Activator Mutagenesis of the Pink scutellum1/viviparous7 Locus of Maize

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The transposable elements Activator/Dissociation (Ac/Ds) were first discovered in maize, yet they have not been used extensively in their native host for gene-tagging experiments. This can be attributed largely to the low forward mutation rate and the propensity for closely linked transpositions associated with Ac and its nonautonomous deletion derivative Ds. To overcome these limitations, we are developing a series of nearly isogenic maize lines, each with a single active Ac element positioned at a well-defined location. These Ac elements are distributed at 10- to 20-centimorgan intervals throughout the genome for use in regional mutagenesis. Here, we demonstrate the utility of this Ac-based gene-tagging approach through the targeted mutagenesis of the pink scutellum1/viviparous7 (ps1/vp7) locus. Using a novel PCR-based technique, the Ps1 gene was cloned and Ac elements positioned precisely in each of the seven alleles recovered. The Ps1 gene is predicted to encode lycopene β-cyclase and is necessary for the accumulation of both abscisic acid and the carotenoid zeaxanthin in mature maize embryos. This study demonstrates the utility of an Ac mutagenesis program to efficiently generate allelic diversity at closely linked loci in maize.

INTRODUCTION

The use of transposable elements as molecular tags for gene isolation (transposon tagging) and characterization has been exploited extensively in plants (reviewed by Kunze et al., 1997). In maize, the lack of an efficient transformation system, the large genome, and the long generation time have made transposable elements particularly attractive for the genetic analysis of gene function. Recently, several reverse genetics resources were developed for maize that promise to speed the elucidation of gene function. Recently, several reverse genetics resources were developed for maize that promise to speed the elucidation of gene function. This can be attributed largely to the low transposition rate (Greenblatt, 1984; Dooner and Belachew, 1989). In studies of Ac transposition from the p and bz loci, ~60% of transpositions are to linked sites, and the majority of these fall within 10 centimorgan (cm) of the donor Ac. Although this propensity for linked transposition limits the use of Ac in random mutagenesis programs, it can be exploited to tag genes that are linked closely to the element. Transpositions generated from donor elements linked closely to target genes have been used to generate Ac- and Dissociation (Ds)-induced alleles of several maize genes (Dellaporta et al., 1988; Hake et al., 1989; DeLong et al., 1993; Colasanti et al., 1998; Shen et al., 2000). However, no systematic study has been performed to examine the efficiency of regional mutagenesis using Ac or Ds in maize.

To examine the utility of Ac in regional mutagenesis, a targeted mutagenesis screen was performed to clone and characterize the Pink scutellum1 (Ps1) gene of maize. The ps1 mutation (Sprague, 1936) conditions pink endosperm and scutellar tissues and results in precocious germination (vivipary) as a result of a block early in the carotenoid/abscisic acid biosynthetic pathway (Figure 1). The Ps1 gene was selected as a target for several reasons. First, an Ac insertion had been mapped to within 4 cm of the Ps1 gene, a genetic distance that is within our target distance of 10 cm. Second, because the Ps1 gene had not been cloned, candidate EST and gene sequences were not available for use in reverse genetics screens, thus providing an opportunity to demonstrate the value of the Ac-tagging approach. Third, ps1 alleles are easily discernible as pink kernels because of the accumulation of lycopene (Neill et al., 1986), enabling a rapid screen of F2 populations for new ps1 insertions.

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.010249.
Geranylgeranyl pyrophosphate

Phytoene (y1)

Phytofluene

ζ-Carotene

Nitrosporene

Neoxanthin (y9)

Lycopene

εLCY

βLCY (y7/ps1)

γ-Carotene

βLCY

α-Carotene

βOH (y3)

εOH

Zeaxanthin

VDE

ZDS

Lutein

IXS (vp92EP8)

Neoxanthin

(9cis-Neoxanthin (vp8)

Xanthoxin

ABA-aldehyde

xanthoxin

AO

ABA

Figure 1. Pathway of Carotenoid and Abscisic Acid Biosynthesis in Maize. The first committed step in the pathway is the formation of phytoene from two molecules of geranylgeranyl pyrophosphate. A series of desaturations (PDS/ZDS) and isomerizations (CRTISO) leads to the formation of the linear carotenoid lycopene. The pathway branches after the cyclization of lycopene by two enzymes. β-Carotene is formed by the action of lycopene β-cyclase (βLCY) at both ends of the lycopene molecule, resulting in two β-rings. α-Carotene is generated through the action of βLCY and lycopene ε-cyclase (εLCY), resulting in an ε-ring and a β-ring. Subsequent hydroxylation and epoxidations lead to the formation of β-carotene derivatives, including abscisic acid (ABA), and α-carotene derivatives such as lutein. Potential blocks in the biosynthetic pathway associated with various maize mutants are shown in parentheses. AO, aldehyde oxidase; CRTISO, carotenoid isomerase; NXS, neoxanthin synthase; pOH, β-ring hydroxylase; εOH, ε-ring hydroxylase; PDS, phytoene desaturase; PSY, phytoene synthase; VDE, violaxanthin deepoxidase; VNCED, 9-cis-epoxy-carotenoid dioxygenase; ZDS, finally, we hoped to identify weak alleles of ps1 that would permit some accumulation of abscisic acid and low levels of lycopene-derived xanthophylls.

RESULTS

Insertional Mutagenesis of the viviparous7 Locus

To generate new ps1 alleles, ~400 transposition events were selected from an Ac element linked closely to the ps1 locus (see Methods). The resulting F1 plants were self-pollinated, and ears were scored for segregation of pink/viviparous kernels. Phenotypic screens of 386 F2 ears resulted in the identification of 7 ears that segregated for putative Ac-induced ps1 alleles (Figure 2). Allelism tests were performed with two of the recovered alleles and the standard ps1 reference allele, ps1-Sprague (ps1-Sp). As shown in Table 1, ~25% of the test-cross progeny displayed a ps1 mutant phenotype when plants heterozygous for the putative Ac insertion allele were crossed to plants heterozygous for the ps1-Sp allele. This test indicated that at least two recessive Pink scutellum1 alleles had been isolated (ps1-m6::Ac [χ2 = 1.49, df = 1, P = 0.22] and ps1-m7::Ac [χ2 = 2.29, df = 1, P = 0.14]). To determine the linkage relationship between the donor Ac (Ac-bti97156) and the ps1 locus, a two-point linkage test was performed, as detailed in Methods. As shown in Table 1, 30 recombinant chromosomes (coarsely spotted aleurone) were detected among 713 chromosomes scored (coarsely spotted plus finely spotted or colorless aleurone), indicating that Ac-bti97156 is within 4 cM of the ps1 locus.

DNA gel blot analysis was performed to identify an Ac-containing restriction fragment length polymorphism that cosegregated with the ps1-m4 mutant phenotype. Because ps1-m4 homozygotes are seedling lethal, approximately two-thirds of segregating F2 plants are expected to be heterozygous for the ps1-m4 allele and one-third are expected to be homozygous for the wild-type (+) Ps1 allele. DNA was isolated from wild-type (ps1-m4/+ or +/+ ) plants that later were self-pollinated to generate segregating F2 populations. F2 ears then were scored for the segregation of ps1 mutants and compared with F1 individuals. DNA gel blot analysis was performed using Ac-specific probes and several methylation-sensitive and -insensitive restriction enzymes. This analysis revealed a 4.8-kb Ac-containing Smal restriction fragment length polymorphism that was present in F1 individuals that carried a ps1-m4 allele but that was absent in F1 plants homozygous for the wild-type Ps1 allele (data not shown).

An inverse PCR protocol then was used to obtain ~450 bp of sequence flanking this insertion, as detailed in Methods. This sequence showed a high degree of sequence similarity to lyco-
and kernels homozygous for wild-type alleles were characterized. Oligonucleotide primers flanking sequences corresponded to the \( \text{Ac} \) insertion site in the donor \( \text{Ac} \) alleles. A unique PCR product was detected for each germinal insertion allele that corresponded to an \( \text{Ac} \) insertion in the appropriate orientation would result in the amplification of an \( \text{Ac-ps1} \) junction fragment. To increase the probability of amplifying these low-copy sequences, two rounds of PCR were performed using nested pairs of \( \text{Ac} \)- and \( \text{Ps1} \)-specific primers. Figure 3B shows the results of an \( \text{Ac} \)-casting experiment using five heterozygous \( \text{ps1} \)-m lines. The \( \text{ps1} \)-flanking sequence was amplified using \( \text{Ps1} \)- and \( \text{Ac} \)-specific primer pairs. A low molecular mass fragment (∼200 bp) was amplified consistently from the \( \text{Ac} \) donor line (Figure 3B, lane 6); however, amplified products were not detected in the \( \text{Ds} \) reporter line (Figure 3B, lane 7). Sequence analysis of 31 PCR products indicated that 23 of them were \( \text{Ac-ps1} \) junction fragments (Figure 4).

Through \( \text{Ac} \) casting, ∼2.2 kb of \( \text{Ps1} \) gene sequence was recovered. Because multiple overlapping PCR fragments were cloned and sequenced from independent \( \text{Ac} \) insertion lines, it was not necessary to perform genomic library screens to recover the complete coding sequence of \( \text{Ps1} \). Furthermore, all \( \text{Ac} \) insertions were generated and maintained in an inbred W22 background; thus, no allelic variation was detected among the various \( \text{Ac} \)-casting products.

**Ps1 Encodes a Lycopene \( \beta \)-Cyclase Homolog**

The maize \( \text{Ps1} \) gene contains a single open reading frame predicted to encode a 53-kD protein that has homology with known plant lycopene \( \beta \)-cyclases (Figure 5). The predicted \( \text{Ps1} \) gene sequence was re-

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**Table 1. Genetic Analysis**

<table>
<thead>
<tr>
<th>Cross</th>
<th>Pink Scutellum</th>
<th>Aleurone Phenotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Full Color</td>
<td>Coarse Spots</td>
</tr>
<tr>
<td>( d\text{Ac ps1-m6}/+ \times \text{ps1-Sp}/+ )</td>
<td>88</td>
<td>35</td>
<td>13</td>
</tr>
<tr>
<td>( d\text{Ac ps1-m7}/+ \times \text{ps1-Sp}/+ )</td>
<td>111</td>
<td>39</td>
<td>17</td>
</tr>
<tr>
<td>Total</td>
<td>199</td>
<td>74</td>
<td>30</td>
</tr>
</tbody>
</table>

*Seed counts combined from two F1 progeny ears for each \( \text{ps1} \) mutant genotype. Allelism tests were performed with the \( \text{ps1-Sp} \) reference allele obtained from the Maize Genetics Cooperation Stock Center (Urbana, IL).*
protein of maize shares several conserved domains and 65 to 68% amino acid identity with other plant lycopene β-cyclases (Hugueney et al., 1995; Cunningham et al., 1996; Pecker et al., 1996). This similarity is most striking in predicted domains that are conserved among all characterized lycopene β-cyclase proteins (Figure 5). By contrast, the similarity between the maize PS1 protein and plant lycopene ε-cyclases is less extensive. For instance, the maize PS1 protein is 31% identical to a lycopene ε-cyclase from Arabidopsis (Figure 5). The high degree of sequence conservation between the maize PS1 protein and several well-characterized plant lycopene β-cyclases strongly suggests that the Ps1 gene of maize encodes lycopene β-cyclase.

Expression Profile of Ps1

Transcripts derived from Ps1 were not detectable by RNA gel blot analysis using up to 10 μg of poly(A⁺) RNA (data not shown). Thus, a reverse transcriptase–mediated (RT) PCR assay was developed to examine the levels of Ps1 transcript in wild-type and ps1 mutant tissues. As shown in Figure 6, Ps1 transcripts were detectable in light-grown leaf tissue, etiolated leaf tissue, roots, and embryos from wild-type seedlings but were less abundant in ps1-m5 mutant embryos (Figure 6, lane 9). Because the primer sequences used in the RT-PCR assay are 3’ to the site of Ac insertion in the ps1-m5 allele, the transcripts detected most likely are derived from somatic excision events. The faint band seen in lane 4 of Figure 6 likely is attributable to contaminating DNA, as revealed in the no-RT control. The bottom gel in Figure 6 shows RT-PCR products using ubiquitin primers and equal amounts of cDNA template as a control for cDNA synthesis. These results indicate that Ps1 transcripts accumulated in several plant tissues and are greatly reduced in ps1 mutant embryos.

Characterization of ps1 Mutant Alleles

It has been shown previously that lutein and zeaxanthin are the major carotenoids that accumulate in wild-type maize kernels, whereas lycopene is the predominant carotenoid in endosperm and embryo tissues of ps1/vp7 mutants (Neill et al., 1986; Lee et al., 1997). To characterize carotenoid accumulation in the various Ac-induced ps1 alleles, HPLC analysis was performed on extracts from individual wild-type and mutant ps1 embryos. As shown in Figure 7, the primary carotenoid that accumulated in wild-type maize embryos at 20 days after pollination was zeaxanthin. Representative HPLC elution profiles are shown for three of the mutant alleles. As shown in Figure 2, ps1-m3::Ac and ps1-m7::Ac alleles condition a relatively weak mutant phenotype, and the ps1-m5::Ac allele conditions a strong mutant phenotype. Comparisons of the retention time and absorption spectrum of the all-trans-lycopene standard with the mutant extracts indicate that the primary carotenoid accumulating in ps1 mutant embryos is all-trans-lycopene. However, putative cis-isomers of lycopene (shoulders off the lycopene peak in Figure 7) also may accumulate in ps1 mutant embryos. Although the predominant carotenoid in embryos carrying the weak ps1 alleles was lycopene, low levels of zeaxanthin also were detected, as confirmed by comparing HPLC retention times, absorption peak maxima, and peak ratios with lutein and zeaxanthin standards (Figure 7). This finding indicates a low level of lycopene β-cyclase activity in the ps1-m3::Ac and ps1-m7::Ac mutant embryos. By contrast, extracts from the severe mutant allele ps1-m5::Ac contained no detectable zeaxanthin (Figure 7).

DISCUSSION

An Ac insertional mutagenesis strategy has resulted in the successful cloning and characterization of the maize Ps1/Vp7 gene. Sequence analysis and HPLC data strongly suggest that the Ps1 gene encodes lycopene β-cyclase. In plants, the linear carotene lycopene is the precursor for cyclic carotenoids (reviewed by Hirschberg, 2001) and lies at the branch point of two pathways (Figure 1). In one branch, the formation of a β-ionone ring at both ends of lycopene through the action of lycopene β-cyclase leads to the formation of β-carotene and its deriva-
tives, including abscisic acid. In the other branch, the coaction of a lycopene \( \varepsilon \)-cyclase and lycopene \( \beta \)-cyclase leads to the synthesis of \( \alpha \)-carotene and its derivatives, including lutein. As-says for higher plant lycopene \( \beta \)-cyclase activity have been developed based on an elegant "color complementation" assay of \textit{Escherichia coli} engineered to produce intermediates in the carotenoid biosynthetic pathway (Hugueney et al., 1995; Cunningham et al., 1996; Pecker et al., 1996). In this \textit{E. coli} system, an Arabidopsis cDNA clone encoding lycopene \( \varepsilon \)-cyclase uses both neurosporene and lycopene as substrates in the formation of the monocarotenoid \( \alpha \)-zeacarotene and \( \delta \)-carotene, respectively (Cunningham et al., 1996).

One scenario consistent with these findings is that lycopene \( \beta \)-cyclase and lycopene \( \varepsilon \)-cyclase constitute an enzyme complex in the thylakoid membranes of plant chloroplasts (Cunningham and Gantt, 1998). In the absence of \( \beta \)-cyclase, the complex is destabilized, resulting in the loss of both \( \varepsilon \)- and \( \beta \)-cyclase activity and the consequent accumulation of lycopene. In maize embryos, zeaxanthin is the primary carotenoid that accumulates and is not dependent on the activity of \( \varepsilon \)-cyclase for its formation (Figure 1). However, in maize leaf tissue, the formation of lutein is dependent on the activities of both lycopene \( \varepsilon \)-cyclase and lycopene \( \beta \)-cyclase (Figure 1). Thus, an examination of leaf tissues from the weak mutant alleles may provide a means of testing the hypothesis that \( \varepsilon \)-cyclase activity is dependent on the accumulation of \( \beta \)-cyclase. If \( \varepsilon \)-cyclase could function in the absence of \( \beta \)-cyclase, \( \delta \)-carotene would be expected to accumulate. Alternately, the accumulation of lycopene in leaf tissues of weak \( \text{ps1} \) mutants would strongly suggest that \( \varepsilon \)-cyclase activity is dependent on the presence of lycopene \( \beta \)-cyclase.

Comparisons of \textit{Synechococcus} lycopene cyclase (Cunningham et al., 1994) and higher plant lycopene \( \beta \)-cyclase proteins (Pecker et al., 1996) strongly suggest the transfer of a lycopene cyclase from an ancient endosymbiont to the nucleus in the eudicot lycopene \( \beta \)-cyclase proteins (Cunningham et al., 1996) and higher plant lycopene \( \beta \)-cyclase enzymes suggests that even subtle perturbations of the amino acid sequence are likely to be detrimental. Indeed, all seven of the \( \text{Ac} \) alleles recovered resulted in seedling-lethal phenotypes, even though the most 3’ insertion was positioned at a site \( \sim 16 \) amino acids from the C terminus of the protein.

Although all \( \text{Ac} \)-induced alleles resulted in seedling-lethal phenotypes, phenotypic differences were observed among the alleles. Five of the seven \( \text{Ac} \) insertion alleles resulted in vivipary, presumably as a result of a failure to accumulate significant pools of abscisic acid (Neill et al., 1986). However, the

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that phenotypic variation is attributable to segregating modifier loci. It also is unlikely that precise Ac excision events that restore Ps1 function are responsible for the phenotypic variation, because the frequency of such functional Ac excision alleles should be independent of the insertion site within the gene. Instead, it is likely that Ps1 transcripts produced in the ps1-m3::Ac– and ps1-m7::Ac–containing lines are capable of directing the synthesis of proteins with some PS1/VP7 activity. This activity is sufficient to prevent precocious germination through the accumulation of abscisic acid.

Comparisons of carotenoid profiles from wild-type and mutant embryos also indicate allelic differences in PS1/VP7 activity. As shown previously (Lee et al., 1997; Tan et al., 1997) and in Figure 7, zeaxanthin is the primary carotenoid accumulating in wild-type maize embryos. In the weak alleles, low levels of zeaxanthin were detected, again suggesting the presence of some PS1 activity in the ps1-m3::Ac– and ps1-m7::Ac–containing lines. Examination of the carotenoid pools in the mutant embryos revealed that the major carotenoid was all-trans-lycopene. However, additional carotenoids, possibly including cis-isomers of lycopene, accumulated to varying levels in the mutant alleles. It is interesting that allele-specific carotenoid profiles also were detected in two tomato carotenoid isomerase mutants (Isaacson et al., 2002), suggesting that HPLC analysis may be an effective tool for identifying weak mutant alleles of enzymes in the carotenoid biosynthetic pathway. In summary, both germination assays and HPLC analyses indicate that complete and partial loss-of-function ps1 alleles were generated through Ac insertional mutagenesis.

Development of an Ac Insertional Mutagenesis Program

An important aim of this study was to develop generalized protocols and techniques for Ac insertional mutagenesis in maize. The Ps1/Vp7 gene was chosen as a target in part for the ease of recovering Ac insertion alleles through phenotypic screens of

Figure 5. Alignment of Plant Lycopene β-Cyclase Enzyme Sequences.

Sequences of predicted lycopene β-cyclase proteins from Arabidopsis thaliana, Lycopersicon esculentum, Narcissus pseudonarcissus, Nicotiana tabacum, Sandersonia aurantiaca, and Zea mays and a lycopene β-cyclase from Arabidopsis are shown. A partial wheat (Triticum aestivum) 5′ EST sequence also is included to reveal N-terminal sequences conserved between maize and wheat. Identical amino acid sequences are shown with white letters on a black background, and gaps are identified by dashes. Previously identified regions of interest (Cunningham et al., 1996) are shown.

TM, transmembrane.
self-pollinated F2 ears. Although the physical distance that separates the donor Ac from the ps1 locus is not known, the genetic distance is <4 cM and is likely to be optimal for targeted mutagenesis programs (Greenblatt, 1984; Dooner and Belachew, 1989). In fact, a 2% recovery of ps1 alleles from the donor Ac (7 alleles per 400 transpositions) was much higher than expected and suggests that the donor Ac may reside very close to the ps1 locus. Recent studies of Ac transposition at the bz locus indicated that Ac displays an insertion site bias favoring sites that are at a close genetic distance, regardless of the physical distance separating the donor Ac and the target site (Fu et al., 2002). This finding likely reflects that fact that Ac insertions occur primarily in gene-rich regions of the genome (Cowperthwaite et al., 2002), where most recombination events are localized (Fu et al., 2001).

In Arabidopsis, the characterization of a number of Ds insertion lines suggested that Ds preferentially inserts in the 5′ regions of genes (Sundaresan et al., 1995). Our examination of 7 germlinal insertion sites and 23 somatic insertion sites revealed no such bias, with insertions occurring throughout the Ps1 gene. Interestingly, two of the seven ps1 alleles recovered contained Ac insertions at identical positions and in the same orientation within the Ps1 gene. In addition, one germlinal insertion and three somatic insertions recovered from Ac casting mapped to the same position, suggesting that these sites may

**Figure 6.** RT-PCR Analysis of Ps1 Transcript Accumulation.

RT-PCR products were fractionated on 1.5% agarose gels and blotted to a nylon membrane. Products were detected using a Ps1-specific probe (top gel) or a ubiquitin probe (bottom gel). RNA was isolated from light-grown seedling leaf tissue (lanes 1 and 2), etiolated leaf tissue (lanes 3 and 4), seedling root tissue (lanes 5 and 6), mature embryos (lanes 7 and 8), and mutant ps1-m5::Ac embryos (lanes 9 and 10). Reverse transcriptase was added to the cDNA synthesis cocktail (odd-numbered lanes) or excluded from the reaction mix (even-numbered lanes).

**Figure 7.** HPLC Analysis of Wild-Type and Mutant ps1 Embryos.

Carotenoids were extracted from embryos at 20 days after pollination and fractionated using reverse-phase HPLC. Spectral characteristics of the elution peaks were compared in wild-type (WT) and mutant (ps1-m3::Ac, ps1-m5::Ac, and ps1-m7::Ac) individuals in addition to the carotenoid standards lutein (Lut), zeaxanthin (Zea), and lycopene (Lyc). The two inset boxes show the absorption profiles of the lycopene and zeaxanthin peaks from the standards and the ps1-m3::Ac mutant.
represent a “hot spot” for Ac insertion. Similar hot spots for Ac insertion have been identified at the p locus of maize (Athma et al., 1992; Moreno et al., 1992), suggesting that Ac may show some target site selection bias. Nevertheless, multiple somatic and germinal insertions were recovered throughout the Ps1 gene, indicating that insertion site preference does not limit the utility of Ac in regional mutagenesis.

To further exploit the utility of Ac in gene characterization, a PCR-based protocol, termed Ac casting, was developed to clone sequences flanking Ac insertions. Ac casting provides a rapid and inexpensive tool for gene walking in maize. The relatively high rate of somatic Ac transposition and the preference for local insertion sites enriches the recovery of sequences near the donor site. The technique was used successfully to obtain both 5' and 3' sequences near the donor site. The technique was useful when the DNA flanking an Ac insertion is sufficiently repetitive that a single-copy or low-copy DNA probe cannot be generated. In these cases, additional flanking sequences derived from Ac casting could be used to identify a unique flanking probe or to precisely place the insertion once the maize genomic sequence is available. Although the fragment lengths recovered were relatively short, ranging from 500 bp to ∼1 kb, the use of long-range Taq polymerases could increase the size of the amplified fragments.

**Future Prospects**

Through the insertional mutagenesis of Ps1, we have demonstrated the utility of Ac as a tool for directed mutagenesis. The uniformity of the inbred lines permits subtle phenotypic comparisons between recovered alleles, and the relatively low forward mutation rate associated with Ac makes it unlikely that the observed phenotypes are caused by insertions in multiple genes. Unlike Mu-induced alleles, germinal revertants can be selected easily with Ac-induced alleles. Furthermore, Ac/Ds excision events often are imprecise (Moreno et al., 1992; Scott et al., 1996), resulting in the creation of novel “footprint” alleles, which can serve as new sources for allelic diversity for the analysis of gene function or for selection in the agronomic improvement of this important crop plant.

**METHODS**

**Genetic Analysis**

Regional mutagenesis of the ps1 locus was performed using Ac-bti97156 as a donor Ac. The Ac and Ds reporters (r-scm3) were maintained in a color-converted W22 inbred line of maize (Zea mays) (Dooner and Keramic, 1971; Kermic, 1984). Most test-cross kernels inherit a single active Ac from the female gametophyte, resulting in a coarsely spotted aleurone. Increases in Ac copy number delay the timing of Ds excision from the p locus, resulting in a finely spotted aleurone (Brutnell and Dellaporta, 1994). Transpositions were selected after a test cross of ∼200 plants homozygous for the Ac-bti97156 insertion by pollen from the Ds reporter line. Approximately 400 putative transposition events were selected as finely spotted kernels. Plants were self-pollinated, and ears were screened for the segregation of pink/viviparous kernel phenotypes.

Allelism tests and linkage analysis were performed through test crosses of plants heterozygous for Ac-bti97156, the donor Ac (dAc), and a mutable ps1 allele (dAc ps1-m/+, r-scm3/r-scm3) to pollen from plants heterozygous for the ps1 reference allele (ps1+Sp+/+, r-g/r-g). To examine the genetic distance separating the dAc and ps1 loci, recombinant chromosomes were scored by monitoring the copy number of Ac using the Ds reporter r-scm3. In the absence of Ac, the Ds insertion is stable, resulting in a colorless aleurone. In the presence of Ac, Ds excisions occur at a time and frequency that is dependent on the Ac copy number in the genome (McClintock, 1948). Increases in Ac copy number result in later excision events and, as a result, smaller, more highly colored aleurone sectors. Transmission of both the dAc and Ac insertions at Ps1 through the female gametophyte results in a finely spotted aleurone as a result of the high Ac copy number. Recombinant chromosomes carrying either the dAc allele or the ps1-m allele transmit a single copy of Ac, resulting in a coarsely spotted aleurone. The recombinant fraction p was estimated as p = s/(s + f + c), where s is the number of coarsely spotted kernels, f is the number of finely spotted kernels, and c is the number of colorless kernels. Errors in the estimate of p arise from unlinked transpositions from either of the two Ac elements or from excisions of Ac without reinserion. Thus, the calculated genetic distance between the dAc and ps1 loci likely is an overestimate of the true genetic distance.

**Extraction and Analysis of Carotenoids**

Total carotenoids were extracted from frozen maize embryos as described previously (Norris et al., 1995) and separated by reverse-phase HPLC using a mobile phase of 0 to 100% B in 45 min at 1 mL/min, where A was acetonitrile:water:triethylamine (9:1:0.1) [v/v/v] and B was ethyl acetate (Goodwin and Britton, 1988). The solid phase consisted of an UltraspHERE ODS5 precolumn (Beckman Coulter, Fullerton, CA) and a Spherisorb S5 ODS1 column (Phase Sep, Norwalk, CT), and the separation was controlled using a Beckman System Gold HPLC apparatus reading at 440 nm. Photodiode array measurements were performed with a 600E controller, a 600 solvent delivery system, and a 990+ diode array detector (Waters, Milford, MA), measuring from 300 to 600 nm at a 2-nm bandwidth and with 4 oversampling, applying the same separation method. The carotenoid standards lutein (γ-carotene-3,3′-diol) and lycopene (β-carotene) were obtained from Sigma (St. Louis, MO). The zeaxanthin standard was obtained from extracts of commercially available capsules (Zeavision, St. Louis, MO).

**Inverse PCR Analysis**

Sequences flanking the Ac elements were isolated using an inverse PCR technique (Ochman et al., 1988). DNA gel blot analysis using a 700-bp Ac-specific internal HindIII-EcoRI restriction fragment (Ac700) identified a 4.8-kb Smal fragment that co-segregated with the ps1-m4:Ac mutant allele. To clone sequences flanking the Ac element, ∼15 μg of genomic DNA was digested with Smal and fractionated on a 0.8% agarose gel. A gel slice that encompassed the DNA fragment containing Ac was excised, and the DNA was extracted using GeneClean III (Qbiogene, Carlsbad, CA) according to the manufacturer’s recommendations. Approximately 50 ng of DNA was self-ligated overnight at 4°C in a 50-μL reaction using T4 DNA ligase (Promega, Madison, WI). A 10-μL aliquot was used directly in the subsequent inverse PCR. Inverse PCR was performed with the Ac-specific primers listed in Table 1. DNA was first denatured at 95°C for 2 min. Amplification was performed for 30 cycles as follows: denaturation at 95°C for 30 s, annealing at 59°C for 1 min, and extension at 72°C for 3 min. At the end of 30 cycles, products were extended at 72°C for 5 min. Products were diluted 50-fold in MilliQ distilled

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water (Millipore, Bedford, MA), and a 10-μL aliquot of diluted PCR products was used in a second round of PCR. DNA products were fractionated on 0.8% agarose gels, and inverse PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA). Products were cloned into pGEM-T Easy vector (Promega) and sequenced using the ABI PRISM Big-Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA). Labeled products were visualized on an ABI377 DNA Sequencer (Applied Biosystems) and assembled using the Lasergene software suite (DNASTAR, Madison, WI).

Ac Casting

Leaf tissue for DNA isolation was collected from the Ps1 Ac-mutable lines by sampling across the width of mature leaves using a hole punch. Fifty hole-punch disks were sampled for each individual plant, and genomic DNA was isolated (Chen and Dellaporta, 1994). The nested Ac-specific primers TBP35, TBP34, JGP2, and JGP3 were used in conjunction with nested Ps1 gene-specific primers to amplify Ac junction fragments from somatic transpositions (see Table 2 for primer sequences). The Ps1-specific primers PS1.8, PS1.4, PS1-10, and PS1-12 were used to clone the 5′ Ps1 sequence, and primers PS1.3 and PS1.7 were used to recover the 3′ Ps1 sequence. The PCR mixture consisted of 10 ng of template DNA, 0.5 μM of each primer, 0.2 mM deoxynucleotide triphosphates, and 2.5 units of Taq polymerase (Promega). PCR cycling conditions consisted of 35 cycles of denaturing at 95°C for 30 s, annealing at 57°C for 1 min, and extension at 72°C for 2 min. After the final cycle, a 10-min extension was performed at 72°C. Ten microliters of a 1:50 dilution of the first-round PCR product was used as a template for the second round of PCR. Amplification products were fractionated on a 0.8% agarose gel and purified using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s protocol. Purified amplification products were ligated into pGEM-T Easy vector (Promega) and sequenced.

Sequence Alignments

Predicted full-length sequences of lycopene β-cyclase proteins from Arabidopsis, tomato, Narcissus pseudonarcissus, Sandersonia aurantiaca, and tobacco and a lycopene ε-cyclase from Arabidopsis were aligned with the predicted PS1 product and a partial translation of a wheat EST sequence with CLUSTAL V using MegAlign software (DNASTAR).

Reverse Transcriptase–Mediated PCR Analysis

Total RNA was isolated from etiolated seedlings, leaves of etiolated seedlings, and wild type and ps1-m5 embryos harvested at 20 days after pollination using TRizol reagent (Invitrogen, Carlsbad, CA). RNA samples were treated with DNase for 30 min at 37°C in a 50-μL reaction consisting of 25 μg of RNA, 5 units of DNase enzyme, and 40 units of RNaseOUT ribonuclease inhibitor (Invitrogen). Four micrograms of DNase-treated RNA was reverse transcribed with oligo(dT)12-18 using the SuperScript First-Strand Synthesis Kit (Invitrogen). Two microliters of the reverse transcriptase (RT) and no-RT (no RT added to the mix) reaction products was subjected to 20 cycles of PCR amplification using PS1 (PS1-15 and PS1-18) or ubiquitin (ZmUb2.1 and ZmUb2.2) gene-specific primer pairs (Table 2). Each PCR cycle consisted of denaturation at 94°C for 45 s, annealing at 57°C for PS1 and 55°C for the ubiquitin primers for 1 min, and extension at 72°C for 1 min. Fifteen microliters of amplification products was fractionated on a 1.5% agarose gel and blotted onto a Hybond-N+ (Amersham Pharmacia Biotech, Piscataway, NJ) membrane. The blots were probed with the radiolabeled Ps1-specific fragment described below. A ubiquitin-specific probe was generated from RT-PCR products using ZmUb2.1 and ZmUb2.2.

Recombinant Inbred Mapping

DNA from 46 individuals derived from a T232 × CM37 inbred population was digested with SacI and probed with the Ps1-specific fragment described below. Polymorphisms were scored and used to determine the map position of Ps1/Vp7 (Burr et al., 1988).

DNA Gel Blot Analysis

Total DNA was extracted from ~1 g of maize leaf tissue as described previously (Chen and Dellaporta, 1994), and 3-μg aliquots were fractionated on a 0.8% agarose gel and transferred to a Hybond-N+ (Amersham) nylon membrane. Digoxigenin (DIG)-labeled DNA probes were synthe-

### Table 2. Primer Sequences

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac primers</td>
<td></td>
</tr>
<tr>
<td>TBP34, second-round PCR (Ac casting)</td>
<td>5′-ACCTCGGATTCGAAATTCGATCGG-3′</td>
</tr>
<tr>
<td>TBP35, first-round PCR (Ac casting)</td>
<td>5′-GTGGGAAACTAGTCTACCCG-3′</td>
</tr>
<tr>
<td>JGP2, first-round PCR (Ac casting)</td>
<td>5′-CGGGCTCCGGATCGCGAAGC-3′</td>
</tr>
<tr>
<td>JGP3, second-round PCR (Ac casting)</td>
<td>5′-ACCGAAGGATGATAGCGACG-3′</td>
</tr>
<tr>
<td>Ps1 primers</td>
<td></td>
</tr>
<tr>
<td>PS1.3, first-round PCR (Ac casting)</td>
<td>5′-CTTCCCCATCAGCCATGCTTCTCC-3′</td>
</tr>
<tr>
<td>PS1.4, second-round PCR (Ac casting); probe synthesis</td>
<td>5′-GGAGAAAGCATGCTGATCGAAAG-3′</td>
</tr>
<tr>
<td>PS1.7, second-round PCR (Ac casting)</td>
<td>5′-CATCCAGACTGTTCTCCTGCGGACC-3′</td>
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<tr>
<td>PS1.8, first-round PCR (Ac casting)</td>
<td>5′-GCTCCGACAGGAGCTGATCGT-3′</td>
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<tr>
<td>PS1-10, second-round PCR (Ac casting)</td>
<td>5′-CAGGTCAAGGAGCTCGAGCTCC-3′</td>
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<tr>
<td>PS1-12, first-round PCR (Ac casting)</td>
<td>5′-GCTCGATGCGCCCAACACGCGAG-3′</td>
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<tr>
<td>PS1-13, probe synthesis</td>
<td>5′-GACATCTCCCGCGGATCGAGGCC-3′</td>
</tr>
<tr>
<td>PS1-15, RT-PCR</td>
<td>5′-CTCGGGATTCGACAGGAGACC-3′</td>
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<tr>
<td>PS1-18, RT-PCR</td>
<td>5′-CTGGCAAGGATCGGCCATCC-3′</td>
</tr>
<tr>
<td>Ubiquitin primers</td>
<td></td>
</tr>
<tr>
<td>ZmUb2.1</td>
<td>5′-TCACACACTCAAGAGGAGGAGC-3′</td>
</tr>
<tr>
<td>ZmUb2.2</td>
<td>5′-TCTGCAAGGATCGGCCATCC-3′</td>
</tr>
</tbody>
</table>
Ac Mutagenesis of the Ps1/Vp7 Locus 883

sized using the Roche PCR DIG Probe Synthesis Kit (Indianapolis, IN) according to the manufacturer’s recommendations. To generate a Ps1-specific probe, DNA from pPS1118 was labeled using primers PS1-13 and PS1.4. Hybridizations were performed using DIG Easy Hyb (Roche) as described previously (Sawers et al., 2002).

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

Accession numbers for the proteins mentioned in this article are as follows: U50739 (predicted full-length lycopene β-cyclase from Arabidopsis), X86452 (lycopene β-cyclase from tomato), X98796 (lycopene β-cyclase from N. pseudonarcissus), X81787 (lycopene β-cyclase from tobacco), AAL92175 (lycopene β-cyclase from S. aurantiaca), US0738 (lycopene ε-cyclase from Arabidopsis), AAY20682 (predicted PS1 product), and BQ608561 (partial translation of a wheat EST sequence).

ACKNOWLEDGMENTS

We thank Phyllis Farmer (Boyce Thompson Institute) and Ben Burr (Brookhaven National Laboratories, Upton, NY) for providing the map position of Ps1. We also acknowledge Sara Lebejko (Monsanto, Mystic, CT) for her assistance in sequencing the Ps1 gene and Mike Edgerton (Monsanto, St. Louis, MO) for helpful discussions. This work was supported by National Science Foundation Grant DBI-0076892 to T.P.B.

Received December 31, 2002; accepted February 10, 2003.

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Activator Mutagenesis of the Pink scutellum1/viviparous7 Locus of Maize
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Plant Cell 2003;15:874-884; originally published online March 4, 2003;
DOI 10.1105/tpc.010249

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