RESEARCH ARTICLE

A Mutation in the pale aleurone color1 Gene Identifies a Novel Regulator of the Maize Anthocyanin Pathway

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By screening for new seed color mutations, we have identified a new gene, pale aleurone color1 (pac1), which when mutated causes a reduction in anthocyanin pigmentation. The pac1 gene is not allelic to any known anthocyanin biosynthetic or regulatory gene. The pac1-ref allele is recessive, nonlethal, and only reduces pigment in kernels, not in vegetative tissues. Genetic and molecular evidence shows that the pac1-ref allele reduces pigmentation by reducing RNA levels of the biosynthetic genes in the pathway. The mutant does not reduce the RNA levels of either of the two regulatory genes, b and c1. Introduction of an anthocyanin structural gene promoter (a1) driving a reporter gene into maize aleurones shows that pac1-ref kernels have reduced expression resulting from the action of the a1 promoter. Introduction of the reporter gene with constructs that express the regulatory genes b and c1 or the phlobaphene pathway regulator p shows that this reduction in a1-driven expression occurs in both the presence and absence of these regulators. Our results imply that pac1 is required for either b/c1 or p activation of anthocyanin biosynthetic gene expression and that pac1 acts independently of these regulatory genes.

INTRODUCTION

The maize anthocyanin pathway provides an excellent system for the study of gene expression. The pathway is well characterized genetically, biochemically, and molecularly, and the product, a purple pigment, is easily scored visually for both the presence and intensity of pigment (reviewed in Holton and Cornish, 1995). We have used a genetic approach to characterize genes that control expression of this pathway in the aleurone layer of the seed. Pigmentation of the maize aleurone requires functional alleles of the biosynthetic genes c2, a1, a2, bronze1 (bz1), and bz2. These biosynthetic genes are expressed in the aleurone only if the C1 allele of the c1 locus and either the B-Peru allele of the b locus or one of several R alleles of the r locus are present (reviewed in Coe and Neuffer, 1977; Styles and Ceska, 1977). Molecular studies have shown that the b and r genes produce homologous basic helix-loop-helix transcription factors (Chandler et al., 1989; Ludwig et al., 1989) that are functionally interchangeable (Goff et al., 1990; Ludwig et al., 1990). The b/r gene product directly interacts with the product of the c1 gene (a Myb transcription factor; Paz-Ares et al., 1986) to induce the expression of biosynthetic genes (Goff et al., 1992). In addition to these regulatory genes, three other genes in maize are known to affect anthocyanin pigmentation in aleurone tissue: anthocyaninless lethal1 (anl1), intensifier1 (in1), and viviparous1 (vp1). The anl1 gene was identified by a recessive lethal mutant that lacks anthocyanin pigmentation of the maize aleurone (Coe et al., 1988), the in1 gene acts as a recessive intensifier/dominant inhibitor of anthocyanin pigmentation in the aleurone (Fraser, 1924), and mutants of vp1 have seeds with colorless aleurones that fail to mature properly (Robertson, 1955). The product of the vp1 gene is required for the expression of C1 (McCarty et al., 1989a; Hattori et al., 1992). Although the anthocyanin pathway in maize has been studied since the beginning of the 20th century, we suspected that more genes affect this pathway than have been identified. Our reasoning derives in part from the identification, in other plants, of genes distinct from the regulatory genes identified in maize (Mol et al., 1996; de Vetten et al., 1997). This discrepancy indicates either that maize exhibits distinct regulation of the pathway or that we have not discovered all of the genes that regulate the pathway. To search for unidentified regulatory genes, we set up a genetic screen that involved looking for maize ears from selfed plants that showed approximately one-quarter pale or colorless kernels instead of the normal 100% darkly colored kernels. In a small-scale screen of ~1000 ears, we identified two novel mutants. One of these mutants appears to identify a new regulator of the maize anthocyanin biosynthetic genes.
RESULTS

Isolation of pale aleurone color1

Approximately 1000 maize plants from stocks with active Mutator (Mu) transposable elements and homozygous for B-Peru, C1, and functional alleles of the biosynthetic genes of the anthocyanin pathway were selfed. The resulting ears were screened for kernels showing an altered anthocyanin pigment phenotype. Two of these ears appeared to segregate pale kernels representing approximately one-quarter of the total (Figure 1A). After planting the pale seeds of each mutant, we noted that plants homozygous for one of the mutants had no apparent growth defect or other phenotype. We designated this mutant pac1-ref, which stands for the reference allele of the pale aleurone color1 (pac1) gene. Aleurone development appeared normal in pac1-ref kernels compared with wild-type kernels. Other than the difference in pigmentation, the pac1-ref aleurone cells showed no differences, including size and position of the vacuoles within the aleurone cells, when compared with cells of wild-type kernels. Plants homozygous for the other mutation, designated pac2-ref, displayed a miniature plant phenotype in which the plants grew to <0.5 meters tall. Heterozygous siblings of this mutant reached ≥1.5 meters in height and were indistinguishable from wild-type plants. These miniature plants were not dwarfs, because the internode length was not abnormally short relative to the other organs of the plant. The miniature plants were infertile, which effectively rendered pac2-ref a lethal mutation. We have chosen to focus on pac1-ref because of its lack of pleiotropic phenotypes and easily scored color phenotype.

The pac1 Mutant Is Not Allelic to Known Anthocyanin Genes

Plants homozygous for pac1-ref were crossed with tester stocks, each with a recessive mutation in one of the anthocyanin genes a1, b, bz1, bz2, c1, c2, red aleurone1 (pr1), and r. Mutations in pr1 result in the loss of flavonoid 3′-hydroxylase enzymatic activity, which leads to the production of pelargonidin, a red pigment, in homozygous pr1 kernels and plants instead of cyanidin, the purple pigment that is normally produced (Larson et al., 1986). Table 1 provides the results of the complementation tests. In all cases, the mutant complemented the testers. In addition, the pac1-ref and pac2-ref mutants complemented each other, indicating that they are not allelic. We did not testcross pac1-ref plants to testers for a2 or vp1. However, the results from the mapping experiment described below rule out allelism of pac1-ref with these two genes.

pac1 Maps to Chromosome 5L

Plants homozygous for pac1-ref, B-Peru, C1, and functional alleles of the biosynthetic genes of the anthocyanin pathway were crossed to a set of B-A translocation stocks. In maize, B chromosomes and translocations between B and normal A chromosomes that carry the B centromere exhibit high levels of nondisjunction in the second meiotic division of male microsporogenesis. This feature allows us to use these translocations to map recessive mutants to particular chromosome arms by looking for phenotypically mutant kernels on an ear pollinated by a B-A-containing stock (Beckett, 1994). The endosperm and aleurone of these phenotypically mutant kernels are hypoploid for the chromosome arm on which the mutant is located, and the seeds contain hyper-
plaid embryos. The pac1 plants were crossed with the full set of plants carrying B-A translocations, and we found that only the B-A translocation between the long arm of chromosome 5 and the B centromere revealed the mutant phenotype (Table 1). All five ears from crosses between pac1-ref plants and TB-5La plants contained mutant kernels. The number of mutant kernels was ~10% of the total kernels on these ears (Table 1). This result indicates that pac1 resides on chromosome 5L because in every cross with TB-5La, nondisjunction revealed plants carrying the recessive mutant phenotype.

To confirm the B-A mapping result and to more precisely map pac1, we localized pac1 relative to pr1, which is also located on chromosome 5L and determines whether the anthocyanin pigment will be red (pr1) or purple (Pr1). The F1 seed from a cross between the pac1-ref pr1 homozygote and a Pac1 pr1 homozygous stock was planted and backcrossed to the original B-Peru, pac1-ref Pr1 stock (see Figure 2 for a diagram of the crosses involved). This backcross was done, rather than a self-cross, for two reasons. First, because the kernels in the pac1-ref stock were colored by B-Peru, whereas the kernel color in the pr1 stock was due to R-r, the F1 plant was heterozygous for both B-Peru and R-r, and thus, a selfed ear would have produced colorless seeds at a frequency of one to 16 due to the doubly recessive b and r class of seeds. These colorless seeds would be very difficult to distinguish from pac1-ref kernels. Second, the pale red kernels from the pac1-ref pr1 recombinant class may have been difficult to distinguish. To avoid these problems, we planted 100 pale kernels from this backcross and testcrossed 79 plants by the Pac1 pr1 stock. Recombination events in the F1 generation were recovered in the backcross as pac1-ref pr1/pac1-ref Pr1 seeds that had a pale purple seed color phenotype. The other recombinant class yielded Pac1 Pr1/pac1-ref Pr1 seeds that had a dark purple phenotype (Figure 2). In contrast, nonrecombinant gametes produced pac1-ref Pr1/pac1-ref Pr1 pale seeds and Pac1 pr1/pac1-ref Pr1 dark purple seeds.

We selected for pac1-ref homozygotes by planting the pale seeds. We were able to determine how many of these seeds carried the recombinant pac1-ref pr1 chromosome by crossing them to the Pac1 pr1 stock. From a total of 79 plants, 27 produced ears with 50% purple/50% red seed, which indicated a recombinant, and 52 had ears with 100% purple seed. This result gives a recombination frequency of 34%. Thus, pac1 and pr1 are linked (\( P < 0.01 \)) and are separated by ~34 centimorgans (cM). Because pr1 is located ~19 cM from the centromere on chromosome 5L, and we know from the B-A mapping that pac1 is on chromosome 5L, it appears that pac1 maps to the telomere proximal region of the long arm of chromosome 5, a region of the maize genome that contains no other known anthocyanin genes.

The pac1-ref Allele Reduces the Pigmentation of the Seed Directed by an Allele of r

The pac1-ref allele was identified as a mutation that produces pale seeds in a stock in which seed pigmentation is directed by the B-Peru allele of the b gene. In addition to B-Peru, several alleles of the r gene, including the R-r allele, can induce anthocyanin pigmentation of the aleurone layer of the seed (Styles et al., 1973). To determine whether the
reduction in anthocyanin pigmentation of the pac1-ref mutant allele was specific to B-Peru-induced pigmentation, we crossed pac1-ref into a b, R-r background (Figure 3 contains details of the crosses involved). After crossing the B-Peru, pac1-ref, r stock with a b, Pac1, R-r stock to create the F1 generation, we grew these seeds and selfed the resulting plants to obtain the F2 generation. The colorless F2 seeds resulting from b and r homozygotes and some of the pac1-ref homozygotes were discarded, and 100 pale-colored seeds (pac1-ref homozygotes) were selected and planted. The resulting plants were testcrossed to b/b, r/r, Pac1/Pac1 plants to determine their b and r genotypes and were self-crossed. Because we selected seeds that were homozygous for pac1-ref, our testcross would reveal whether pac1-ref can reduce R-r-directed seed color. If pac1-ref affects R-r seed color, then pale seeds should yield some plants containing the R-r allele. Because of the male-specific imprinting displayed by R-r (Kermicle, 1970), we could distinguish the R-r-directed seed color from that of B-Peru in the ears resulting from the testcross. The imprinting of R-r results in mottled kernels when it is transmitted through the male rather than the solid-colored kernels produced when R-r is transmitted through the female gametophyte. The B-Peru allele produces uniformly dark seeds regardless of its parental origin.

Two plants gave 100% mottled seed in the ears from the outcross to the b and r tester. Both of these plants produced 100% pale seeds in the ears from the self-cross, and these ears looked very similar to those from plants homozygous for B-Peru and pac1-ref (an example of each is shown in Figure 1B). Nine additional plants gave three-quarters pigmented seeds in the testcross, with approximately one-third of pigmented seeds being mottled. This outcome is expected for plants that are heterozygous for both B-Peru and R-r. The pac1 genotype was confirmed by testcrossing the progeny from the selfed ears to the B-Peru, pac1-ref stock. This result indicates that pac1-ref prevents the normal dark pigmentation produced by R-r as well as that produced by B-Peru and thus that pac1-ref is not specific to B-Peru-induced pigmentation.

Because the R-r allele also colors two other seed tissues, the embryo and the scutellum, we could assess whether pac1-ref affects the pigmentation of these two tissues. Scutellum color was reduced in a manner similar to that of the aleurone. The scutellum is a diploid, zygotic tissue, and the observation that pac1-ref alters pigment in this tissue indicates that the activity of pac1 is not confined to the tripliod endosperm tissues. Unlike the scutellum, pigmentation of the embryo was very similar to that of wild-type seeds.

The pac1-ref Allele Does Not Noticeably Affect the Anthocyanin Pigmentation of Plant Tissues Induced by the B-I or r-r Alleles

The B-Peru allele does not confer strong pigmentation on the vegetative tissues of the plant. Thus, to determine whether the pac1-ref allele is specific to the seed, we crossed pac1-ref into a stock containing the B-I allele, which produces strong pigmentation of most of the vegetative tissues of the plant. To generate a stock homozygous for pac1-ref and containing B-I, F1 progeny (B-Peru/B-I, Pac1/pac1-ref) were selfed, and we selected the colorless seeds. Colorless seeds resulted from the following genotypes: B-Peru/(B-Peru or B-I) and pac1-ref/pac1-ref or B-I/B-I (the B-I allele does not confer seed color with any pac1 genotype). The F2 plants derived from colorless seeds were scored for plant color to identify whether B-I was present and testcrossed to B-Peru/B-Peru and pac1-ref/pac1-ref. Eight plants with at least one copy of B-I and homozygous for pac1-ref were identified from 29 plants. The color of the pac1-ref/pac1-ref plants was within the range seen with the Pac1/Pac1 and Pac1/pac1-ref siblings. To confirm the result, we separated 40 progeny from a cross of a B-I/B-I and Pac1/pac1-ref plant to the B-Peru/B-Peru and pac1-ref/pac1-ref plant by seed color, and they were plated out. The 20 plants resulting from pale seeds (B-I/B-Peru and pac1-ref/pac1-ref) did not differ significantly in plant color from the 20 plants that resulted from dark seeds (B-I/B-Peru and Pac1/pac1-ref).

To determine whether pac1-ref affects the pigmentation of the anthers that is directed by the r-r allele of r, we generated pac1-ref homozygous plants that carried at least one
copy of r-r. These plants were produced by a scheme of crosses similar to those used to generate the B-Peru, pac1-ref stock. These r-r and pac1-ref/pac1-ref plants had the same intensity of anther pigmentation as seen in wild-type plants. These results show that Pac1 is not required for normal expression of anthocyanin pigments in most of the vegetative tissues of the plant.

The pac1-ref Allele Reduces the RNA Levels of the Biosynthetic Genes a1, bz1, and c2 but Does Not Reduce b or c1 RNA Levels

From the results of the genetic experiments, we have shown that pac1 is not allelic to any known anthocyanin gene, that it affects seed pigment activated by both B-Peru and R-r, and that it does not affect anthocyanin expression directed by the B-I and r-r alleles that function in vegetative plant tissues. These results suggest several possibilities for the action of pac1. One of these possibilities is that pac1 is a regulator of the anthocyanin pathway. Based on the genetic data, it could act independently of B-Peru and C1 on the target genes or it could act on one of the regulatory genes. An example of the latter possibility is provided by vp1 activation of C1 transcription. To determine whether pac1 affects the production of anthocyanin pigments by affecting the expression of the regulatory or biosynthetic genes, we measured the RNA levels of the regulatory genes b and c1 and three of the biosynthetic genes, c2, a1, and bz1. These three biosynthetic genes encode the enzymes chalcone synthase (c2), dihydroflavonol reductase (a1), and UDP-glucose:flavonoid 3-O-glucosyltransferase (bz1), which represent the first step, the last shared step with other flavonoid pathways, and the last biosynthetic step, respectively, in the maize anthocyanin pathway.

Ears were harvested 35 days after pollination, and the crowns of pale (B-Peru/B-Peru and pac1-ref/pac1-ref) kernels and dark (B-Peru/b and Pac1/pac1-ref) kernels were collected. Total RNA was prepared, and 10 μg of this RNA was used in each RNase protection reaction. Figure 4 shows the results of six RNase protection experiments. For the first experiment, a1, b, c1, and actin probes were used; for the second, b, c2, and actin probes were used; and for the third, bz1 and actin probes were used. In each experiment, the results from the anthocyanin probes were normalized against the actin RNA level. The three experiments revealed that pac1-ref kernels had significantly less a1, bz1, and c2 RNA compared with the heterozygous kernels. These results indicate that pac1-ref reduces pigmentation by reducing the expression of the biosynthetic genes.

In addition, the pac1-ref/pac1-ref kernels had no reduction in b and c1 RNA levels compared with the heterozygous kernels. This result indicates that pac1-ref does not reduce pigmentation by reducing the expression of the known regulatory genes of the pathway. The slightly higher b and c1 RNA levels in the pac1-ref homozygous kernels are probably due to the dosage of B-Peru and/or background effects, which are examined in the Discussion section. Because pac1-ref kernels had markedly reduced expression of the biosynthetic genes but normal expression of the regulatory genes, pac1 represents a new regulator of the anthocyanin pathway in maize.

The pac1-ref Allele Affects Both B/C1 Activation and P Activation of the a1 Promoter

We have shown that pac1 affects the expression of multiple anthocyanin biosynthetic genes and is thus a possible regulator of these genes. Because the pac1-ref allele reduces the pigmentation and RNA levels of the anthocyanin structural genes, we hypothesize that the wild-type allele, Pac1, encodes a positive regulator. In maize, the first steps of the anthocyanin pathway, including the c2 and a1 genes, are identical to the first steps of the phlobaphene pigment pathway controlled by the p gene. In the aleurone, B-Peru, in combination with C1, normally induces expression of the a1 gene, which represents the last shared step in the anthocyanin and phlobaphene pathways. In pericarp tissue, the a1 gene is induced by the P-rr allele of the p gene or by certain alleles of b and r. In addition, expression of either P or B/R and C1 in tissues that are not normally pigmented by these genes induces the expression of the endogenous genes and of chimeric reporter constructs containing the a1 gene promoter (Grotewold et al., 1994; Tuerck and Fromm, 1994).

To test whether pac1-ref is specific to B/C1 activation, we introduced a reporter construct consisting of the a1 promoter driving expression of the firefly luciferase (luc) cDNA into either Pac1, b, C1 kernels or pac1-ref, b, C1 kernels. To determine whether the a1 promoter was induced, we introduced the a1::luc reporter, together with either the B-Peru and C1 cDNA constructs driven by the cauliflower mosaic virus (CaMV) 35S promoter or with the P cDNA driven by the CaMV 35S promoter. We tested 50 ng of the regulatory constructs with 2.5 μg of the reporter construct. In this experiment, a clear defect in a1-driven luciferase expression was evident in the pac1-ref kernels in both the B/C1 and P cotransformed samples (Figure 5). In addition, the level of luciferase expression in the absence of any regulatory genes was reduced in pac1-ref aleurones relative to the wild-type aleurones. We also performed experiments using 20 ng of DNA of each of the regulatory gene constructs and 2.5 μg of DNA of the a1::luc reporter. In these experiments, a defect in B/C1 induction was clearly seen in the pac1-ref kernels, but the P induction differences were too small to reveal any reduction in the pac1-ref kernels (data not shown). We found that the bombardment of the a1::luc reporter construct by itself into B-Peru, C1, Pac1 aleurones produced a luciferase expression level similar to that produced by b, C1, Pac1 kernels bombarded with the a1::luc reporter and 20 ng of each of the CaMV 35S B and C1 regulatory gene constructs (data not shown). Because the activation of the a1 promoter by
both B/C1 and P is affected, the activity of pac1 is not specific to the B/C1 regulation of the a1 promoter.

**DISCUSSION**

The pac1-ref mutant allele defines a new regulator of the anthocyanin biosynthetic genes, pac1. It maps to chromosome 5L and affects both B-Peru- and R-r-directed expression of anthocyanin pigmentation of the aleurone and scutellum. Like several other genes in the anthocyanin pathway, pac1 shows an apparent tissue specificity. All of the known alleles of b and r show tissue specificity (Coe, 1979). The expression of c1 is restricted to the seed (Hanson et al., 1996), and most alleles of pl (for purple plant), a paralog of the c1 gene, are expressed only in the plant (Cone et al., 1993). Like b and r, c1 and pl produce homologous protein products that are functionally interchangeable. The recessive intensifiers in1 and a3, which may encode negative regulators, also show tissue specificity, with in1 affecting anthocyanin expression only in the seed and a3 affecting expression only in the plant tissues (Coe, 1985; Styles and Coe, 1986). In contrast, most of the biosynthetic genes show no limits of expression other than those imposed by the regulatory genes. An exception to this pattern is c2. Both the c2 and white pollen1 (whp1) genes encode the enzyme chalcone synthase, and they display incompletely overlapping patterns of expression. The known C2 alleles are strongly expressed in both the aleurone and vegetative plant tissues. In contrast, Whp1 is normally not expressed in the aleurone, but it is expressed in the plant to a level sufficient to partially cover the loss of c2 function (Coe et al., 1981).

We have shown that pac1 is involved in the regulation of anthocyanin biosynthetic gene expression (and possibly part of the phlobaphene pathway), but we do not know whether pac1 is specific to the anthocyanin pathway, as the regulators b, c1, pl, and r are thought to be. The lack of other phenotypes in the pac1 mutant suggests that pac1 is a specific regulator of anthocyanin genes. However, be-

![Image](https://via.placeholder.com/150)
cause we have only one allele, it is possible that this allele is defective only for some of the functions of the native gene. For instance, the vp1 gene has such alleles; a class of vp1 alleles that are defective for C1 activation but have no viviparous phenotype has been identified (McCarty et al., 1989b). The isolation of other pac1 alleles and the cloning of the pac1 gene should allow us to determine whether pac1 is a specific regulator of the anthocyanin pathway.

The pac1 gene appears to regulate different parts of the entire anthocyanin pathway: c2 is at the beginning of the pathway, a1 is at the branch point with the phlobaphene pathway, and b2 is at the last step of anthocyanin pigment synthesis. The RNA levels of all three of these biosynthetic genes were dramatically reduced in pac1-ref kernels. We suspect that the same reductions occur with the other anthocyanin biosynthetic genes. Given that the pac1-ref allele appears to affect the ability of the p gene product to induce the a1 promoter, pac1 may in fact affect more than just the genes in the anthocyanin pathway.

The pac1-ref allele affects the activation of the a1 promoter by both b/c1 and p in transient assays. We have not formally tested for the effect of the pac1-ref allele on anthocyanin or phlobaphene pigmentation of the pericarp, where p is normally expressed. However, based on the fact that pericarp is a maternal tissue and thus more akin to the vegetative parts of the plant than to the aleurone, we suspect that it would have no effect, because pac1-ref does not affect anthocyanin expression in any of the vegetative tissues that we have examined. In theory, we could have detected pac1-ref effects in B-I-mediated pericarp pigmentation. However, this detection was not possible for two reasons: first, the pericarp color is variable with respect to different maize stocks, and second, it depends on the presence of the strong Pl-Rhoades allele of pl. Our B-1 pac1-ref stocks all contained PI’-mahogany, the weak, paramutant derivative allele of PI-Rhoades (Hollick et al., 1995).

Biosynthetic gene RNA levels are dramatically reduced by pac1-ref, but the b and c1 RNA levels are actually slightly higher in pac1-ref kernels than in wild-type kernels (Figure 4). Although it is possible that there is opposite regulation of the regulatory genes by pac1 or feedback regulation through the pathway, we think other explanations are more likely. The apparent increase of b and c1 RNA in pac1-ref kernels is most likely due to background effects but may also reflect the difference in dosage of the B-Peru allele in the wild-type and pac1 samples. In the RNase protection experiments, the dosage of C1 was the same in both the wild-type kernels and pac1-ref kernels, but the dosage of B-Peru differed in the two samples. Because the wild-type sample resulted from an outcross of the pac1-ref plants to a standard b and r tester stock and the B-Peru and pac1-ref plants were used as male, there is only one copy of B-Peru in the wild-type endosperm (and thus aleurone cells) versus the three copies of B-Peru in the pac1-ref kernels.

Another possible factor in the difference in C1 and possibly B-Peru RNA levels in the two samples is the slight difference in the backgrounds of the two samples. The wild-type sample was an outcross to a b tester stock that is derived from a different genetic background than the pac1-ref stock. Because the expression of many phenotypic traits, including anthocyanin expression, can vary considerably in different lines of maize, the difference in backgrounds may be the cause of the slight differences in b and c1 RNA levels.

Assuming that the lesion in the pac1-ref allele results in reduced expression or no expression of the normal gene product, the low level of a1::luc expression in pac1-ref kernels even in the presence of the B/C1 or P proteins indicates that pac1 is required for either B/C1 or P activation of a1. In addition to the reduction of a1::luc reporter expression when cotransfected with the regulatory gene cDNAs in pac1-ref aleurones, we also saw a reduction of expression in pac1-ref kernels when the a1::luc reporter gene was transfected without the regulatory genes. These data, combined with the modest induction by b/c1 or p even in the absence of pac1 function, suggest that the Pac1 gene product is not required for B/C1 and P to bind the promoter.

The reduction in luciferase expression is not due to pac1-ref affecting the CaMV promoter used to drive the regulatory gene cDNA constructs because the transformation control, the Renilla luciferase cDNA, is also driven by the same promoter. Thus, any pac1-ref effect on CaMV 35S activity would have altered the levels of expression of all the genes. This alteration would not have affected the ratio of luc expression to Renilla luc expression because both the regulatory genes and the transformation control are driven by the CaMV 35S promoter. Because we saw a change in the ratio of firefly luc activity, driven by the a1 promoter, to Renilla luc
activity, driven by the CaMV 35S promoter, and because Renilla luc levels were similar in both wild-type and pac1-ref aleurones, we conclude that the reduction of expression seen in pac1-ref aleurones is specific to the activation of the a1 promoter in this assay.

Based on our data, it seems likely that the normal product of the pac1 gene acts independently of the B/C1 and P proteins to produce its effects on the activation of the a1 promoter. Because the other anthocyanin promoters have strong similarities in sequence and structure to the a1 promoter (Lesnick and Chandler, 1998), the pac1 product probably affects activation of the other anthocyanin biosynthetic gene promoters similarly. Our data do not enable us to determine whether the pac1 product interacts directly with the promoters or mediates its effects through other factors. A provocative hypothesis is that pac1 encodes the factor, or part of a factor, that interacts with a conserved region that has been found in the a1, a2, bz1, and bz2 promoters and is required for their expression but is not always required for C1 or P protein binding to the promoter (Lesnick and Chandler, 1998). We plan to test this hypothesis by cloning pac1 and characterizing its gene product.

METHODS

Genetic Stocks and Nomenclature

A purple-seeded stock homozygous for functional alleles of the a1, a2, bronze1 (bz1), bz2, c1, c2, and red aleurone1 (pr1) genes and for the B-Peru allele of b and the nonfunctional r-g allele of r was crossed to stocks with active Mutator (Mu) transposable elements (Patterson et al., 1991). Mu activity was assayed in the previous generation by a DNA gel blot assay for methylation of the Mu1 element (Chandler and Walbot, 1986). Mature ears from self-pollinated plants were screened for altered anthocyanin pigment. Anthocyanin mutant tester stocks, the R-r stock, and the B-A mapping stocks were obtained from the Maize Genetics Coop stock center (University of Illinois, Urbana, IL). Consistent with maize genetics nomenclature, wild-type and dominant alleles of genes are capitalized (e.g., Pac1), and the pac1 mutant allele is referred to as pac1-ref, which stands for the original reference allele. The R-r allele used consists of multiple components (reviewed in Coe et al., 1988). The capitalized first R refers to the R-S component, which confers kernel color (in this case, r represents the lack of kernel color). The second, lowercase r (-r), refers to the plant color component (in this case, -r represents the lack of plant color). Unless otherwise noted, all stocks are homozygous for the functional C1 allele of c1 and for dominant, functional alleles of all anthocyanin biosynthetic genes.

RNA Extraction from Aleurone Tissue and Quantification of a1, b, bz1, c1, and c2 RNA

The crowns of kernels 35 days after pollination were shaved off with a razor blade. The amount of starchy endosperm in the shaved crowns was minimized. RNA was prepared as described for endosperm tissue (Wessler, 1994). Quantification of specific messages was by an RNase protection assay (Melton et al., 1984; Gilman, 1993). Antisense probe RNA was in vitro transcribed with α-32P-UTP and unlabeled ATP, CTP, and GTP; gel purified; and hybridized with 10 μg of total RNA. The following probes were used: actin, 232 bp of the maize actin2 gene from the SsII site to theSacI site; a1, 361 bp from the Sall site to the Kpnl site; b, 315 bp from the AvaiI site that is 475 bp from the 5′ end to theSacI site at 790 bp; bz1, 355 bp of exon 2 sequence from SacI to SmaI; c1, 388 bp from the PstI site 751 bp from the 5′ end to the 3′ EcoRII site; and c2, 438 bp from BamHI to Nael. All experiments involved hybridizing 50,000 or 100,000 cpm of one or more anthocyanin gene probes together with 100,000 cpm of the actin probe. Hybridization was performed in 80% formamide, 40 mM Pipes, pH 6.4, 400 mM NaCl, and 1 mM EDTA at 50°C overnight. The hybridized RNA was digested with 0.4 μL of T1 RNase (Life Technologies, Gaithersburg, MD) for 45 min at 33°C, incubated with 30 μg of proteinase K in a final concentration of 0.5% SDS at 37°C for 15 min, and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (49:49:1); the aqueous phase was precipitated with 5 volumes of ethanol. The precipitate was washed with 70% ethanol and resuspended in sequencing loading dye, denatured at 85°C, and loaded on a 6% polyacrylamide–8M urea sequencing gel. The gel was visualized on a Storm 8000 PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and the protected bands for each probe were quantified, corrected for background, and normalized to the actin-protected band.

Transient Transfection and Assay of a1 Promoter Activity in pac1 and Wild-Type Kernels

Mature wild-type (Pale aleurone color1 [Pac1], b, C1, and r-g) and mutant (pac1-ref, b, C1, and r-g) kernels were prepared, and DNA was introduced by particle gun bombardment (Selinger et al., 1998). The plasmids used were pA1luc, which has the a1 promoter fused to luciferase (Klein et al., 1989), p35SBP cDNA and p35SC1 cDNA, which have the B-Peru and C1 cDNAs, respectively, fused to the cauliflower mosaic virus (CaMV) 35S promoter (Goff et al., 1990), p35SRluc, which has the Renilla luc cDNA fused to the CaMV 35S promoter (Lorenz et al., 1991; Selinger et al., 1998), and pZmp, which has the P cDNA driven by the CaMV 35S promoter (Sainz et al., 1997). In each precipitation, 2.5 μg of pA1luc and 0.1 μg of p35SRluc were used alone or were mixed with 20 or 50 ng each of p35SC1 cDNA and p35SBP cDNA or 20 or 50 ng of pZmp. Luc assays were performed with the Promega dual luciferase assay kit and read on a luminometer (model TD 20/20; Turner Designs, Sunnyvale, CA). After subtracting background values from the nonbombarded samples, the ratio of firefly luc activity to Renilla luc activity was determined, and this ratio was normalized to the wild-type pA1luc alone ratio, which was set to 1.0. Normalized ratios were averaged, and the standard error of the mean was determined.

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REFERENCES


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