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Mutations in the *pale aleurone color1* Regulatory Gene of the *Zea mays* Anthocyanin Pathway Have Distinct Phenotypes Relative to the Functionally Similar *TRANSPARENT TESTA GLABRA1* Gene in *Arabidopsis thaliana*[W]

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The *pale aleurone color1* (*pac1*) locus, required for anthocyanin pigment in the aleurone and scutellum of the *Zea mays* (maize) seed, was cloned using *Mutator* transposon tagging. *pac1* encodes a WD40 repeat protein closely related to anthocyanin regulatory proteins ANTHOCYANIN11 (AN11) (*Petunia hybrida* [petunia]) and TRANSPARENT TESTA GLABRA1 (TTG1) (*Arabidopsis thaliana*). Introduction of a *35S-Pac1* transgene into *A. thaliana* complemented multiple *ttg1* mutant phenotypes, including ones nonexistent in *Z. mays*. Hybridization of *Z. mays* genomic BAC clones with the *pac1* sequence identified an additional related gene, *mp1*. PAC1 and MP1 deduced protein sequences were used as queries to build a phylogenetic tree of homologous WD40 repeat proteins, revealing an ancestral gene duplication leading to two clades in plants, the PAC1 clade and the MP1 clade. Subsequent duplications within each clade have led to additional WD40 repeat proteins in particular species, with all mutants defective in anthocyanin expression contained in the PAC1 clade. Substantial differences in *pac1*, *an11*, and *ttg1* mutant phenotypes suggest the evolutionary divergence of regulatory mechanisms for several traits that cannot be ascribed solely to divergence of the dicot and monocot protein sequences.

INTRODUCTION

Flavonoids are involved in many important processes, including auxin transport (Brown et al., 2001; Peer et al., 2001), attraction of pollinators (Mol et al., 1998), defense against predators (Dooner et al., 1991; Grotewold et al., 1998), and protection against UV damage (Stapleton and Walbot, 1994). The visible yet dispensable nature of many flavonoid molecules, especially anthocyanins, has enabled the identification of many biosynthetic genes and their regulators, providing an excellent model for investigations of gene regulation across a wide variety of plants. The flavonoid pathways have both similarities and differences in regulation in different species.

The anthocyanin pathway in *Zea mays* (maize) is one of the most thoroughly investigated branches of flavonoid metabolism. The products of the pathway are red and purple pigments that are easily scored and not required for *Z. mays* growth and reproduction, properties that have aided in the identification of many anthocyanin biosynthetic genes and several regulators of the pathway (reviewed in Dooner et al., 1991). Two classes of regulatory genes encode basic helix-loop-helix (bHLH) and MYB transcription factors (Paz-Ares et al., 1987; Chandler et al., 1989; Ludwig et al., 1989; Cone et al., 1993). The bHLH (BOOSTER1 [B] or RED1 [R]) and MYB (PURPLE PLANT1 [Pl1] or COLORED ALEURONE1 [C1]) proteins coordinately activate transcription of the biosynthetic genes through protein–protein interactions (Goff et al., 1992). The multiple members of each class are functionally redundant, and typically only one of the *b* or *r* genes and one of the *c1* or *pl1* genes are expressed in any given tissue. In aleurone, *b* and *r* alleles expressed in the seed (Styles et al., 1973) act in combination with *c1*, which directly binds to the promoters of the biosynthetic genes, activating transcription (Sainz et al., 1997; Lesnick and Chandler, 1998).

Comparison of anthocyanin regulation in several dicot species (*Petunia hybrida* [petunia], *Antirrhinum majus* [snapdragon], and *Arabidopsis thaliana*) with that in *Z. mays* reveals similarities and interesting differences. In both *Z. mays* and dicots, conserved enzymatic genes are activated in groups or modules, with each group or module responsible for flux through specific branches of the flavonoid pathways leading to anthocyanin or proanthocyanidin pigmentation. These groups or modules are regulated in each species by similar sets of regulatory proteins, including bHLH and MYB proteins. Differences include the presence of species-specific enzymes at certain positions in the pathway and differences in the grouping of genes into modules. In dicots, anthocyanin biosynthetic genes are divided into early and late biosynthetic genes (Martin and Gerats, 1993). By contrast, many of the functionally analogous early and late biosynthetic genes in *Z. mays* are coordinately controlled as a single module with no division

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between early and late biosynthetic gene regulation (reviewed in Mol et al., 1998). *Z. mays* and dicots also may differ with respect to regulation of the regulatory genes. In dicots, some regulatory proteins are capable of increasing the RNA levels of some of the other regulatory genes. Several examples of this have been reported, including the activation of the *TRANSPARENT TESTA8* (*TT8*) bHLH gene by the TRANSPARENT TESTA GLABRA1 (TTG1) protein in *A. thaliana* and the induction of *TT8* expression by ectopic expression of the *TT2* MYB gene (Nesi et al., 2000, 2001; Spelt et al., 2000). This contrasts significantly with *Z. mays*, in which the identified regulators of the biosynthetic genes are not under each other's control (Goff et al., 1990). Significant differences between the regulatory programs in dicots and monocots also can be illustrated in comparisons of regulatory genes in one species with homologous genes in another species, combined with a consideration of whether their functions are identical. As an example, the bHLH encoding *anthocyanin1* (*an1*) and *TT8* genes in dicots (Nesi et al., 2000; Spelt et al., 2000) share stronger similarity with the *Z. mays intensifier1* (*in1*) gene (Burr et al., 1996) than with the *Z. mays b* and *r* genes. This is significant because *an1* and *TT8* are positive activators of the pathway, as are *b* and *r*, but *an1* and *TT8* are more closely related by sequence to *in1*, which functions as a negative regulator. Thus, evolution of the modules of flavonoid biosynthetic genes and the regulation of these modules has taken significantly different routes in the dicot and monocot species investigated. The result is that the regulatory framework, including the hierarchical organization of the regulators' functions, cannot be predicted solely from homology.

In a screen for new regulators of the *Z. mays* anthocyanin pathway (Selinger and Chandler, 1999), the *pale aleurone color1* (*pac1*) locus was identified by a mutation that resulted in pale aleurone color. Determination of the RNA levels of several of the biosynthetic genes established that *pac1* is required for normal RNA levels of the biosynthetic genes. By contrast, *pac1* is not required for normal RNA levels of the regulatory genes *b* and *c1*. Either B or R bHLH proteins require PAC1 for full activation of the biosynthetic genes. Different alleles of *b* and *r* that confer different tissue-specific patterns of pigmentation require *pac1* function in the aleurone and scutellum but not in husk, sheath, tassel, and anthers (Selinger and Chandler, 1999).

We report the cloning and characterization of the locus encoding the PAC1 protein. The PAC1 sequence is most similar to AN11 in *P. hybrida* and TTG1 in *A. thaliana* (de Vetten et al., 1997; Walker et al., 1999). All three encode WD40 repeat proteins involved in regulation of anthocyanin pigmentation, although the precise means by which this regulation is accomplished varies among species. In addition to anthocyanin production, other species-specific traits require the function of this class of WD40 repeat proteins. To begin to understand the evolutionary context for species-specific differences in anthocyanin gene regulation, we complemented the *ttg1* mutant phenotypes using a *35S-Pac1* transgene, further investigated *pac1* mutant phenotypes in *Z. mays*, isolated another gene encoding a related WD40 repeat protein from *Z. mays* (*mp1*), and characterized the phylogenetic relationships among members of this WD40 repeat protein family.

RESULTS

Identification and Characterization of a *Mu1* Insertion Cosegregating with the *pac1* Mutant Phenotype

The *pac1-ref* allele was isolated in a screen for pale aleurone color mutants in a line that contained active *Mutator* (*Mu*) transposons (Selinger and Chandler, 1999). When crossed with an active *Mu* line, dark purple sectors (spots) in *pac1-ref* aleurones were observed, suggesting the presence of a *Mu* transposable element in the *pac1-ref* allele that was capable of excision and restoration of *pac1* gene activity. By contrast, no spots were observed in progeny from crosses between *pac1-ref* and active *Activator* and *Suppressor-mutator* lines. A cosegregation analysis of *Mu* elements and the *pac1-ref* phenotype was used to clone the *pac1* gene as follows. *pac1-ref* plants that had lost *Mu* activity (assessed as an absence of spotting) were outcrossed for several generations to non-*Mu* plants to dilute the number of *Mu* elements. One of the resulting plants (heterozygous for *pac1-ref* and wild-type alleles) was self-pollinated and displayed the expected 3:1 purple:pale seed phenotype. A linked marker and the *pac1-ref* anthocyanin phenotype were used to identify homozygous wild-type and homozygous *pac1-ref* progeny (see Methods). DNA gel blot analysis using *Mu* element probes revealed a *Mu1* hybridizing band cosegregating with the *pac1-ref* phenotype in 11 plants (Figure 1A) and absent in three homozygous wild-type siblings. Sequence flanking this element was obtained using inverse PCR (see Methods), resulting in the sequencing of 1588 bp, including 199 bp of *Mu1* sequence, 1380 bp of the putative *pac1* locus, and a 9-bp duplication at the site of *Mu1* insertion. To confirm that the putative *pac1* sequence recovered was adjacent to the cosegregating *Mu1* element and not some other *Mu1* element, a probe was derived and designated SacI/SmaI 700 (Figure 2). DNA gel blot analysis revealed colocalization of this probe and the cosegregating *Mu1* band and an additional 1380-bp band in wild-type plants (data not shown).

The *pac1-2* and *pac1-3* Alleles Confirm the Identification of the *pac1* Locus

Two new alleles of *pac1* (*pac1-2* and *pac1-3*) were isolated by crossing *pac1-ref* plants to plants from an active *Mu* line and screening the resulting seed for the pale aleurone phenotype. Subsequent crosses were used to segregate these new alleles away from the *pac1-ref* allele. The *pac1-2* allele displayed a spotted phenotype in active *Mu* lines, suggesting excision of an element and restoration of wild-type gene activity. To determine unequivocally that the *Mu1* cosegregating sequence cloned from the *pac1-ref* allele identified the *pac1* locus, DNA gel blot analysis was performed using the SacI/SmaI 700 probe and DNA from plants with the *pac1-2* allele. This experiment revealed an ∼2700-bp band consistent with a 1300-bp insertion within the same 1380-bp fragment as the insertion associated with the *pac1-ref* allele (Figure 1B). The other new allele, *pac1-3*, displayed a pale aleurone phenotype with no detectable spotting in the presence of active *Mu*. DNA gel blot analysis using the SacI/SmaI 700 probe and DNA from plants with the *pac1-3* allele

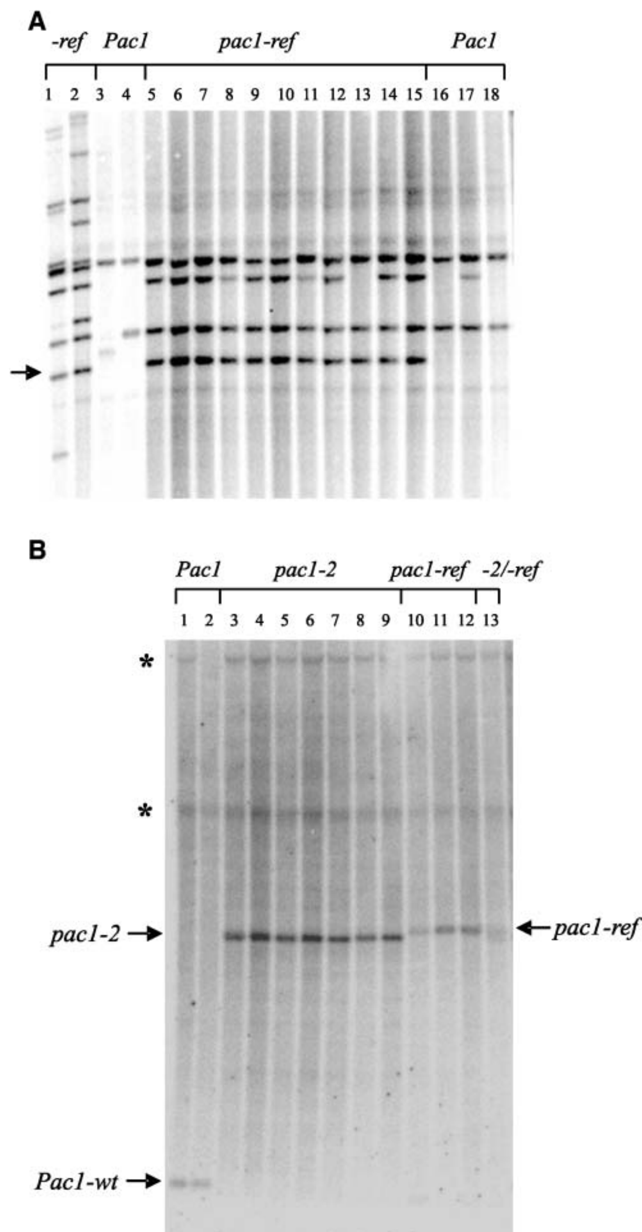

Figure 1. DNA Gel Blot Analysis of *pac1* Mutant Alleles.

DNA samples for both blots were digested with EcoRI and HindIII.

(A) Identification of a 2.8-kb *Mu1* hybridizing DNA fragment cosegregating with the *pac1-ref* mutant allele (arrow). Lanes 1 and 2, plants known to be *pac1-ref*; lanes 3 and 4, plants known to be *Pac1*; lanes 5 to 15, *pac1-ref* in family used for inverse PCR; lanes 16 to 18, *Pac1* siblings of *pac1-ref*. An internal *Mu1* fragment was used as the probe.

(B) Identification of the insertion in the *pac1-2* allele. Lanes 1 and 2, *Pac1*; lanes 3 to 9, *pac1-2*; lanes 10 to 12, *pac1-ref*; lane 13, *pac1-2*/*pac1-ref*. Hybridization was with the SacI/SmaI 700 probe and DNA flanking the *Mu1* insertion (Figure 2). Asterisks indicate fragments cross-hybridizing with the SacI/SmaI 700 probe that are not from the *pac1* locus.

revealed a hybridizing band slightly smaller than that of the wild type (data not shown).

Regions of the *pac1-2* and *pac1-3* alleles were PCR amplified and sequenced (see Methods). Sequence from the *pac1-2* allele revealed a *Mu* element insertion within the same open reading frame (ORF) interrupted in the *pac1-ref* allele. The *Mu* element in the *pac1-2* allele had characteristics of a *MuDR* element with a significant internal deletion, and it therefore was designated *dMuDR* (see Methods). Amplification of the *pac1-3* allele revealed a 25-bp deletion within the *pac1* ORF resulting in a frameshift starting at putative amino acid 203, altering the next 20 amino acids and introducing a premature stop codon. The locations of the *Mu* element insertions in the *pac1-ref* and *pac1-2* alleles and the deletion in the *pac1-3* allele are indicated in Figure 2. Taken together, characterization of these mutants firmly establishes the identification of the *pac1* locus.

Analysis of *pac1* Genomic Structure and mRNA Expression

To characterize a wild-type allele of the *pac1* gene, BAC clones containing the gene were identified in the Clemson University Genomics Institute (CUGI) B73 genomic BAC library (see Methods). Comparison of fragments produced by restriction digestion and restriction mapping of wild-type genomic DNA revealed that the BAC insert chosen for sequencing had the same restriction map as *pac1* wild-type genomic DNA. The resulting *pac1* sequence was deposited to GenBank (accession number AY115485).

An ORF of 353 amino acids within a single exon spans the region containing the two *Mu* insertions and the *pac1-3* deletion (Figure 2). To further characterize the gene's structure, 3′ rapid amplification of cDNA ends (RACE) was performed using RNA isolated from immature tassel (see Methods). Thirteen 3′ RACE clones were isolated, sequenced, and compared with the genomic locus, revealing an intron in the genomic sequence starting a few base pair 3′ of the ORF's stop codon as well as five alternative poly(A) addition sites (indicated by arrows in Figure 2). The detection of another gene in the genomic sequence within 1 kb upstream of the *pac1* start codon and the lack of significant ORFs in the sequences between the two coding regions suggest the identification of the complete *pac1* ORF.

To determine which tissues express *pac1*, RNase protection experiments were performed using the pac1-rp-300 probe (see Methods; Figure 2) and *actin1* as an internal control. Low levels of *pac1* transcript were detected in RNA from all wild-type tissues tested (Figures 3A and 3B; Methods). The expression levels varied no more than threefold in all tissues, and the amount of signal resulting from our protections was roughly one-third to one-half of that seen in protections with *b* and *c1* (data not shown), suggesting that *pac1* is expressed more ubiquitously but at a lower level than either the bHLH or MYB transcription factors. Similar results were observed (data not shown) using the pac1-rp-3′ probe, which spans the splice site of the two *pac1* exons (see Methods; Figure 2).

Examination of transcript levels from mutant *pac1* alleles revealed substantial differences when compared with the wild

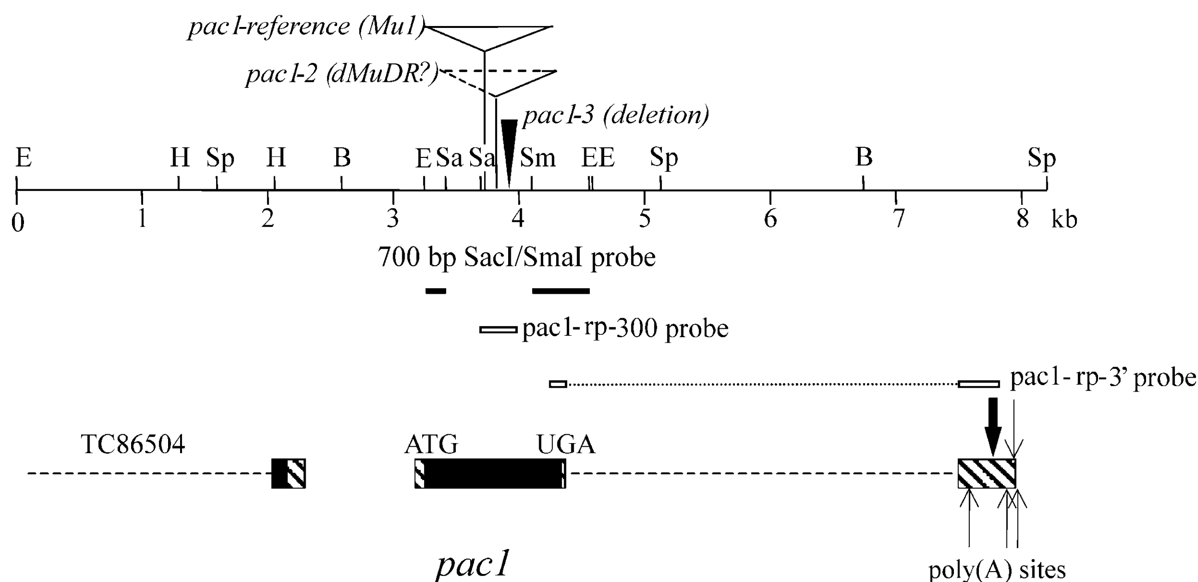

Figure 2. The *pac1* Locus and Probes.

All open reading frames in this genomic fragment are indicated by closed boxes, untranslated regions by hatched boxes, and introns by dashed lines. The *pac1* transcription unit spans two exons separated by a 3017-bp intron (dashed line). Large and small arrows indicate relative abundance of sequenced poly(A) sites. The location of *Mu* insertions in the *pac1-ref* and *pac1-2* alleles and the *pac1-3* deletion are depicted at the top of the figure. The 9-bp duplication and *Mu1* insertion for *pac1-ref* begins at codon 165. Sequence from *pac1-2* indicates a *Mu* insertion at codon 192. The *pac1-3* deletion begins at codon 203. The 700-bp SacI/SmaI probe was obtained by inverse PCR and is indicated by solid lines. Open boxes indicate probes for RNase protections. Part of a small putative zinc finger protein is located <1000 bp upstream of the *pac1* initiator ATG site. Transcripts for this upstream gene are described in TIGR contig TC86504. B, BamHI; E, EcoRI; H, HindIII; Sa, SacI; Sm, SmaI; Sp, SpeI.

type. When using the pac1-rp-300 probe (Figure 3A), which spans the locations of the insertions and the deletion, a smaller 180-base fragment (asterisk in Figure 3A) was observed when *pac1-3* RNA was used, consistent with internal cleavage to a smaller product because of a lack of complementary sequence of the probe and the *pac1-3* transcript in the region of the *pac1-3* deletion. *pac1-ref* (Figure 3A) and *pac1-2* (data not shown) RNA protected substantially less pac1-rp-300 probe than the wild type and did not reveal any significant smaller products, suggesting that the region covered by the pac1-rp-300 probe is either not well transcribed or is substantially altered for these two alleles. We favor the latter explanation because levels of *pac1* RNA for all three alleles did not differ from those of the wild type when the pac1-rp-3′ probe was used (data not shown). Occasionally, a trace of protected RNA that is similar in size to the wild type is observed in *pac1-ref* and *pac1-2* samples (signal normalized to *actin1* for *pac1-ref* husk in Figure 3A is 13% of that of wild-type sibling husk), suggesting that in a minority of transcripts in at least some tissues, the *Mu* elements may be spliced out or that the samples may contain sectors of tissue with somatic excisions of the *Mu* elements.

The PAC1 Protein Is a Homolog of AN11 and TTG1

In a Basic Local Alignment Search Tool (BLASTp) search (Altschul et al., 1997) using the PAC1 sequence as a query, TTG1 (*A. thaliana*) and AN11 (*P. hybrida*) were identified as the strongest hits, each with e-110 expect values. Significantly, AN11 and TTG1 are involved in the expression of several traits in their respective plants, including anthocyanin production. Figure 4 shows an alignment of PAC1, AN11, and TTG1. All three are well conserved, with the noticeable exception of the most N-terminal portion of PAC1. The most similar *Z. mays* protein identified to date, MP1, which is discussed in detail later, shares less homology with PAC1 than do AN11 and TTG1. In summary, PAC1, a regulator of anthocyanins in *Z. mays*, is a homolog of proteins previously identified as regulators of the anthocyanin pathway in *P. hybrida* (de Vetten et al., 1997) and *A. thaliana* (Walker et al., 1999).

The PAC1 Protein Complements *ttg1-1* and *ttg1-13* Phenotypes in *A. thaliana*

Because of the high similarity of PAC1 with TTG1 and the involvement of both proteins in anthocyanin pigmentation, the ability of PAC1 to complement *ttg1* mutants was investigated. Of particular interest was whether the *Z. mays* PAC1 protein could complement not only *ttg1* defects in anthocyanin production but also other *ttg1* defects as well. A PAC1 expression vector, designated *35S-Pac1*, which included a gene encoding resistance for the herbicide Basta, was transformed into plants homozygous for one of two *ttg1* mutant alleles. Both *ttg1* alleles result in severe defects in trichome differentiation, anthocyanin pigmentation, seed coat pigmentation, and seed coat mucilage (Koorneef, 1981; Larkin et al., 1999). The molecular basis of the mutant phenotypes are a premature stop codon 25 amino acids

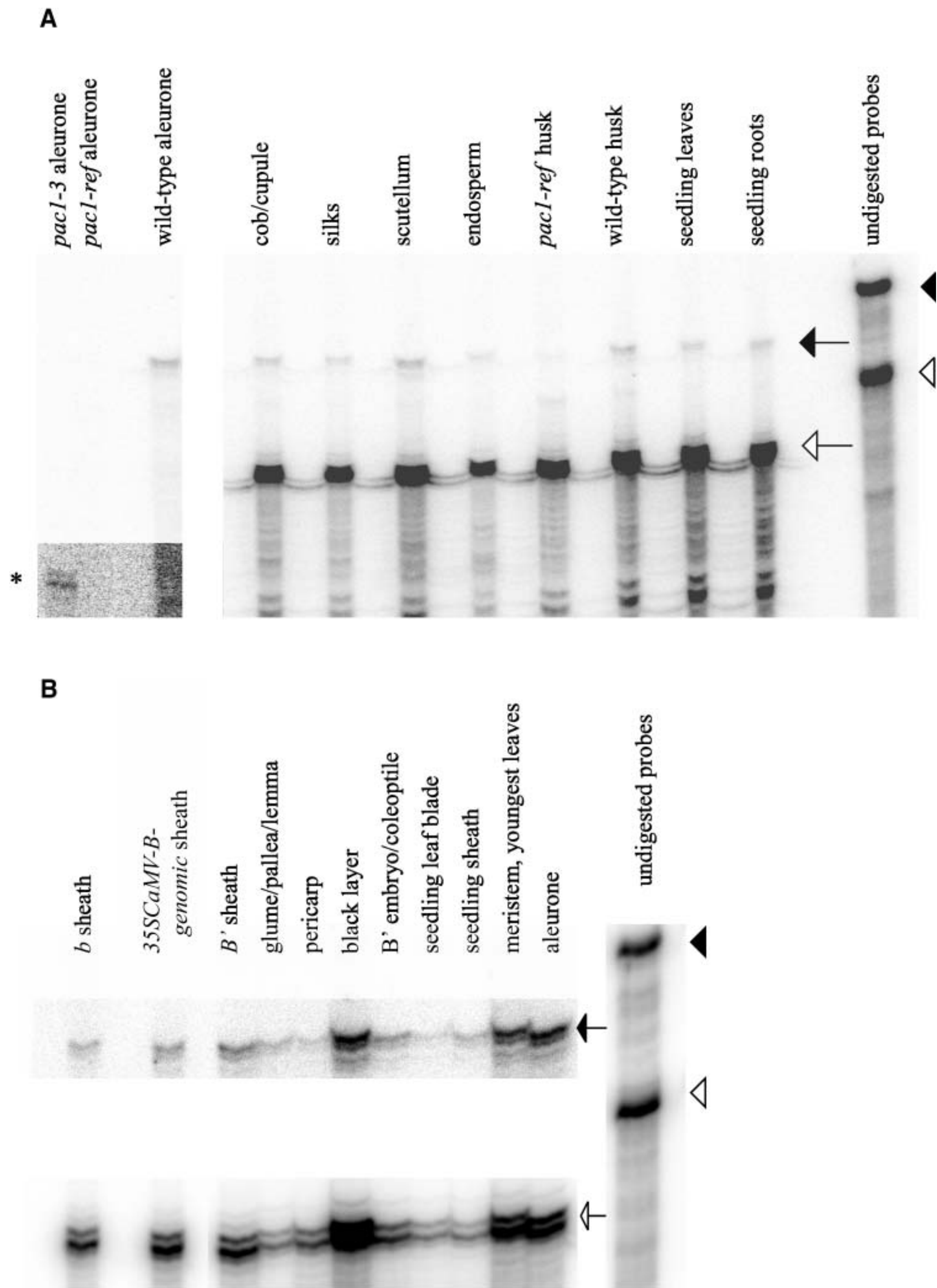

Figure 3. RNase Protections Using the pac1-rp-300 Probe.

Arrowheads indicate undigested probes, and arrows indicate probes protected by sample RNA (closed, *pac1*; open; *actin1*).

(A) The pac1-rp-300 probe was protected in the absence of the probe for *actin1* for the first three samples, which are from aleurones of *pac1-3*, *pac1-ref*, and a wild-type control. The remaining samples include both *actin1* and *pac1* probes. The asterisk in the panel at left, which contains a longer exposure, shows the expected protected product for the *pac1-3* allele. The *pac1-ref* and wild-type husks were from plants segregating in the same family. All other plant parts were from homozygous wild-type individuals.

(B) All samples were from *Pac1* wild-type individuals. The levels were adjusted upward in Adobe Photoshop for the *pac1* protected probe because the signal was weak compared with *actin1*.

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PAC1 : -------------MDPPKPPSS-VASSSGPET-PNPHAFTCELPHSIYALAFSPVAP----VLASGSFLEDLHNRVSLLSFDPVRPSAASFRALPALSFDHPYPPTK
TTG1 : -------------MDNS---------APDSLS-RSETAVTYDSPYPLYAMAFSSLRSSSGHRIAVGSFLEDYNNRIDILSFDS---DSMTVKPLPNLSFEHPYPPTK
AN11 : -------------MENSS--------QESQHL-RSENSVTYDSTYPIYSMAFSSFPTP-RRRIAVGSFIEELNNRVELLSFNE---ETLTLNPIPNLSFDHPYPPTK
MP1  : MGGVGEGDAWADQEQGNGGGSRGV-GGGGGEAKRSE-IYTYEAAWHIYAMNWSVRRDK-KYRLAIASLLEQVTNRVEVVQLDE---ASGDIAPV--LTFDHQYPPTK

PAC1 : LQFNPR----AAAPSLLASSADTLRIWHTPLDDLS------------------DTAPAPELRSVLDNRKASSEFCAPLTSFDWNEVEPRRIGTASIDTTCTVWDIDR
TTG1 : LMFSPPSLRRPSSGDLLASSGDFLRLWEIN-----------------------EDSSTVEPISVLNNS-KTSEFCAPLTSFDWNDVEPKRLGTCSIDTTCTIWDIEK
AN11 : LMFHPNP--IKSNNDILASSGDYLRLWE-------------------------VKESSIEPLFTLNNS-KTSEYCAPLTSFDWNEVEPKRIGTSSIDTTCTIWDVEK
MP1  : TMFMPDP--HALRPDLLATSADHLRIWRIPSSDDAEDGAASANNNNGSVRCNGTQQPGIELRSELNGN-RNSDYCGPLTSFDWNDADPRRIGTSSIDTTCTIWDVER

PAC1 : GVVETQLIAHDKAVHDIAWGEAGVFASVSADGSVRVFDLRDKEHSTIVYES------------------PRPDTPLLRLAWNRSDLRYMAALLMDSSAVVVLDIRAP
TTG1 : SVVETQLIAHDKEVHDIAWGEARVFASVSADGSVRIFDLRDKEHSTIIYES------------------PQPDTPLLRLAWNKQDLRYMATILMDSNKVVILDIRSP
AN11 : GVVETQLIAHDKEVYDIAWGEAGVFASVSADGSVRIFDLRDKEHSTIIYES------------------PTPDTPLLRLAWNKQDLRYMATILMDSNKVVILDIRSP
MP1  : EAVDTQLIAHDKEVYDIAWGGAGVFASVSADGSVRVFDLRDKEHSTIIYESGSGGSSGGGSNSGAGDGGXASPTPLVRLGWNKQDPRYMATIIMDSPKVVVLDIRYP

PAC1 : GVPVAELHRHRACANAVAWAPQATRHLCSAGDDGQALIWELPETAAAVPAEG---------------IDPVLVYDAGAEINQLQWAAAHPDWMAIAFENKVQLLRV
TTG1 : TMPVAELERHQASVNAIAWAPQSCKHICSGGDDTQALIWELPTVAGPNG------------------IDPMSVYSAGSEINQLQWSSSQPDWIGIAFANKMQLLRV
AN11 : AMPVAELERHQASVNAIAWAPQSCRHICSGGDDGQALIWELPTVAGPNG------------------IDPMSMYSAGAEINQLQWSPAQRDWIAIAFSNKLQLLKV
MP1  : TLPVVELHRHHAPVNAIAWAPHSSCHICTAGDDMQALIWDLSSMGTGSNGSGNGNGNTAAGAAAEGGLDPILAYTAGAEIEQLQWSATQPDWVAIAFANKLQILRV
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Figure 4. Alignment of PAC1, TTG1, AN11, and MP1.

Amino acids conserved in all four proteins are indicated in black. Gray indicates identity in two or three of the proteins.

from the C terminus of the protein in *ttg1-1* (Walker et al., 1999) and a deletion of the entire coding region in *ttg1-13* (Larkin et al., 1999). Figure 5 and Table 1 show the results of these complementation experiments. Significantly, and presented in more detail below, when any *ttg1* mutant phenotype was complemented, complementation of all of the other tested *ttg1* mutant phenotypes was observed.

To determine if the *35S-Pac1* transgene complements the *ttg1* defect in trichomes, all primary transformant (Basta-resistant) seedlings were screened for trichomes. Trichomes were observed in 72% of the resistant seedlings (Figures 5A and 5B, Table 1), whereas no trichomes were observed in any nontransformed controls (*ttg1-1* and *ttg1-13* parental lines and nontransgenic mutant lines resulting from the floral dip). To determine if anthocyanin defects in *ttg1* mutants were complemented, mature plants were examined (Table 1). Ten out of fourteen mature Basta-resistant plants had increased anthocyanin pigments, whereas four showed no complementation. The four anthocyanin noncomplemented plants also lacked trichomes and, importantly, yielded only Basta-sensitive progeny that were not complemented for any *ttg1* phenotype, suggesting that these four plants either escaped initial Basta selection and were not transformed or were subsequently silenced for transgene activity.

Examination of the seed phenotypes of *35S-Pac1 ttg1* plants showed that all 10 primary regenerant Basta-resistant plants yielded brown seeds upon self-pollination (Figure 5C), indicating complementation of the *ttg1* proanthocyanidin defect in the maternally derived seed coat (Table 1, Figure 5C). Similarly, seeds from Basta-resistant plants were positive for the presence of mucilage on the seed coat using ruthenium red staining (Figure 5E). By contrast, little to no mucilage was detected in the seed coats of nontransformed *ttg1* mutants (Figure 5D), and control seeds were yellow, indicating a lack of proanthocyanidins in all *ttg1* mutant controls (Figure 5C).

To examine the transmission of complemented phenotypes resulting from expression of *35S-Pac1* in *ttg1* plants, we planted seed obtained from self-pollination of 9 of the 10 complemented lines grown under nonselective conditions. In all lines, when one phenotype was complemented, all phenotypes were complemented: anthocyanins, trichomes, proanthocyanidins, and seed coat mucilage (root phenotypes associated with *ttg1* mutants are difficult to score, and complementation of the *ttg1* root defects therefore was not examined rigorously).

pac1 Affects Anthocyanin Regulation in *Z. mays* Seedling Roots but Does Not Affect Root Morphology or Trichomes

The observations that *pac1* was expressed in all *Z. mays* tissues tested prompted a further examination of *pac1* mutant phenotypes in a variety of *Z. mays* tissues. Consistent with what was previously reported (Selinger and Chandler, 1999), anthocyanin pigmentation of above ground tissues, mature husk, sheath, tassel, or anthers was not obviously affected in comparisons of *pac1* and wild-type plants. However, in roots, a noticeable reduction in anthocyanin pigmentation was observed in *pac1* mutants. To score this effect, a 1 to 8 root color score was devised, in which a score of 1 corresponded to the absence of anthocyanins, and a score of 8 corresponded to the highest anthocyanin levels observed in this experiment (for examples of root color scores of 8 and 4, see Figures 5F and 5G, respectively). The roots of 11 mutant *pac1-2* plants had an average color score of 2.1 (SD = 1.1, range: 1 to 5). By contrast, nine wild-type siblings had an average color score of 7.2 (SD = 2.1, range: 4 to 8), indicating that *pac1* function is required for strong activation of the anthocyanin pathway in roots.

Because *TTG1* function in *A. thaliana* is required for normal root (Galway et al., 1994) and trichome development, possible effects of the *pac1* mutant on *Z. mays* root and trichome

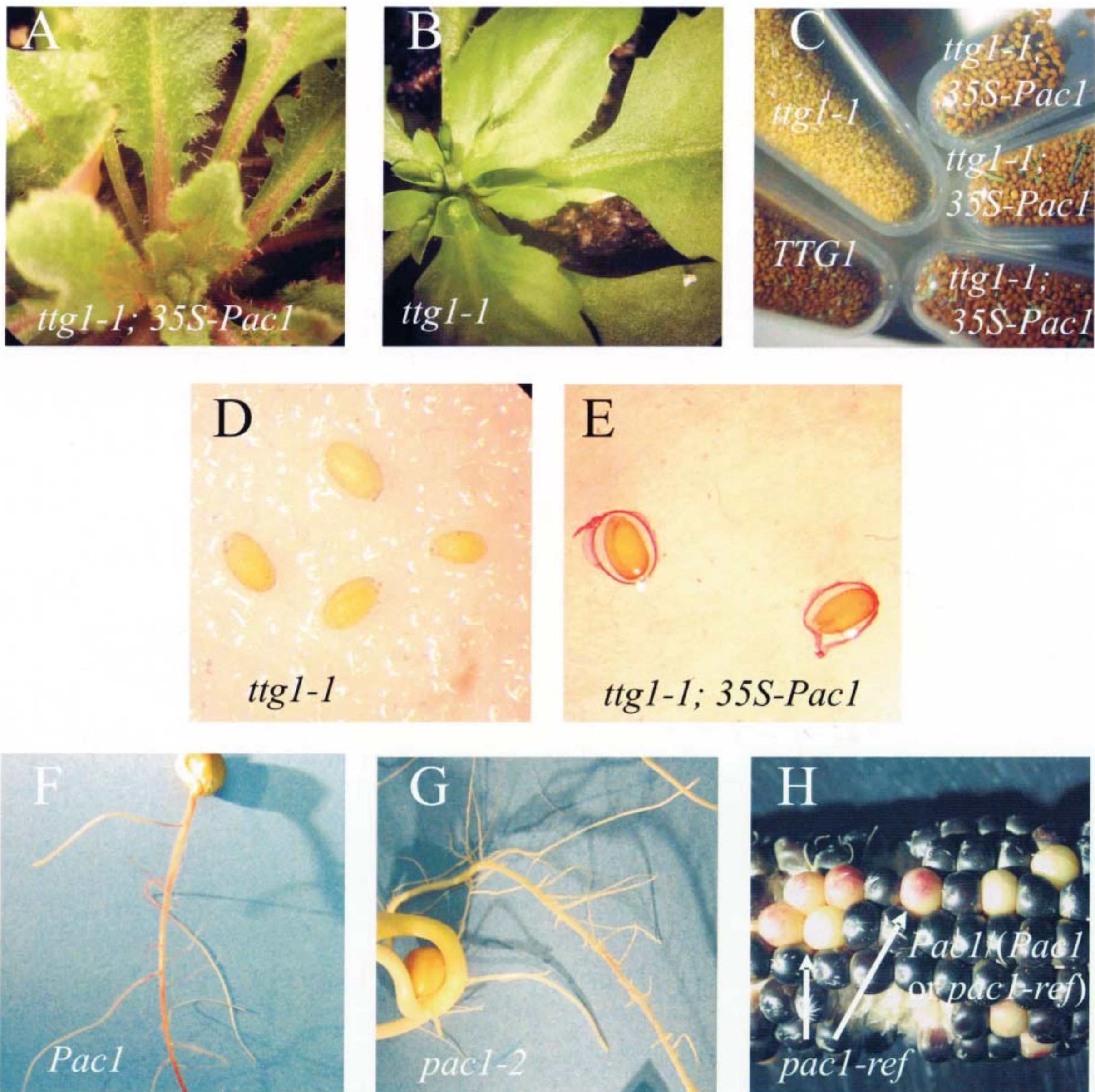

Figure 5. Phenotypes of *35S-Pac1* Complemented *ttg1-1* Mutants and *pac1* Mutants.

(A) and **(B)** Anthocyanin, trichome, and leaf phenotypes are complemented by the *35S-Pac1* transgene. A *ttg1-1* plant containing the *35S-Pac1* transgene **(A)** and a nontransgenic sibling **(B)**.

(C) The 35S-*Pac1* transgene restores wild-type seed pigmentation to *ttg1-1* mutants. Mature seed phenotypes of wild-type (*TTG1*) Columbia, *ttg1-1* in the Columbia background (yellow seed), and three independent *ttg1-1* lines containing the *35S-Pac1* transgene.

(D) and **(E)** Mucilage production is restored by the *35S-Pac1* transgene. Ruthenium red staining of *ttg1-1* Columbia **(D)** and *ttg1-1*; *35S-Pac1* seeds **(E)**.

(F) and **(G)** Root phenotypes in wild-type **(F)** and *pac1-2* **(G)** sibling seedlings expressing the anthocyanin regulatory gene *r*. Plant in **(F)** received a root anthocyanin score of 8. Plant in **(G)** was from the most darkly pigmented seed in the *pac1-2* category and received a root anthocyanin score of 4.

(H) *in1*; *pr1*; *R* ear segregating *Pac1*/(*Pac1* or *pac1-ref*) and *pac1-ref* kernels. All *pac1-ref* kernels were pale.

development were investigated. *Z. mays pac1-2* and wild-type siblings showed no differences in root morphology or abundance of root hairs (data not shown). Likewise, no *pac1* mutant effects were demonstrated in *pac1-ref* and wild-type siblings with respect to architecture and spacing of the two trichome classes in *Z. mays*, prickle hairs and macrohairs, on mature leaf blades. In summary, *pac1* mutant phenotypes in *Z. mays* differ significantly from *ttg1* mutant root and trichome phenotypes in *A. thaliana*, in that *pac1* mutants do not display defects in root and trichome development that are characteristic of *ttg1* mutants.

Table 1. Phenotypes of Primary *35S-Pac1* Transformants while under Basta Herbicide Selection

Allele (Background)[a]	Number of Seedlings with Trichomes (Total Scored)[b,c]	Number of Plants with Trichomes Yielding Brown Seed and Mature Plant Anthocyanins (Total Scored)
ttg1-1 (Columbia)	26 (39)	6 (6)
ttg1-1 (Landsberg erecta)	13 (15)	3 (3)
ttg1-13	12 (16)	1 (1)

[a] The *ttg1-13* allele was partially introgressed from the RLD ecotype into Columbia.

[b] Number of trichomes per first true leaf ranged from 4 to 38 trichomes with an average of 17.7 trichomes (SD of 7.3, $n = 30$ plants, all alleles pooled). No trichomes were seen in several hundred nontransformed *ttg1* mutants.

[c] All plants lacking trichomes as seedlings lacked anthocyanins as mature plants. The four such plants that were followed to silique shatter yielded yellow seed, consistent with no proanthocyanidin synthesis.

In addition to effects on anthocyanin pigmentation, significant *pac1* effects on height were noticed with all three *pac1* mutant alleles. We examined each mutant allele relative to their wild-type siblings and each showed a significant difference (data not shown). The pooled data demonstrated that wild-type plants were 17 cm taller on average ($n = 55$; mean, 139 cm; SE = 13) than *pac1* mutant plants ($n = 55$; mean, 122 cm; SE = 15; *t* test, $P < 0.001$). There was no significant corresponding decrease in the number of leaves (means: the wild type, 17.5 with SE of 1; mutant, 17.4 with SE of 1.2). Thus, the wild-type function of *pac1* is required for normal stature with the loss of function leading to shorter plants. This difference is fully recessive because heterozygous individuals had equivalent heights as wild-type individuals (27 confirmed heterozygotes had a mean of 141 cm with a SE of 7, and 11 homozygotes had a mean of 140 cm with a SE of 9).

Genetic Interactions of *pac1* with Other *Z. mays* Anthocyanin Regulatory Loci

The bHLH and MYB proteins encoded by *b* and *c1* loci are required for activation of the anthocyanin pathway in *Z. mays*. It was demonstrated previously (Selinger and Chandler, 1999) and verified (this study, data not shown) that *b* and *c1* RNA levels are similar in *pac1-ref* and wild-type individuals, suggesting that *pac1* does not influence the expression of either of these genes at the transcriptional or RNA levels. To test for the possibility that *pac1* expression is influenced by *b* and/or *c1* expression levels, RNase protections were performed using the pac1-rp-300 probe and RNA from adult sheaths, a tissue with no *c1* expression (Cone et al., 1993). *pac1* RNA levels were assayed in sheaths of plants with a nonfunctional allele of *b* producing no anthocyanin pigmentation (*b*), a functional allele of *b* producing a moderate amount of anthocyanin pigmentation (*B*'), and a strongly expressed functional transgenic allele of *b* producing intense anthocyanin pigmentation (*35SCaMV-B-I-genomic*; C.C. Carey and V.L. Chandler, unpublished data). There was no change in *pac1* RNA levels in response to increasing *b* expression, indicating that *pac1* expression is not under the control of *b* (Figure 3B). Although expression of *pac1* mRNA does not require expression of *c1*, we have not formally ruled out the possibility that the MYB gene *pl1* may regulate *pac1* in tissues such as sheath and leaf in place of *c1*. However, because most *pl1* alleles are not expressed in the aleurone, there is no absolute requirement of *pac1* for *pl1* expression. In conclusion, *pac1* regulates neither *b* nor *c1* at the RNA level, and *pac1* expression does not require *b* or the exclusive action of *c1* or *pl1*.

The *in1* gene (Burr et al., 1996) encodes a bHLH protein in *Z. mays* with the most similarity to TT8, the bHLH protein in *A. thaliana* thought to function like *Z. mays* B and R in activation of anthocyanin and proanthocyanidin biosynthetic genes. The fact that *in1* mutations result in very intense pigmentation in the aleurone suggests that IN1 acts as a repressor (Burr et al., 1996) or diverts metabolic precursors into an alternative pathway (Nesi et al., 2000). Because of the established relationship of some of the bHLH regulators with WD40 repeat proteins, we examined the genetic interaction of *pac1* and *in1* by creating a line that was homozygous *R*; *in1*; *pr1* (*red aleurone*, a locus encoding flavonoid 3′-hydroxylase function [Larson et al., 1986]) and heterozygous for *pac1-ref*. Two ears resulting from self-pollination of these plants displayed one-quarter pale and spotted kernels (consistent with somatic excision of the *Mu1* element in the *pac1-ref* allele) and three-quarters dark kernels (Figure 5H). DNA gel blot analysis verified that all pale seeds tested were homozygous for the *pac1-ref* allele, and purple seeds were either heterozygous or wild-type for *pac1* (Methods). Therefore, the function of PAC1 cannot simply be the removal of the IN1 putative repressor molecule to allow full function of R, otherwise all *in1* mutant seed would have been dark, regardless of *pac1* genotype. Rather, *pac1* is epistatic to *in1* and PAC1 function is required for the activation of the anthocyanin pathway in aleurones in the absence of IN1 function.

Identification and Characteristics of the WD40 Repeat Gene Family Related by Sequence to the Plant Anthocyanin Regulators

It is intriguing that *pac1* mutants only affect anthocyanin pigmentation in a subset of tissues, given the homology of PAC1 with AN11 and TTG1 and that mutations in the *an11* and *TTG1* genes eliminate anthocyanin pigmentation entirely. Likewise, it is interesting that *pac1* mutants do not affect traits such as root hairs and trichomes as might have been predicted given the homology of PAC1 with TTG1. One explanation for these observations is the presence of *Z. mays* genes with shared, partially redundant functions. Consistently, DNA gel blot analysis using SacI/SmaI 700 revealed DNA fragments that hybridized but that were independent of the *pac1* locus (Figure 1B, fragments marked with asterisks). In addition, two clones were identified from the CUGI B73 BAC library, which contained an ORF of the WD40 repeat protein family identical to a sequence obtained from an EST database and previously named MP1 (Hernandez et al., 2000). Consistent with there being two separate groups of related WD40 repeat proteins, the MP1 protein is more similar

in sequence to ATAN11A and ATAN11B (de Vetten et al., 1997) (68% identity) from *A. thaliana* than to PAC1 (58% identity) or TTG1 and AN11. Using *mp1* DNA sequence as a probe for stringent DNA gel blot analysis, no hybridization was observed to *pac1* nor to the fragments that cross-hybridized with SacI/SmaI 700, suggesting that the two other fragments identified with *pac1* probes using DNA gel blot analysis may represent other genes.

Sequence analysis and phylogenetic trees of PAC1 and MP1 proteins and their homologs in several species could shed light on the evolutionary history of these WD40 repeat proteins and set the foundation for studies addressing the redundant or divergent functions of these proteins. To that end, sequences of the two *Z. mays* proteins PAC1 and MP1 were used to identify homologs in GenBank, the Plant Genome Database, and the Index for Genomic Research (TIGR) gene indices from all plant species and a representative sampling of other eukaryotes. In all cases, proteins identified as homologs were more similar to PAC1 and MP1 than to other WD40 repeat proteins in their respective genomes. As a general rule, homologous sequences had TBLASTN expect values of e-25 and higher significance, whereas several more distantly related WD40 repeat sequences were excluded at expect values of e-10 and lower significance.

Seventy-five deduced protein sequences, including those of AN11 and TTG1, were assembled into a multiple alignment (see supplemental data online). The multiple alignment, which included 30 full-length sequences, suggested that there are at least two classes of this WD40 repeat protein family in plants. Ninety-seven amino acids of the N-terminal sequence of 46 proteins (31 species) were used to generate the representative phylogeny shown in Figure 6 (see Methods). The tree shows three well-supported clades, with two of the clades consisting solely of plant proteins and the third consisting of the animal proteins except nematode. The PAC1 clade includes PAC1, AN11, and TTG1, for which anthocyanin mutant phenotypes have been identified, whereas the MP1 clade includes MP1, ATAN11A, and ATAN11B, for which no mutant phenotypes have been described. Thus, the phylogenies and multiple alignments support a scenario in which an ancestral gene duplication in a plant ancestor led to the PAC1 clade (containing known anthocyanin regulators) and the MP1 clade of proteins. In several plant species, there were subsequent duplication events in either or both clades. *Chlamydomonas reinhardtii* and *Ceratopteris richardii* (fern) appear to have sequences shared with both the MP1 and PAC1 clades, such that they are classified into neither clade.

In contrast with plants, there are generally one or two homologous genes in most non-plant species, with a well supported clade within the animals. Nematode, with three loci that are quite divergent from the other animal family members,

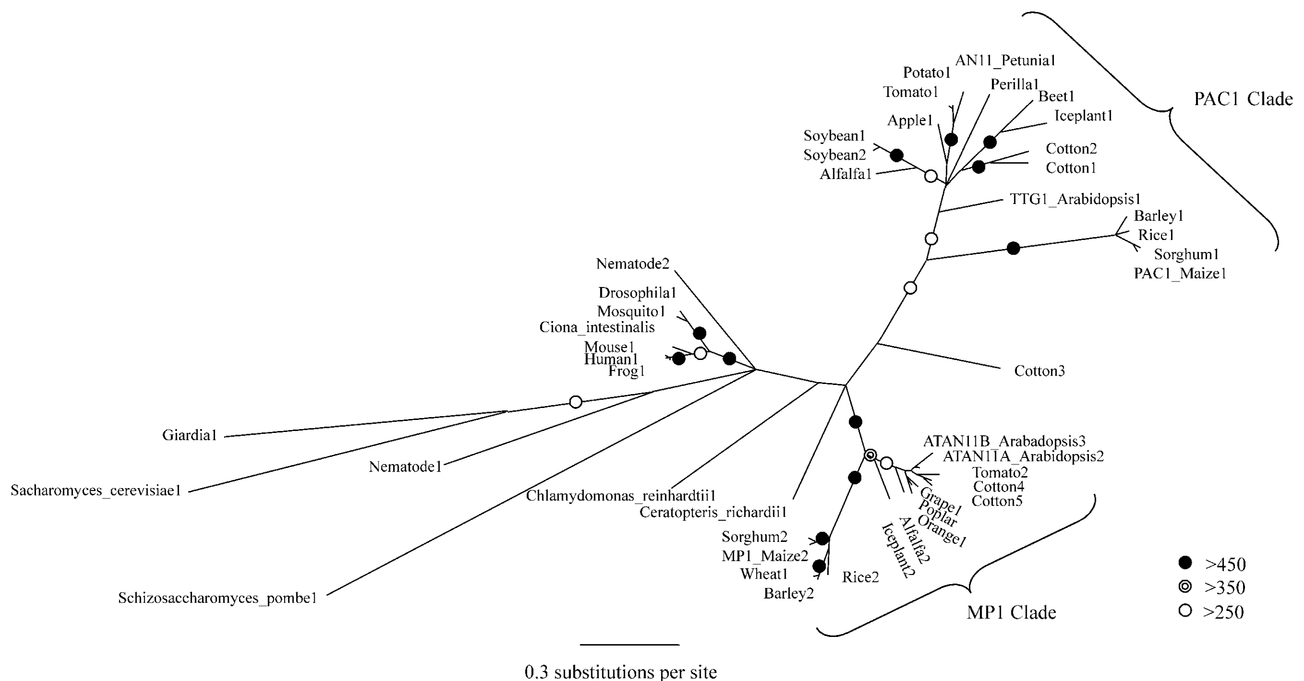

Figure 6. Phylogenetic Relationships of PAC1 and MP1 Homologs.

Three major clades of PAC1 and MP1 homologs are apparent. The PAC1 clade consists of PAC1, AN11, and TTG1. The MP1 clade contains MP1, ATAN11A, and ATAN11B. A third clade consists of animal species within a grouping of non-plant species. The consensus unrooted tree is depicted, with leaf and branch lengths representing distance derived from maximum likelihood analysis. Leaves that were minimal in length have been exaggerated to emphasize position on branches. Five hundred trees were generated by bootstrapping. Number of trees supporting each branch point is as presented in the key, with the exception that branch points with <250 supporting trees are unmarked. The bar indicates a length representing 0.3 amino acid substitutions per site.

had the most representatives of this WD40 repeat subfamily. No functional assignment in any of the non-plant species has been made nor could any function be inferred from investigating coexpression patterns of the *Saccharomyces cerevisiae* homolog with other transcripts in publicly available microarray databases or from investigating protein interaction databases. In summary, several completely sequenced genomes in non-plant species were searched, revealing that fewer of these WD40 protein family members are found in non-plant species and that these form a group distinct from the two clades in plants.

DISCUSSION

WD40 Anthocyanin Regulatory Proteins Are More Conserved Than Other Classes of Anthocyanin Regulators

The 353–amino acid PAC1 protein has high similarity to WD40 repeat proteins encoded by AN11 and TTG1 both in terms of sequence and with respect to common function because they are all regulators of the anthocyanin pathway. This high degree of conservation between species contrasts with what is observed with the other two classes of regulators (bHLH and MYB). With respect to amino acid sequence, PAC1 shares 62% identity with AN11 and TTG1 as well as with the proposed anthocyanin regulator PFWD in *Perilla frutescens* (Sompornpailin et al., 2002). By contrast, only 30% identity is evident in pair-wise comparisons of any of the following bHLH homologs: B/R in *Z. mays* (Chandler et al., 1989; Ludwig et al., 1989), IN1 in *Z. mays* (Burr et al., 1996), TT8 in *A. thaliana* (Nesi et al., 2000), GLABRA3 and MYC146 in *A. thaliana* (Payne et al., 2000), AN1 in *P. hybrida* (Spelt et al., 2000), JAF13 in *P. hybrida* (Quattrocchio et al., 1998), and DELILAH in *A. majus* (Goodrich et al., 1992). Given the number of developmental programs in which PAC1, AN11, and TTG1 are implicated, it is likely that a large number of interacting partners combined with the pressures of conserving the seven-bladed propeller structure contributes to the high degree of conservation among these three proteins. Modeling of protein secondary structure and a consideration of the location of *ttg1* point mutations suggest that these proteins can form seven-bladed propeller structures typical of other WD40 repeat proteins (see supplemental data online).

A *pac1* Transgene Functionally Complements *ttg1* Mutants

The *35S-Pac1* transgene complements all *ttg1* mutant phenotypes, including those resulting from developmental programs that differ significantly between *Z. mays* and *A. thaliana* (trichomes) and are nonexistent in *Z. mays* (seed coat mucilage), demonstrating that PAC1 contains most if not all functional competencies characteristic of TTG1 in spite of the evolutionary distance between monocots and dicots. One interpretation of these results is that there are no distinct domains within these WD40 repeat proteins responsible for function in distinct pathways. Consistent with this, Larkin et al. (1999) showed that all weak and strong mutant alleles of *ttg1* affected trichome, root, and anthocyanin phenotypes. Complementation of *ttg1* mutants has been observed using an *an11* transgene (C.T. Payne, F. Zhang, and A.M. Lloyd, unpublished data), and expression of PFWD from *P. frutescens* (Sompornpailin et al., 2002) in wild-type *A. thaliana* plants results in supernumerary trichomes and enhanced anthocyanin pigmentation, suggesting a role for PFWD in regulation of these traits in *P. frutescens*. As a result, sequence comparison between PAC1 clade proteins should facilitate determination of residues structurally required for function in all pathways.

Is a Protein Homologous to PAC1 Required for *Z. mays* Trichome, Root Hair, and Tissue-Specific Regulation of the Anthocyanins?

In *Z. mays*, the most obvious phenotypes resulting from the *pac1* mutations are reduced anthoycanin pigmentation in a subset of normally pigmented tissues. Importantly, this is in contrast with *A. thaliana ttg1* mutants, in which anthocyanins are eliminated from all tissues throughout the plant. Shortened stature is observed in plants with *pac1* mutant alleles, whereas no such effect is reported in *ttg1* or *an11* mutants in *A. thaliana* or *P. hybrida*. Significant and additional contrasts include the *A. thaliana ttg1* mutant effects on proanthocyanidins, trichomes, seed-coat mucilage, and root hair development and the absence of these effects in *Z. mays pac1* mutants. In *P. hybrida*, *an11* mutants affect anthocyanins, proanthocyanidins, seed-coat cell morphology, and flower pH, but trichome and root development are unaffected. Thus, many different processes are variously affected by these mutations in different species, with pertubation of anthocyanin pigmentation being the common phenotype. This raises a central evolutionary question: Does the diversification of the nonanthocyanin functions result from evolution of the protein sequences, or does the diversification of other functions reflect evolution at another level, such as the presence or absence of redundantly functioning proteins and/or a difference in the regulatory networks controlling these processes in different species?

Based on the ability of the *pac1* transgene to complement the *ttg1* mutant phenotypes, it appears that the differences in nonanthocyanin functions among these species is not the result of differences at the protein sequence level but is the result of differences in organization of the genome or in the mechanisms of regulation of these traits. Gene duplications resulting in redundantly functioning WD40 repeat proteins with different tissue-specific expression patterns within the same organism could explain the differences in phenotypes observed in *Z. mays*, *P. hybrida*, and *A. thaliana*. The observation that an ancestral duplication led to PAC1 and MP1 clade proteins suggests that proteins from each class still may share common function, in which case, MP1 may functionally replace or be primarily responsible for normal anthocyanin pigmentation in some tissues and also may be responsible for trichome and root development.

Evidence arguing against PAC1 and MP1 clades retaining some common functionality is that several *A. thaliana* programs are apparently affected when *ttg1* is mutant, despite the presence and presumed function of two MP1 clade proteins (ATAN11A and ATAN11B). ESTs representing at least one of

these proteins are as widespread and as abundant as ESTs representing *TTG1*, suggesting that MP1 and PAC1 clade proteins may not be functionally equivalent in *A. thaliana*. Evidence arguing that PAC1 and MP1 clade proteins may retain the capacity for common functionality is transient activation experiments in *P. hybrida*, in which expression of a luciferase reporter driven by an anthocyanin biosynthetic gene promoter was enhanced by coexpression of ATAN11A and even the *Homo sapiens* (human) homolog from the non-plant clade (de Vetten et al., 1999). Our observation of some, albeit reduced, anthocyanin pigmentation in *pac1* mutant tissues, such as roots, suggests that different tissues in *Z. mays* vary quantitatively in their requirement for PAC1 protein function. Again, this would be in marked contrast with *A. thaliana*, in which all tissues appear to absolutely require TTG1 protein function for anthocyanin pigmentation. We consider three possibilities to explain these differences. First, MP1 may regulate the anthocyanin pathway in some *Z. mays* tissues, such as husk and sheath, and may function in the regulation of trichome developmental programs. Second, it is possible that an as yet unsequenced PAC1 homolog in *Z. mays* may serve one or more of these functions. Third, it is possible that regulation of anthocyanins in husk, sheath, and anthers is distinct from regulation in *Z. mays* aleurones, scutella, and roots, such that a WD40 repeat protein is not required. Clearly, the identification of other homologs and an understanding of MP1 function will be required to elucidate requirements for WD40 repeat protein function.

Models for WD40 Repeat Protein Interactions with bHLH and MYB Proteins

The bHLH and MYB proteins in the *Z. mays* anthocyanin pathway were shown to physically interact several years ago (Goff et al., 1992). When Lloyd et al. (1992) showed that the *Z. mays r* gene could complement *ttg1* mutant defects, they suggested that *TTG1* may encode or activate a bHLH protein in *A. thaliana*. The cloning of genes encoding AN11 and TTG1 (de Vetten et al., 1997; Walker et al., 1999) and other regulatory proteins established that genetic interactions between bHLH, MYB, and WD40 repeat proteins are required for anthocyanin regulation and trichome development. In addition to the physical interaction between bHLH and MYB proteins demonstrated by Goff et al. (1992), bHLH and MYB physical interactions have been found between regulators of *A. thaliana* trichome development (Payne et al., 2000) as well as between proteins purported to be regulators of the anthocyanin pathway in *P. frutescens* (Sompornpailin et al., 2002). In *A. thaliana* and *P. frutescens*, interactions between bHLH and the PAC1 clade also were found. Thus, a reasonable hypothesis is that a triad of MYB, bHLH, and WD40 proteins form a complex required for regulation of several additional traits, including root hair development and seed coat mucilage. A simplistic view of this complex would entail a bHLH at the center of this complex that interacts with a WD40 repeat protein on one side and a MYB protein on the other side. Although this interpretation may be correct, there are several complexities. First, in *A. thaliana* siliques, the expression of the anthocyanin and proanthocyanidin regulatory bHLH, *TT8*, requires the function of *TTG1* (Nesi et al., 2000). Therefore, *TTG1* appears to function upstream of the bHLH. Second, in *P. hybrida*, the AN2 and AN4 putative MYB proteins activate accumulation of the bHLH *an1* RNA (Spelt et al., 2000). Therefore, in these dicots, some of the regulatory genes appear to be under the regulation of other regulatory proteins. One possibility is that once each protein is expressed, it is capable of interacting with the others. There could be benefits for the evolution of such a feedforward loop in which the formation of the regulatory triad could additionally upregulate the expression of the regulated regulator (*TT8* or *an1*), thereby helping to lock in expression of the anthocyanin pathway. In *Z. mays*, it is striking that we have tested for and found no evidence that any of the regulators regulate each other. The *Z. mays* bHLH, MYB, and PAC1 clade WD40 repeat proteins appear to be regulated independently, and expression of all three are required for anthocyanin pigmentation in *Z. mays* kernels and roots. Thus, although a general role for the bHLH, MYB, and WD40 repeat proteins as regulators of the anthocyanin pathway has been conserved in monocots and dicots, the mechanisms of regulation of these regulators has been reorganized.

In summary, PAC1 clade WD40 repeat proteins in *Z. mays*, *P. hybrida*, *A. thaliana*, and possibly *P. frutescens* are required in their respective organisms not only for anthocyanin pigmentation but also for expression of several different traits, with the exact traits varying by species. The PAC1 protein functionally complements *ttg1* mutant phenotypes in *A. thaliana*, demonstrating that PAC1 clade proteins have likely retained all of their functional competency since the divergence of monocots and dicots. This result suggests that differences in the regulation of anthocyanin and proanthocyanidin pathways in *Z. mays*, *P. hybrida*, and *A. thaliana* include not only differences in the organization of the biosynthetic genes that are regulated by these bHLH, WD40 repeat, and MYB proteins but also differences in the regulation of the regulators of these genes. Finally, mutants of the PAC1 clade WD40 repeat proteins (PAC1, TTG1, and AN11) suggest the primary importance of this clade for anthocyanin regulation.

METHODS

Genetic Nomenclature

Genes are indicated by lower case italic letters. Dominant wild-type *Z. mays* alleles are indicated by italic letters with the first letter capitalized. Lower case italic letters indicate recessive *Z. mays* alleles. Homozygous alleles are indicated with a single designation, for example, *pac1-ref* refers to an individual homozygous for the *pac1-ref* allele. *Pac1*/(*Pac1* or *pac1-ref*) indicates a wild-type allele of *pac1* in combination with either a wild-type or mutant allele of *pac1*. Proteins are indicated by non-italic capital letters. In *A. thaliana*, *TTG1* refers to a wild-type allele, and *ttg1-1* and *ttg1-13* refer to recessive mutant alleles.

Origin and Analysis of the *pac1-ref*, *pac1-2*, and *pac1-3* Alleles

Isolation of *pac1-ref* stocks was as described by Selinger and Chandler (1999). Seed used for inverse PCR cloning of *pac1-ref* was from a self-pollinated *pac1-ref*/*Pac1*; *in1*; *R-r*; *b* plant. Certain alleles of *r* or *b* are required to see *pac1* phenotypes in the aleurone. In this work, observation of the *pac1* phenotype was facilitated by either the *R-r* allele of *r*, the

B-Peru allele of *b*, or both. We isolated the *pac1-2* allele using a directed tagging scheme as follows: *b; R-r*; *pac1-ref* plants were crossed by *B-Peru*; *r-r*; *Mu* plants, resulting in the identification of a pale aleurone kernel heterozygous for the newly identified *pac1-2* allele and the *pac1-ref* allele. Similarly, we isolated the *pac1-3* allele using a directed tagging scheme as follows: *B-Peru/B-I*; *pac1-ref* plants were crossed to *B-Peru; Mu* plants, resulting in the identification of a pale aleurone kernel heterozygous for the newly identified *pac1-3* allele and the *pac1-ref* allele. The *pac1-2* and *pac1-3* alleles were segregated from the *pac1-ref* allele by a series of outcrosses and self-pollinations and confirmed by DNA gel blot analysis using the 700-bp SacI/SmaI probe (Figure 2).

Mapping the *pac1* Locus

The *pac1* locus was previously mapped to the distal end of the long arm of chromosome 5 (Selinger and Chandler, 1999). To facilitate cloning of the *pac1* locus, a *Pac1/pac1-ref* plant derived from a heterogeneous background was self-pollinated and progeny plants used for mapping. We identified a linked molecular marker for distinguishing *pac1-ref* and *Pac1* alleles by screening several simple sequence repeat (SSR) markers on chromosome 5. Parental *pac1-ref* and *Pac1* lines displayed a size polymorphism (140 and 170 bp, respectively) using the SSR primers Phi087 (forward, 5′-GAGAGGAGGTGTTGTTTGACACAC-3′) and Phi087 (reverse, 5′-ACAACCGGACAAGTCAGCAGATTG-3′) (Maize Genetics and Genomics Database, http://www.maizegdb.org). Plants from pale and dark seeds in the mapping population were screened for linkage with *pac1*. All 25 plants from pale seed were positive only for the 140-bp product, suggesting a distance of <3.9 centimorgan from the phi087 SSR marker. Ten plants from dark seed were positive for both the 140- and 170-bp products, and three plants only produced the 170-bp product. Subsequent probing with *Mu1* (described below and in Results) confirmed the presence of the *pac1-ref* cosegregating band in 11 plants from the pale seed and its absence in the three wild-type plants, from which only the 170-bp Phi087 PCR product was obtained.

Cosegregation Analysis and PCR Identification of the *pac1-ref*, *pac1-2*, and *pac1-3* Alleles

DNA samples from plants homozygous for *pac1-ref* or *Pac1* were digested with EcoRI and HindIII, blotted, and then probed with a *Mu1*-specific sequence from plasmid p*A/B*5 (Talbert et al., 1989). To recover DNA flanking the *Mu1* element, *pac1-ref* DNA was digested with EcoRI and self-ligated under very dilute conditions to obtain circular products. Inverse PCR of the *pac1-ref* locus made use of primers vc141 (5′-CGCACGGGAACGGTAAACGGGGACAGAAAAC-3′) and vc143 (5′-TGACAGAGACACGAGACGAAACAAGCTGAAGG-3′) within the *Mu1* element, resulting in amplification across the terminal inverted repeats into the DNA flanking the element. PCR products were used as templates for a second reaction with primers vc140 (5′-AGTTTGGCTGTC-GCGTGCGTCTCCAAAACAG-3′) and vc144 (5′-AGTTTGGCTGTCGCG-TACGTCTCTAAAACAG-3′), which are nested toward the ends of the *Mu1* element relative to vc141 and vc143. A 1588-bp product was present in homozygous mutant samples and absent in homozygous wild-type control samples. GenBank accession numbers AY442344 and AY442345 for *pac1-ref* were derived from this product. Part of this 1588-bp product was designated the SacI/SmaI 700 probe and used for hybridization to DNA gel blots. The *pac1-2* allele was amplified from *pac1-2* genomic DNA using a primer common for many *Mu* element termini (rm005, 5′-CATTTCGTCGAATCCCCTTCC-3′) and a primer in the *pac1* locus (vc293, 5′-GGTACAGCCGCCGCCGTCTCAG-3′). The sequence obtained from the *pac1-2* locus (GenBank accession number AY442262) included 28 bp of sequence best matching the ends of *MuDR*, and DNA gel blot analysis revealed a BamHI site in the vicinity of the insertion. The size (∼1400 bp) of the band visualized with DNA gel blot analysis using *pac1-2* DNA, SacI/SmaI 700 probe, and EcoRI/HindIII enzymes suggests that the *pac1-2* allele contains a *MuDR* element with a significant internal deletion that preserved one of the BamHI sites in *MuDR*. The *pac1-3* allele (GenBank accession number AY442261) was amplified from *pac1-3* genomic DNA using two primers in the *pac1* locus: vc181 (5′-CAACCGCAAGGC-CTCCTCCGAGTTCTG-3′) and vc189 (5′-GTACACTAGCACAGGATC-AATCC-3′).

Identification and Sequencing of BAC Clones Containing the *pac1* and *mp1* Loci

BAC filters from the CUGI *Zea mays* National Science Foundation B73 library were hybridized according to directions (http://www.genome.-clemson.edu/groups/bac/protocols/bacmanual.html) with the 700-bp SacI/SmaI probe. We ordered several of the strongest hybridizing clones and screened them by PCR amplification for the *pac1* locus using primers vc178 (5′-CGCCTTTGAGAACAAGGTCCAG-3′) and vc182 (5′-AGAGCTGGAATTAAAGTTCAATTCCTTGC-3′). Positive clones included ZMMBBb0174B16, ZMMBBb0176H10, ZMMBBb0213M14, ZMM-BBb0231M22, ZMMBBb0124P19, ZMMBBb0156C21, and ZMMBB-b0227P08. Three overlapping subclones from clone ZMMBBb0124P19 were isolated, sequenced, and assembled into a contig of 8239 bp (GenBank accession number AY115485). To identify potential homologs of *pac1*, we examined additional BAC clones hybridizing to the 700-bp SacI/SmaI probe using restriction mapping and DNA gel blot analysis, hybridizing sequentially with the 5′ and 3′ halves of the *pac1* coding region. Several clones, which hybridized strongly to both halves of the coding region, were shown by restriction mapping not to be *pac1*. To determine if these BAC clones contained genes homologous to *pac1*, they were subjected to additional restriction mapping, subcloning, and sequencing. Clone ZMMBBb099L23, which was subcloned and sequenced, and clone ZMMBBb0139C12, which was shown to be overlapping with ZMBBb099L23 based on its restriction pattern, both contain the *mp1* locus (GenBank accession number AY339884), a gene previously identified by Hernandez et al. (2000) as having homology with *an11*. Erich Grotewold provided us with the *mp1* EST sequence, enabling verification that the genomic clones represented the same gene as *mp1*.

RNA Analysis

Total RNA was prepared using TRIzol reagent (Invitrogen Life Technologies, San Diego, CA) for husk, sheath (after pollination), and immature tassel (2 cm in length) or the endosperm RNA isolation method described by Wessler (1994) for aleurone, scutellum, black layer, endosperm, and pericarp all at 23 to 25 d after pollination. RNA from freshly emerged silks, pallea/lemma, and cob/cupules from the same ear and 3-week-old seedling root, leaf, sheath, and inner leaf/meristem also were prepared using the endosperm RNA isolation method. 3′ RACE of immature tassel and aleurone total RNA was done according to instructions in the 5′/3′ RACE kit from Boehringer Mannheim (Roche Diagnostics, Indianapolis, IN) using *pac1* primer vc177 (5′-TGATCTGGGAACTGCCTGAGAC-3′) or vc178 (5′-CGCCTTTGAGAACAAGGTCCAG-3′). 3′ RACE was more successful with immature tassel than with aleurone and was therefore the source of all 3′ RACE clones. RNase protections were as described by Selinger and Chandler (1999) using antisense probes designed against the *pac1* cDNA. The pac1-rp-3′ probe resulted from the 3′ RACE reactions using the *pac1* primer vc177 and therefore spans the splice site for the two *pac1* exons and ends at the most common poly(A) site. The pac1-rp-300 probe, which spans the locations of all three *pac1* mutations, was generated by PCR from wild-type DNA using primers

vc554 (5′-TCACCTCCTTCGATTGGAAC-3′) and vc555 (5′-GCCATA-TAGCGGAGGTCAGA-3′). In all protections, 5 to 10 ug of total RNA and the internal controls *actin1* (Selinger and Chandler, 1999) or *ubiquitin2* (Dorweiler et al., 2000) were used.

Complementation of *ttg1* Mutants

A *pac1* genomic fragment was cloned into a binary expression vector by replacing GUS in the GSA1131 vector (http://www.chromdb.org/) using NcoI and BamHI sites engineered into the *pac1* transcript at the start and stop codons using primers vc280 (5′-CATGCCATGGACCCACCCAAG-CCGCC-3′) and vc281 (5′-GCGGGATCCTCAGACCCTAAGAAGCTG-GACC-3′). The source of the template was a subclone of BAC ZMMBBb0124P19 and, therefore, the B73 allele. The GSA1131 vector included a Basta resistance gene for herbicide-resistant selection of transformants. The *Agrobacterium tumefaciens*–mediated floral dip method (Clough and Bent, 1998) was used to transform the *ttg1-1* and *ttg1-13* lines described in Larkin et al. (1999), and the *ttg1-1* stock (CS89) from Koornneef (1981) was obtained from the ABRC. The former stocks were predominantly in the Columbia background, and the latter stock was in the Landsberg *erecta* background. A total of 32,000 seeds were planted. The transformation efficiency was ~0.2% for each line. Lines that were propagated segregated 60 to 81% Basta resistance with a median of 71%, suggesting that the propagated lines segregated a single transgenic locus in the expected 3:1 ratio. Trichome phenotypes were observed in *35S-Pac1 ttg1* regenerants from all three *ttg1* mutant lines. Medium used for plates was MS (Sigma, St. Louis, MO) with 3% (w/v) sucrose and 4 g of phytagar. For selection, Basta was used at a concentration of 10 μg/mL. Seed was imbibed for 3 or 8 d at 4°C and then plated. Seedlings were transplanted into soil at leaf stages 4 to 8 for the first planting of transformed seed or at leaf stages 2 to 4 for the second planting of transformants. In most experiments, controls included parental mutant lines as well as seeds resulting from the *A. tumefaciens*–mediated floral dip that did not appear to be transformed. Together, these controls are referred to as nontransformed. Plants for all experiments were grown in an 18-h-light/6-h-dark cycle with fluorescent lighting. Ruthenium red staining of seed coat mucilage was as described by Penfield et al. (2001).

Z. mays Seedling Root Color Score

A *pac1-2/Pac1 B-Peru* plant heterozygous for a functional and nonfunctional *r* allele was self-pollinated, resulting in purple and pale spotted seeds. Fourteen purple (*Pac1⁻*) and 14 pale (*pac1-2*) spotted seeds were germinated in paper towels moistened with tap water and grown in the dark until roots were 15 cm in length on average, at which point they were scored for pigment and harvested for DNA. Because the *r* allele responsible for root pigmentation was heterozygous with an allele incapable of function in the root, progeny were screened using DNA gel blot analysis for the presence of restriction length polymorphisms indicative of the root-functioning *r* allele. We excluded all seedlings that did not carry the functional *r* allele, leaving 11 *pac1-2* mutant and 9 wild-type *Pac1* or *Pac1/pac1-2* plants. All excluded seedlings had a root color score of 1. The *pac1* genotypes were confirmed by DNA gel blot analysis.

Generation of the *in1*; *pr1*; *R-r* Stock Segregating *pac1-ref* and *Pac1*

An *in1*; *pr1*; *R-scm* (a revertant of the *R-mb* allele giving full seed color; Maize Genetics and Genomics Database, http://www.maizegdb.org) plant was crossed with a *pac1-ref*; *In1*(wt); *R-r* plant. All resulting seeds were dark. Plants derived from this seed were self-pollinated, generating red, pale, and dark seed. Red seeds were planted (*pr1 Pac1*/*pr1* [*Pac1* or *pac1-ref*]; *In1*/[*In1* or *in1*]), and the resulting plants self-pollinated. Dark progeny seeds were planted (*pr1* [*Pac1* or *pac1-ref*]/*pr1* [*Pac1* or *pac1-ref*]; *in1*) and self-pollinated. Two of these self-pollinated plants (ds1432-4 and ds1474-1) generated ears that were segregating one-quarter pale and three-quarters dark. The phi087 SSR marker polymorphism and DNA gel blot analysis was used to determine *pac1* wild-type and mutant genotypes. All assayed pale seed from one ear (ds1432-4) were *pac1-ref*; whereas all assayed dark seed from the same ear were heterozygous or wild-type for *Pac1*. Similar results were obtained with the other ear (ds1474-1).

Bioinformatics and Construction of Alignments/Phylogenies

Publicly available sequences were collected from GenBank through NCBI (http://www.ncbi.nlm.nih.gov/), from the Plant Genome Database (http://www.plantgdb.org/), and from the gene indices (Quackenbush et al., 2001) at the TIGR Gene Index Databases, Institute for Genomic Research, Rockville, MD (http://www.tigr.org/tdb/tgi) from May to July, 2003. We aligned an initial set of 33 homologs using CLUSTAL W at Baylor College of Medicine (http://searchlauncher.bcm.tmc.edu/) with the default Dayhoff percent accepted mutation weight matrix. The alignment was fine-tuned manually using GeneDoc (http://www.psc.edu/biomed/genedoc/). The remaining sequences were added, manually aligned, and have been made available in Fasta format (see supplemental data online). A region that included the first 131 amino acids of PAC1 was selected to construct the alignment used in the phylogeny, resulting in the use of 46 unique coding regions from 31 species, from which all regions of insertions and deletions were removed leaving each sequence with 97 amino acids (indicated by shading or the use of lower case letters in the PAC1 sequence in the supplemental data online). The phylogenies were constructed using the PHYLIP package of programs (http://evolution.genetics.washington.edu/phylip.html) (Felsenstein, 1989) and Protml (Adachi and Hasegawa, 1996). Fitch, Kitsch, and neighbor joining programs all were used as documented in the PHYLIP package of programs, with each giving approximately the same tree topology. For the results presented in Figure 6, 500 bootstraps were performed with neighbor joining to derive a consensus tree that was used as an input for the Protml program with default options to derive distance measurements. Bootstrap values for the consensus tree from neighbor joining are indicated along the branches for values >250. The *Oryza sativa* (rice) and *Z. mays pac1* loci are syntenous, as determined using the Gramene Web site (http://www.gramene.org/).

Accession and Sequence Identifier Numbers

GenBank accession numbers for *pac1* and *mp1* are as follows: AY115485 (*pac1*), AY442344 and AY442345 (*pac1-ref*), AY442262 (*pac1-2*), AY442261 (*pac1-3*), and AY339884 (*mp1*).

TIGR gene indices, GenBank, and Plant Genome Database report, accession, identifier numbers, and scientific names are as below for sequences used in the phylogeny. Each is further commented on in the supplemental data online. TIGR gene indices reports: TC73929 (Barley1 *Hordeum vulgare*), TC66955 (Potato1 *Solanum tuberosum*), TC126206 (Tomato1 *Lycopersicon esculentum*), TC146713 (Soybean1 *Glycine max*), TC146716 (Soybean2 *G. max*), TC93713 (Alfalfa1 *Medicago truncatula*), TC17885 (Cotton1 *Gossypium hirsutum*), TC15388 (Cotton2 *G. hirsutum*), TC185139 (TTG1_Arabidopsis1 *A. thaliana*), TC5440 (Iceplant1 *Mesembryanthemum crystallinum*), TC16648 (Cotton3 *G. hirsutum*), TC21234 (Chlamydomonas_reinhardtii1 *C. reinhardtii*), TC10687 (Grape1 *Vitis vinifera*), TC89552 (Alfalfa2 *M. truncatula*), TC17903 (Cotton4 *G. hirsutum*), TC52375, TC51908, and GenBank BE592997 (Sorghum2 *Sorghum bicolor*), TC78949 (Barley2 *H. vulgare*), TC142332 (Frog1 *Xenopus*

Laevis), TC804879 (Mouse1 *Mus musculus*), THC1438474 (Human1 *H. sapiens*), TC153935 (Drosophila1 *Drosophila melanogaster*), TC36782 (Mosquito1 *Anopheles gambiae*), TC75759 (Nematode1 *Caenorhabditis elegans*), TC26097 (Ciona_intestinalis1 *Ciona intestinalis*), and TC11950 (Saccharomyces_cerevisiae1 *S. cerevisiae*).

GenBank accession and GenInfo numbers: BG560471 GI 13589469 (Sorghum1 *S. propinquum*), AY115485 (PAC1_Maize1 *Z. mays*), AP004178 GI 15718435 (Rice1 *O. sativa* range 71305 to 72372), AB059642 GI 14270084 (Perilla1 *P. frutescens*), AF220203 GI 6752885 (Apple1 *Malus x domestica*), U94748 GI 2290531 (AN11_Petunia1 *Petunia x hybrida*), BQ087012 GI 20046213 (Ceratopteris_richardii1(Fern) *C. richardii*), U94746 GI 2290527 (ATAN11A_Arabidopsis2 *A. thaliana*), X97488 GI 1495264 (ATAN11B_Arabdidopsis3 *A. thaliana*), BE033994 GI 8329003 (Iceplant2 *M. crystallinum*), AF530912 GI 22324808 (Cotton5 *G. hirsutum*), AY339884 (MP1_Maize2 *Z. mays*), AP004772 GI 18844992 (Rice2 *O. sativa* range 96686 to 97918), CB611069 GI 29550682 (Orange1 *Citrus sinensis*), BF098437 GI 10904147 (Tomato2 *L. esculentum*), BU825070 GI 23996202 (Poplar1 *Populus tremula x P. tremuloides*), CAB02116.2 GI 7160713 (Nematode2 *C. elegans*), T39719 GI 7490148 (Schizosaccharomyces_pombe1 *Schizosaccharomyces pombe*), and EAA38483 GI 29246903 (Giardia1 *Giardia lamblia*).

Plant Genome Datebase EST contig numbers: BVSVtuc03-04-08. 2097 (Beet1 *Beta vulgaris*) and TAtuc02-12-22.2204 (Wheat1 *Triticum aestivum*).

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers AY115485, AY339884, AY442261, AY442262, AY442344, and AY442345.

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