determined for only a very small number of plant introns (Liu and Filipowicz, 1996; Simpson et al., 1996).

We propose that exon skipping in ap3-1 is due to two factors that affect the strength and balance of interactions among splicing factors. The first is the poor match to the consensus 5′ splice site for intron 5 that results from the ap3-1 mutation. The second is the suboptimal location of the branch point for intron 4 (located at position −19). Neither one of these factors alone is sufficient to cause an Ap3 mutant phenotype. The branch point consensus located at position −19 by itself does not cause a defect in splicing, because this branch point is likely utilized in wild-type flowers. Similarly, the ap3-11 mutation creates a new branch point in intron 4 at position −33, but ap3-11 flowers retain the ap3-1 mutation at the 5′ splice site for intron 5. Presumably, the Ap3-1 mutant phenotype results from a combination of the suboptimal intron 4 branch point and poor 5′ splice consensus for intron 5. Specifically, the mutation in the 5′ splice site for intron 5 likely affects the binding of the U1 snRNA to the 5′ splice site. The suboptimal branch point...
at position −19 could affect the binding of either the U2 snRNP to the branch point sequence or a U2AF65-like protein to the AU-rich sequence 3′ to the branch point. Both defects together likely result in the failure of proper interactions of the splicing machinery that occur across exon 5; specifically, the SR proteins may be unable to bridge the weak interactions that take place at both the 5′ splice site for intron 5 and the branch point for intron 4. As a result, exon 5 is not recognized by the splicing machinery, and exon skipping frequently results. This model for the function of the ap3-11 suppressor strongly suggests that exon definition models of intron–exon recognition are operative in plants as well as in animals.

In this study, the ap3-11 mutation was only analyzed together with the ap3-1 mutation (i.e., as an ap3-11 ap3-1 double mutant). We predict that a plant that contained the ap3-11 mutation but was wild type at the position of the ap3-1 mutation would have wild-type flowers. Experiments with mammals in which poor matches to consensus 5′ splice sequences were mutated to closer matches to the consensus resulted in increased efficiency of splicing (Grabowski et al., 1991; Kuo et al., 1991). We also predict that mutating the −3, +3, +5, and +6 positions of the 5′ splice site of intron 5, which have nonconsensus bases, would also suppress the ap3-1 mutation by making the 5′ splice site a closer match to the consensus sequence. It was somewhat surprising that we did not isolate these classes of intragenic suppressor mutations; this could be due either to the failure of these 5′ splice site mutations to suppress or to a bias in the types of mutations generated by ethylmethane sulfonate.

The other factor that may enhance exon skipping is the small size of exon 5 (42 bases). In mammals, it has been demonstrated that internal deletion of an exon to <50 bases resulted in exon skipping (Dominski and Kole, 1991); this effect could be suppressed by making mutations in the 5′ and 3′ splice consensus sequences that resulted in a closer match to consensus sequences. The low level of exon skipping that was observed in studies analyzing the splicing of a synthetic double intron construct in tobacco leaf cells might be explained partially by the large size of the exon (294 bases) that was used in these studies (McCullough et al., 1996).

Because it is now possible to map branch point sequences in Arabidopsis (Liu and Filipowicz, 1996; Simpson et al., 1996), it is possible to test this model to determine whether ap3-1 and ap3-11 utilize alternative branch points.

METHODS

Mutagenesis and Suppressor Screening

ap3-1 (Arabidopsis thaliana) seeds were imbibed in 0.15 or 0.3% ethylmethane sulfonate overnight. The mutagenized seeds were then sown on soil and grown at 16°C (the permissive temperature for ap3-1). M1 plants were allowed to self-fertilize at 16°C. M2 seeds were collected in families, sown on soil and grown at the nonpermissive temperature of 23°C, and screened for plants that exhibited a weak Ap3 phenotype (e.g., restoration of male fertility and/or petal development). In our screen, we isolated four intragenic suppressors from different M2 families. One of these suppressors (isolate 98-1) was studied in detail and is referred to in this study as ap3-11. The T3 ap3-11 suppressor was isolated in a slightly different genetic background (i.e., wild type at the GLABROUS1 [GL1] locus), whereas the other three suppressors were isolated in the gl1-1 background. For the three suppressors isolated in the gl1-1 background, we cannot rule out that these putative independent isolates may have resulted from some sort of contamination or mixing of samples.

Construction of an ap3-11 Partial Genomic Library

Genomic DNA was isolated from ap3-11 plants, digested with EcoRI and ligated to λ DASHII/EcoRI arms, and packaged with the GigapackII XL packaging extract according to the manufacturer's instruction (Stratagene, La Jolla, CA). A 14-kb EcoRI fragment containing the AP3 gene was isolated from this ap3-11 partial genomic library. The AP3 region was subcloned into plasmids and sequenced on an Applied Biosystems 373 automated DNA sequencer (ABI/Perkin-Elmer, Foster City, CA) using primers in the AP3 region.

DNA Sequencing of ap3-1 Suppressors

The exons, intron–exon boundaries, and promoter sequences for the various ap3-1 suppressors were amplified via polymerase chain reaction (PCR) from small-scale genomic DNA preparations (Edwards et al., 1991; Yi and Guerinot, 1996) using pairs of AP3-specific primers. PCR products were directly sequenced using one of the PCR primers.

RNA Isolation and Reverse Transcriptase–PCR

Total RNA was isolated from wild-type [Landsberg erecta (Ler)], ap3-11, and ap3-1 inflorescences grown at 16, 23, or 29°C by using the method of Verwoerd et al. (1989). First-strand cDNA was synthesized with Moloney murine leukemia virus reverse transcriptase (RT; Gibco BRL, Gaithersburg, MD), using oligo(dT)12 as a primer. PCR reactions were performed using cDNA template and AP3 oligonucleotides located in exons 2 (5′-TTGGGCACTCAATAGG-3′) and 7 (5′-GAACGTAGCTGAATCTCC-3′). PCR conditions were as follows: 94°C for 1 min followed by 30 cycles of 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 1.5 min. Agarose gels containing the RT-PCR products were digitally photographed with an Eagle Eye II photodocumentation system (Stratagene). The digital images from the Eagle Eye were opened, and the intensity of the bands was compared using Photoshop 4.0 (Adobe, Mountain View, CA). The RT-PCR experiments were repeated several times, and in all experiments the following were true: (1) for ap3-1 grown at 16°C, the ratio of full-length to deleted product was always >1; (2) for ap3-1 grown at 23 and 29°C, the ratio of full-length to deleted product was always <1; (3) for ap3-11 grown at all three temperatures, the ratio of full-length to deleted product was always >1; and (4) for ap3-11 grown at all three temperatures, the ratio of full-length to deleted product was always greater than the ratio observed for ap3-1 grown at 16°C.
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