DOI: 10.1534/genetics.110.126136

Identification of the *Pr1* Gene Product Completes the Anthocyanin Biosynthesis Pathway of Maize

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Manuscript received December 22, 2010
Accepted for publication February 22, 2011

ABSTRACT

In maize, mutations in the *pr1* locus lead to the accumulation of pelargonidin (red) rather than cyanidin (purple) pigments in aleurone cells where the anthocyanin biosynthetic pathway is active. We characterized *pr1* mutation and isolated a putative F3′H encoding gene (*Zmf3′h1*) and showed by segregation analysis that the red kernel phenotype is linked to this gene. Genetic mapping using SNP markers confirms its position on chromosome 5L. Furthermore, genetic complementation experiments using a CaMV 35S::ZmF3′H1 promoter–gene construct established that the encoded protein product was sufficient to perform a 3′-hydroxylation reaction. The *Zmf3′h1*-specific transcripts were detected in floral and vegetative tissues of *Pr1* plants and were absent in *pr1*. Four *pr1* alleles were characterized: two carry a 24 TA dinucleotide repeat insertion in the 5′-upstream promoter region, a third has a 17-bp deletion near the TATA box, and a fourth contains a *Ds* insertion in exon1. Genetic and transcription assays demonstrated that the *pr1* gene is under the regulatory control of anthocyanin transcription factors *red1* and *colorless1*. The cloning and characterization of *pr1* completes the molecular identification of all genes encoding structural enzymes of the anthocyanin pathway of maize.

THE flavonoid biosynthetic pathway is ubiquitous in higher plants and leads to the synthesis of a variety of pigmented and nonpigmented compounds. The pigmented flavonoid metabolites have been used as phenotypic markers in many model plant species (Holton and Cornish 1995; Chopra *et al.* 2006) and have proven to be an excellent tool to study the genetic, molecular, and biochemical processes underlying the regulation of tissue-specific gene expression patterns (Koes *et al.* 2005). Flavonoids have important biological functions during the growth and development of a plant (Stafford 1990; Shirley 1996; Dixon and Steele 1999; Taylor and Grotewold 2005) and have many pharmacological and dietary benefits for humans and animals (Miyagi *et al.* 2000). Flavonoids are produced through the phenylpropanoid pathway (Figure 1A), and depending upon the genetic constitution of the plant system, naringenin has several different fates including formation of anthocyanins, flavans, flavones, condensed tannins, and phlobaphenes (Winkel-Shirley 2001). In maize, purple and red anthocyanins are derived from 3-hydroxyflavonoids (Styles and Ceska 1989) and their tissue-specific accumulation is regulated by pairs of duplicated transcription factors *red1/booster1* (*r1/b1*) and *colorless1/purple plant1* (*c1/pl1*). The *R1* and *B1* genes encode bHLH transcription factors (Ludwig *et al.* 1990; Goff *et al.* 1992) and *C1* and *PL1* encode MYB-homologous DNA binding domain proteins (Cone *et al.* 1993). Anthocyanin accumulation in aleurone requires the joint action of R1 and C1, while B1 and PL1 together are needed for anthocyanin biosynthesis in vegetative plant parts (Chandler *et al.* 1989).

We are interested in sorghum and maize 3-deoxyanthocyanidins, 3-hydroxyanthocyanidins, and C-glycosyl flavones that have a role in tolerance to fungal pathogens (Snyder and Nicholson 1990) and insect pests (Snook *et al.* 1994). It was shown that several flavonoid branches leading to these compounds require the activity of a flavonoid 3′-hydroxylase in sorghum and maize (Cortes-Cruz *et al.* 2003; Boddu *et al.* 2004). Similarly, formation of anthocyanidins in the kernel aleurone requires the activity of a flavonoid 3′-hydroxylase (F3′H), and this activity has been attributed to the functional *red aleurone1* locus, also known as *purple aleurone1* and designated as *pr1* in maize (Larson *et al.* 1986). Since the early 20th century, the *pr1* locus has been used as a marker in maize genetics for the identification,

Supporting information is available online at http://www.genetics.org/cgi/content/full/genetics.110.126136/DC1.

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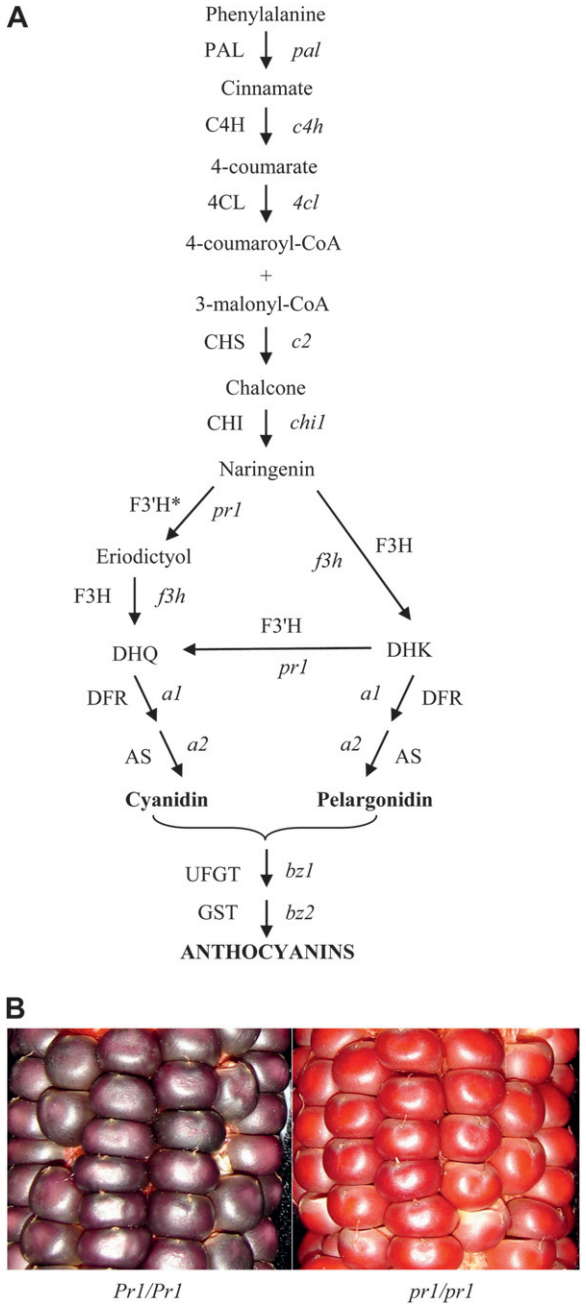

FIGURE 1.—Biosynthesis and accumulation of anthocyanins in maize. (A) Phenylpropanoid biosynthetic pathway leading to the production of anthocyanins. Genes (enzymes) in the pathway are: *pal* (PAL), phenylalanine-ammonia lyase; *c4h* (C4H), cinnamic acid hydroxylase; *4cl* (4CL), 4-coumaryl: CoA ligase; *c2* (CHS), chalcone synthase; *chi1* (CHI), chalcone isomerase; *f3h* (F3H), flavanone 3-hydroxylase; *pr1* (F3′H), flavonoid 3′-hydroxylase (* = based on enzyme activity assay); *a1* (DFR), dihydroflavanone reductase; *a2* (AS), anthocyanidin synthase; *bz1* (UFGT), UDP-glucose flavonoid 3-*O*-glucosyltransferase; and *bz2* (GST), glutathione S-transferase. (B) Close-up photos show red and purple aleurones in kernels of *pr1* and *Pr1* ears, respectively.

characterization, and mapping of several loci. During the mid 20th century, genetic and biochemical studies established that the red and purple aleurone color difference is due to the presence (*Pr1*) or absence (*pr1*) of a hydroxyl group in the pigment and that a single gene is responsible for this phenotype (MCCLARY 1942; ZARUDNAYA 1950; COE 1955). Maize F3′H is a NADPH-dependent cytochrome P450 enzyme, which can act on a wide range of substrates: the flavonols, flavones, and flavanones, all of which are intermediates in the flavonoid biosynthetic pathway (LARSON and BUSSARD 1986). Although the availability of mutants at different catalytic steps has led to the isolation of most of the structural genes required for maize anthocyanin biosynthesis (DOONER *et al.* 1991), the sequence of the *pr1* gene has remained elusive (CONE 2007). The *pr1* mutants produce a red pigment, pelargonidin, due to the failure to hydroxylate the B ring of dihydrokaempferol (DHK) (FORKMANN 1991) to yield the purple cyanidin pigment produced in maize lines carrying a functional *Pr1* allele (Figure 1B). Using mutants of *pr1*, LARSON *et al.* (1986) demonstrated that the *pr1* locus may encode or regulate an F3′H-mediated conversion of DHK to dihydroquercitin (DHQ) *in vitro*. To define the role of the *pr1* gene in maize anthocyanin biosynthesis, we isolated and characterized a putative maize *f3′h1* (*Zmf3′h1*) sequence. Our results provide the historic missing link between red and purple anthocyanin accumulation in maize. Analysis of *Zmf3′h1* and its transcriptional regulation in distinct tissue types was performed to understand the intermediate steps leading to the synthesis of anthocyanins in maize. Gene expression results and genetic data presented here demonstrate that the *pr1* gene is regulated by transcription factors that control the synthesis of anthocyanins in silk, husk, and aleurone tissues.

MATERIALS AND METHODS

Maize genetic stocks: Seeds of the following maize inbred lines and genetic stocks were kindly provided by the Maize Genetics Cooperation Stock Center (US Department of Agriculture-Agricultural Research Service, University of Illinois, Urbana, IL): W23 (genotype *P1-wr c1 r-g*), W22 (*Pr1 A1 C1 R1*), Mp708 (*pr1 c1 r1 p1*), MGS14273 (*pr1 A1 A2 C1 R1*), MGS130543 (*pr1 A1 A2 C1 R1*), MGS14284 (*pr1 A1 A2 C1 R1*), MGS131036 (*c1 R1-g b1 pl1*), MGS14633 (*c1 R1-r B1 Pl1*), MGS167054 (*r1-g C1 b1 pl1*), and MGS14638 (*r1-r C1 B1 Pl1*). To develop F_2 populations, plants of *pr1-MGS14273*, *pr1-MGS130543*, and *pr1-MGS14284* alleles were crossed by W22 and progenies were grown from the selfed F_1 plants. F_2 populations segregated 3:1 for purple to red aleurone, indicating the recessive nature of all *pr1* alleles studied. Segregating plants were used for RNA expression and cosegregation analysis using PCR-based polymorphism. To study the regulation of *pr1* by anthocyanin regulatory genes, crosses were made between *pr1-MGS14273* and *c1-MGS131036*, *c1-MGS14633*, *r1-MGS167054*, and *r1-MGS14638* alleles. The F_2 progenies were grown from the selfed F_1 plants. The mutant stocks of the regulatory genes *c1* and *r1* carried functional alleles of the biosynthetic genes required for anthocyanin synthesis.

To create the *Ds* insertion allele described in this study, testcross progeny were generated by crossing females carrying a recessive *pr1* allele (*pr1 A1 C1 R1 y1*; kindly provided by

Dr. Erik Vollbrecht) by closely linked hemizygous *Ds* pollen donors (Ds/+). The inbred W22 *Ds* donor was estimated to be ~3.8 Mb, or 1.8 cM distal to *pr1*, and carried a single copy of *Ac-im* (Conrad and Brutnell 2005) (*Pr1 Ds/+; A1; C1; r1; Y1; Ac-im/+*). Putative *pr1* loss-of-function alleles were identified as kernels with exceptional red aleurone among the darkly purple-pigmented aleurones of sibling progeny. One insertion allele was confirmed and named as *pr1-Ds1:W22*.

Genomic, cDNA cloning, and sequence characterization: During the onset of this project a full-length maize *f3′h* sequence was not available in the GenBank. Putative *f3′h* sequences (http://www.ncbi.nlm.nih.gov) were used to design PCR primers to isolate the full-length *Zmf3′h1* sequence. Oligonucleotides ZF3F2 and ZF3R2 (see supporting information, Table S2 for primer sequences) were based on the alignment of the maize partial EST sequence (accession no. BG873885) with sorghum *f3′h* sequence (Boddu *et al.* 2004) and used to PCR amplify a 387-bp fragment (F387). Fragment FR is a partial genomic sequence from the 5′ half of the gene, which was PCR amplified using forward primer OSF1 (designed from the rice *f3′h* gene) and reverse primer ZMR4. All DNA fragments used as probes were labeled with α-^{32}P-dCTP using the Prime-a-Gene Labeling System (Promega, Madison, WI). A λ-FIX II (Stratagene, La Jolla, CA) library prepared from seedling leaf DNA of maize inbred line W23 was screened to isolate the full-length *Zmf3′h1* gene (accession no. HQ699781). The full-length maize *f3′h1* cDNA was obtained by performing reverse transcription (RT)–PCR using gene-specific primers SBF12 and SBR22 on total RNA isolated from maize silks of W23. Standard PCR buffer and reaction conditions were followed with the modified annealing temperature of 60° for 2 min, followed by polymerization at 72° for 2 min. All DNA sequencing reactions were performed at the Pennsylvania State University's Nucleic Acid Facility, using a method of dye primer cycle sequencing, and reactions were analyzed on the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequence assembly and analysis was performed using tools available from the NCBI at www.ncbi.nih.gov (Altschul *et al.* 1990). Conserved domain searches were done using the NCBI CD Search tool at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi.

Multiple sequence alignment: Amino acid sequences were aligned using the ClustalX program (version 1.81; multiple alignment parameters: gap opening 10, gap extension 0.20; DNA weight matrix: IUB; Protein weight matrix: Gonnet series) (Thompson *et al.* 1994). Additional F3′H amino acid sequences included: *Arabidopsis thaliana* (AF271651), *Callistephus chinensis* (AF313488), *Glycine max* (AF499731), *Matthiola incana* (AF313491), *Oryza sativa* (AC021892), *Pelargonium* × *hortorum* (AF315465), *Perilla frutescens* (AB045593), *Petunia hybrida* (AF155332), *Torenia hybrida* (AB057673), and *Sorghum bicolor* (AY675075).

PCR amplification: Positions of PCR primers used for the successful identification of polymorphisms in *Pr1* and *pr1* alleles are shown in Figure 3 (see Table S2 for primer sequences). The standard reaction (50 μl) contained 0.1 μg of genomic DNA, 25 μl of GoTaq green master mix (Promega), and 200 nM each of the two oligonucleotide primers. A typical reaction consisted of 35 cycles of denaturation (1 min, 94°), annealing (1 min, 57°), and extension (2 min 30 sec, 72°) in a thermal cycler (MJ Research). PCR products from both mutant and wild-type *pr1* alleles were cloned in pCR2.1-TOPO cloning vector (Invitrogen, Carlsbad, CA) and sequenced. Nucleotide sequences were aligned using ClustalW program (Larkin *et al.* 2007).

Mapping of *Zmf3′h1* gene: A single nucleotide polymorphism (SNP)-based assay was developed to map the *Zmf3′h1* sequence. The following primers, 5′-AGGTGGACGGGTT CCGCATC-3′ and 5′-GTATGCCTCCTCCATGTCTAGC-3′ were used to amplify and sequence a segment of the *Zmf3′h1* gene from DNA of the inbred lines Tx501, NC7A, and Mp708. Examination of the sequence alignments revealed a C-to-G polymorphism in Tx501 relative to NC7A and Mp708. The primer 5′-GATGAGCTCGAAGTCGCT-3′ was designed to interrogate the polymorphic site by primer extension. SNP markers were genotyped with the SNaPshot system (Applied Biosystems, Foster City, CA) and assayed on an ABI 3700 as previously described (Vroh Bi *et al.* 2006). The genotypes for SNP were derived from 346 (Tx501 × NC7A) F_2 individuals and 246 (Tx501 × Mp708) F_2 individuals obtained from previously characterized quantitative trait locus mapping populations (Cortes-Cruz *et al.* 2003). The linkage maps were constructed with Mapmaker/Exp, version 3.0 (Whitehead Institute, Cambridge MA).

RNA gel blot analysis: For RNA isolation, silk and husk tissues were collected at the silk emergence. To isolate total RNA, tissues were ground in liquid nitrogen and then extracted using Tri-Reagent (Molecular Research Center). The RNA was separated on denaturing gels and RNA gel blot hybridizations were performed following standard methods described previously (Boddu *et al.* 2006).

RT–PCR expression assay: Kernel aleurones were peeled from endosperm tissues at 24–28 days after pollination (DAP) from *pr1* segregating plants, *c1* and *r1* mutants, and from wild-type W22 inbred line. RNA was isolated from three biological replicates of each line and three samples were analyzed from each replicate. To isolate total RNA, tissues were ground in liquid nitrogen and then extracted using Tri-Reagent (Molecular Research Center, Cincinnati, OH). First strand cDNA was synthesized using SuperScript II cDNA synthesis system (Invitrogen) and oligo-dT primer using conditions as described by the manufacturer. One microgram of total RNA from aleurones was used for each reverse transcription reaction. First strand cDNA was diluted to a final volume of 100 μl with sterile ddH_2O. Five microliters of first strand cDNA was PCR amplified using gene-specific primers. Plasmid DNA that contained the complete *Zmf3′h1* genomic sequence was used as an internal control. This amplified a PCR product of 996 bp *vs.* the observed 771-bp product amplified from *Zmf3′h1* cDNA. Primers used for detection of anthocyanin gene transcripts are shown in Table S1 (Piazza *et al.* 2002). PCR reactions were performed in a total volume of 25 μl with GoTaq green master mix (Promega) using the manufacturer's instructions. Conditions were as follows: an initial denaturation of 94° for 4 min, denaturation 94° for 1 min, annealing 55°–60° for 1 min, and extension 72° for 1 min 30 sec, repeated for 30 cycles, and a final extension step of 72° for 10 min. PCR products were analyzed on 1.0% agarose gel.

Extraction, TLC, and HPLC analysis of flavonoid compounds: Thin layer chromatography (TLC) was carried out using silica 60 plates (Sigma Aldrich, St. Louis, MO) and a mobile solvent containing ethyl acetate:formic acid:acetic acid:water (100:11:11:27, v/v) (Holton 1995; Dong *et al.* 2001). Approximately 2 g of chopped aleurones and endosperms were allowed to soak in 1 ml of methanol with 1% HCl for 48 hr before transferring the extracts to a fresh tube. A total of 30 μl extract was spotted per lane. For high performance liquid chromatography (HPLC) analysis of dihydroflavonols, maize silk samples (300 mg) taken at the time of silk emergence were imbibed in 1-ml mixture of methanol:HCl (99:1,v/v) and compounds were allowed to leach for 48 hr at 4°. The anthocyanin pigments were extracted by grinding 300 mg of aleurone tissue in 1 ml of 80% methanol. Extracts were acid hydrolyzed with an equal volume of 4 N HCl to prepare aglycones (Burbulis *et al.* 1996; Nyman and Kumpulainen 2001). The extracts were then filtered through 0.45 μm Acrodisc LC 13-mm syringe filters (Gelman Laboratory, Ann Arbor, MI). Reverse phase HPLC analysis was performed on a Shimadzu

high-performance liquid chromatograph (Shimadzu, Columbia, MD) using an Ascentis C18 column (25 cm × 4.6 mm, 5 μm; Supelco, Bellefonte, PA). Pigment separation was performed at 35° by gradient elution using 0.2% formic acid (solution A) and 100% methanol (solution B) at a flow rate of 1 ml/min. The injection volume was 50 μl and spectral measurements were taken over a wavelength range of 230–550 nm, which is known to detect flavonoid compounds (Grotewold *et al.* 1998). Flavonoid standards of cyanidin and pelargonidin were purified from maize husk and pelargonium flowers, respectively.

Arabidopsis seed stocks, growth conditions, and stable transformation: For genetic complementation experiments, seeds of Landsberg *erecta* and *transparent testa7* (*tt7*) mutants were obtained from the Arabidopsis Biological Resource Center at Ohio State University, Columbus, OH. The mutant *tt7* used in this study was in a Landsberg *erecta* background. Seeds were surface sterilized with 10% bleach and germinated in pots filled with steam-sterilized Metro Mix 300. Soil was misted with water after seeding, then covered with plastic wrap, and kept in a cold room at 4° for 3–5 days to break seed dormancy and to promote uniform germination. Plastic wrap was removed and pots were transferred to growth chamber and maintained at 22° and 70% relative humidity (RH). Plants were kept under short day conditions (10 hr light and 14 hr dark) for the first 3 weeks and then transferred to a greenhouse under a long day cycle (16 hr light and 8 hr dark) to induce flowering. An overnight-grown *Agrobacterium tumefaciens* strain GV3101 carrying a CaMV 35S::ZmF3′H1 construct cloned into a pSR3000 vector was harvested by centrifugation and suspended in 200-ml solution containing 5% sucrose and 0.002% Silwet l-77 (Lehle Seeds, Round Rock, TX). Plants were transformed using the floral dip method (Clough and Bent 1998), in which each plant inflorescence was immersed for at least 10 sec. Transformants were screened on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) that contained 50 μg/ml kanamycin and 100 μg/ml ampicillin. Green seedlings were transferred to soil mix and grown in a growth chamber at 22° with a 10 hr light and 14 hr dark cycle.

Spectrophotometric analysis: Seeds of Landsberg *erecta*, *tt7* and T_2 seeds from two independent transformation events were germinated on minimal medium containing 3% sucrose and 0.5% (w/v) agar. These were kept at 25° in continuous light; seedlings were collected after 10 days, and pigments were extracted as described in Dong *et al.* (2001). Briefly, seedlings were homogenized in 1.5 ml of 1% (v/v) HCl in methanol, then 1 ml of double-distilled water was added, and chlorophyll was separated from the extracts with chloroform. Pigment analysis was done on UV spectrophotometer, UV-mini 1240 (Shimadzu) and measurements were taken over wavelength range of 400–600 nm. HPLC analysis of extracts was performed with the same column and solvent program used for aleurone anthocyanin analysis.

Dissection of *pr1* promoter: The analysis of the *pr1* promoter for the location and distribution of *cis*-regulatory sequence elements was performed using the Plant Care database (http://bioinformatics.psb.ugent.be/webtools/plantcare.html/) and MetInspector (Genomatrix, Munich, Germany) (Quandt *et al.* 1995). The *cis*-binding sites were identified on the basis of their similarity with sites present in promoters of other anthocyanin genes as well as similarity with MYB and MYC protein binding sequences (Sainz *et al.* 1997).

RESULTS

Isolation of a putative flavonoid 3′-hydroxylase from maize: To identify the locus encoding flavonoid 3′-hydroxylase from maize, λ-phage libraries were screened with probes designed from putative partial *Zmf3′h1* sequences from the maize genome (see materials and methods). The isolated *Zmf3′h1* sequence corresponds to GRMZM2G025832, an annotated gene in B73 RefGen_v2 (chromosome 5: 180038179–180040249, www.maizegdb.org). Comparison between *Zmf3′h1* [W23] and GRMZM2G025832 shows 92% identity for gene sequence and 95% similarity for peptide sequence.

Sequence alignments with other plant F3′H's showed that the isolated sequence encodes a predicted F3′H peptide of 517 amino acids (Figure 2). Overall, the maize F3′H shares ~55% amino acid identity with dicot F3′H's and high sequence similarity with monocot F3′H's: 78% with rice, 90% with sugarcane, 65% with barley, and 91% with sorghum. The phylogenetic tree (data not shown) shows that maize F3′H resides in the same clade as other monocots such as rice and the closely related species sorghum and is clearly divergent from the dicot clade that includes Arabidopsis and Matthiola. Conserved cytochrome P450-dependent monoxygenase domains, hallmark of F3′H's, were observed in the putative maize F3′H sequence. These domains include a heme-binding site (HBS), oxygen-binding site (OBS), and the characteristic hydrophobic membrane anchor present at the amino terminus. A dicot F3′H-specific motif (GGEK) with unknown function had previously been reported (Brugliera *et al.* 1999; Toda *et al.* 2002), and this sequence was found to be modified to GGSH in maize and sorghum F3′H (Boddu *et al.* 2004).

Characterization of the lesion in *pr1* mutants: To characterize the lesion present in three different mutant alleles of *pr1* (*pr1-MGS14273*, *pr1-MGS130543*, and *pr1-MGS14284*), we used a PCR-based approach. Using *Pr1* gene-specific primers, we detected polymorphisms between wild-type and mutant *pr1* alleles (Figure 3A). Sequencing results indicated the presence of a 24 TA dinucleotide repeat insertion in the upstream promoter region of *pr1-MGS14273* and *pr1-MGS130543*, and a 17-bp deletion near the TATA box of *pr1-MGS14284*. Inbred MP708, which carries a recessive allele of *pr1*, also showed the presence of 24 TA dinucleotide repeats. PCR screening of the genic region did not show any sequence polymorphisms between *Pr1* and *pr1* alleles. This suggests that the mutant *pr1* alleles may be defective due to these insertion and deletion events in the 5′ region. To determine whether the isolated *Zmf3′h1* sequence is linked to the *pr1* locus, we developed a population segregating for *Pr1* and *pr1* by crossing *pr1* plants with W22 inbred. A total of 40 segregating plants from each population were characterized for PCR-based polymorphisms. A 1:2:1 ratio for *Pr1/Pr1:Pr1/pr1:pr1/pr1* showed a molecular cosegregation of the polymorphisms defined for each of the tested alleles (*pr1-MGS14273*, *pr1-MGS130543*, and *pr1-MGS14284*) with the *pr1* mutant phenotype.

To unambiguously confirm the identity of the *pr1* locus, an independent *pr1* allele was characterized,

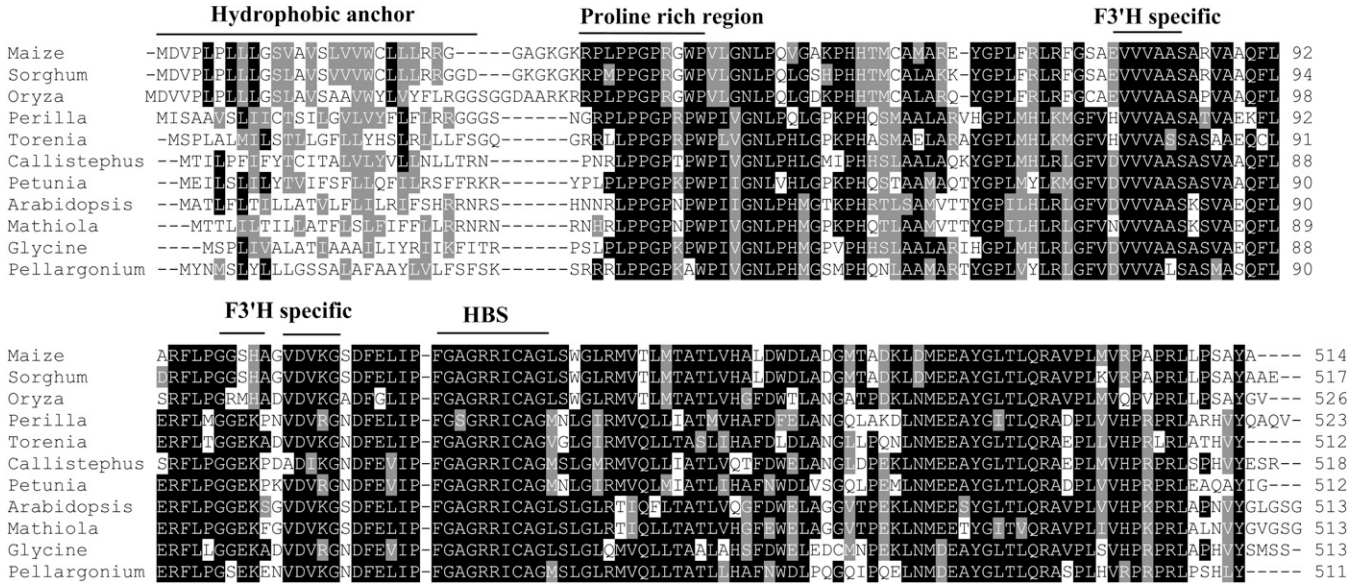

FIGURE 2.—Multiple sequence alignments of deduced amino acid sequences of F3′H from maize and other plant species using the ClustalX program. Lightly shaded areas are F3′H-specific sequences and darkly shaded regions are conserved domains found in the CYP450 proteins. Other regions shown are: hydrophobic anchor, proline-rich region, and heme-binding site (HBS).

which resulted from a *Ds* insertional mutagenesis. Following a directed tagging experiment (see MATERIALS AND METHODS) five candidate *pr1* kernels were identified from 37 test-cross ears (~7400 kernels). Genomic DNA was extracted from four of these individual seedlings (one did not germinate) and used as templates in a series of PCR assays containing multiple *pr1* gene-specific and *Ds*-specific primer pairs (see Table S2 for primer sequences), in a screen similar to that described previously for the *Ds* tagging of *bz2* (AHERN *et al.* 2009). The primer set MGR1/JSR01 amplified a product that indicated a single *Ds* insertion at *pr1*. Complementary PCR products were amplified using the additional primer sets MGR2/JSR01 and MGF1/JSR05. The latter

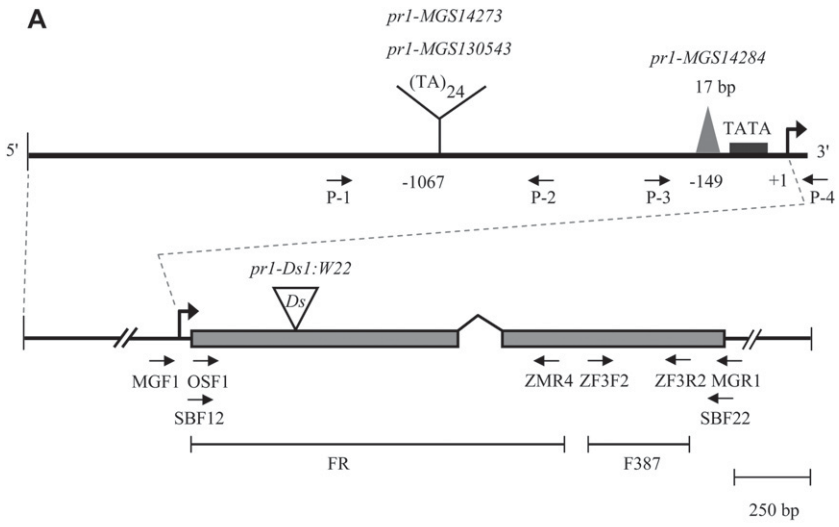

FIGURE 3.—Characterization of *Zmf3′h1*, isolation of *pr1* insertion, and deletion alleles. (A) A segment of λ-clone shows *Zmf3′h1* and its 5′- and 3′-flanking sequences. FR and F387 are the probes used to screen the genomic library. Shaded boxes represent two exons that are joined by a bent line, which corresponds to the single intron of the *Zmf3′h1* gene. Enlarged 5′-flanking region shows the location of 24 dinucleotide repeats and a deletion near the TATA box in mutant *pr1* alleles. Positions of TA repeats and a 17-bp deletion are marked with respect to transcription start site (shown as bent arrow and marked +1). The position of the *Ds* insertion in exon 1 is shown and allele's name is indicated. Small arrows below the map illustrate the orientation and position of the PCR primers. (B) Self-pollinated ear of a plant with the genotype *pr1::Ds/pr1-ref.* Note the purple revertant kernel (arrow), indicating a potential excision event of *Ds*, which would result in the restoration of *Pr1* gene expression.

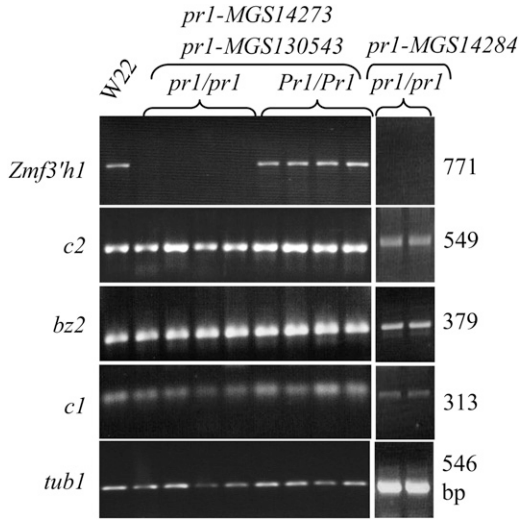

FIGURE 4.—Mutant *pr1* alleles do not accumulate *Zmf3′h1* transcripts in aleurone tissues. Expression of *Zmf3′h1*, *c2*, *bz2*, and *c1* in aleurones was detected by RT–PCR from three *pr1/pr1* mutant alleles and *Pr1/Pr1* wild-type plants. Purple aleurones from W22 were used as positive control and α-*tubulin1* was used as a loading control.

two products, representing the regions flanking either side of the *Ds* insertion site, were purified and sequenced. Both sequences independently confirmed the insertion of a *Ds* element in the first exon of the *Zmf3′h1* gene and thus confirmed the identity of the *pr1* locus. This seedling was transplanted, grown to maturity, and self-pollinated to create an ear segregating for the *Ds* insertion at *pr1* (Figure 3B). The identification of a *Ds* insertion in the *Zmf3′h1* gene that cosegregated with the *pr1* mutant phenotype thus confirmed the molecular identity of the *pr1* locus. The position of the *Ds* insertion in the *pr1* sequence is shown in Figure 3A.

The *Zmf3′h1* gene sequence maps to the *pr1* locus on chromosome 5L: A genetic linkage map was constructed for chromosome 5 containing an *Zmf3′h1* SNP based on the scoring of 346 and 246 individuals for the (Tx501 × NC7A) F_2 and (Tx501 × Mp708) F_2 populations, respectively (Figure S1). The position of the *Zmf3′h1* SNP corresponds to the genetic RFLP-placed position of the *pr1* locus (MaizeGDB, http://www.maizegdb.org) and interestingly, it was also detected as a major QTL for the synthesis of apimaysin *vs.* maysin in these same populations (CORTES-CRUZ *et al.* 2003). Therefore, these genetic mapping results conclusively show that the *Zmf3′h1* gene sequence isolated in this study corresponds to the *pr1* locus on chromosome 5L.

The *Zmf3′h1* transcripts are absent in tissues accumulating red anthocyanins: RT–PCR and RNA gel blot analysis of steady state transcription of *Zmf3′h1* were performed for kernel aleurone, young maize silk, and husk tissues (Figure 4, Figure S2) collected from plants segregating for *Pr1* or *pr1* alleles. *Pr1* plants produced ears with all purple kernels and accumulated *Zmf3′h1* transcripts. *Zmf3′h1*-specific transcripts could not be detected in RNA from homozygous *pr1* plants that produced ears with red kernels. RT–PCR using gene-specific primers for other flavonoid pathway genes as well as for *c1* and *r1*, the anthocyanin regulatory genes, showed steady state transcripts of these genes in both *Pr1* and *pr1* plants. Similarly, RNA gel blots were stripped and rehybridized with probes of other genes required for anthocyanin biosynthesis. C1, a transcription factor required for the expression of genes encoding structural enzymes of the anthocyanin pathway, as well as the enzyme-encoding genes *c2* and *bz2*, were expressed in both functional and mutated *pr1* plants. In summary, these results show that *Zmf3′h1*-specific transcripts were absent in mutant *pr1* plants while expression of other genes of the pathway were detected.

***Zmf3′h1* is required for the formation of cyanidin:** Methanolic extracts of *pr1* and *Pr1* endosperms and aleurones were separated using TLC (Figure 5A). *pr1* aleurones accumulated red pelargonidin glycosides, while *Pr1* showed the presence of purple cyanidin glycosides. None to very little cyanidin pigment was detected in the *pr1* plants. While the yellow tetrahydroxychalcone can sometimes be detected in the extracts, numerous other compounds in the pathway are colorless and were not detected in our TLCs (HOLTON and CORNISH 1995; BRUGLIERA *et al.* 1999). HPLC analysis of silk methanolic extracts from lines containing the mutant *pr1* allele revealed the expected DHK peak at 12 min, while lines containing *Pr1* showed a peak at 8 min, the peak expected for DHQ as measured at 230 nm (Figure 5B). HPLC analysis of acid hydrolyzed extracts from the aleurone tissues at 530 nm revealed that the *pr1* plants accumulated mostly the pelargonidin (not shown). *Pr1* plants accumulated high levels of cyanidin (data not shown). Overall, chromatography results show that *Pr1* is required for the formation of DHQ from DHK and subsequently downstream cyanidin synthesis, which causes the purple pigmentation in the aleurone tissue in *Pr1* plants. The accumulation of the respective anthocyanidins and dihydroflavonols in the mutant and wild-type plants confirms that the *pr1* gene is responsible for the formation of 3′, 4′-hydroxylated flavonoids.

***Zmf3′h1* complements Arabidopsis *tt7* mutant phenotype:** The *A. thaliana* mutant *tt7* is defective in F3′H activity (SHIRLEY *et al.* 1995; PEER *et al.* 2001). Mutant seeds have a yellow coat color when compared to wild-type seeds with a brown seed coat and *tt7* seeds fail to accumulate brown tannins in the testa (KOORNNEEF 1990; SHIRLEY *et al.* 1995). In addition, *tt7* seedlings do not produce anthocyanin pigments in the cotyledon or hypocotyl when grown on a nitrogen-deficient minimal medium (HSIEH *et al.* 1998). The development of anthocyanin pigments in wild-type Arabidopsis seedlings in response to stress has been used to study the anthocyanin biosynthetic pathway (GROTEWOLD *et al.* 1998). We have used this assay to ascertain the role of isolated *Zmf3′h1* in the development of anthocyanins.

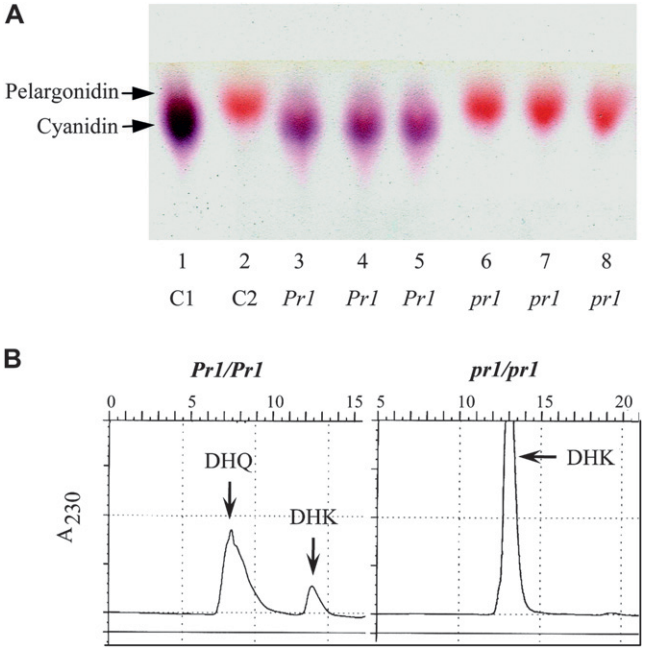

Figure 5.—*pr1* aleurones produce pelargonidin, while *Pr1* aleurones produce cyanidin. (A) TLC of extracts from *Pr1* or *pr1* kernels. Samples shown in each lane are: 1, control C1 for cyanidin; 2, control C2 for pelargonidin; 3–5, *Pr1*; and 6–8, *pr1*. (B) HPLC analysis of silk methanolic extracts from sibling plants carrying *pr1* or *Pr1*. Extracts were analyzed at wavelength 230 nm for DHK and DHQ. *pr1* shows single peak representing DHK, while *Pr1* has two peaks representing both compounds; thus DHK is converted into DHQ through the action of F3′H (Figure 1).

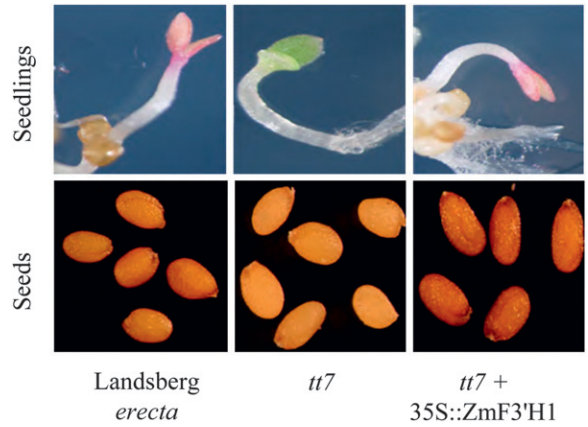

Figure 6.—Complementation of *tt7* mutant with *Zmf3′h1* resulted in accumulation of pigments in seedling and seed coat. Seedling and seed phenotypes are shown for wild-type Landsberg *erecta*, *tt7* mutant, and *35S*::ZmF3′H1-complemented *tt7*.

tt7 plants were transformed with the 35S::ZmF3′H1 transgene (see materials and methods). The T_1 seeds were screened on kanamycin-containing medium, and resistant plants were grown to maturity in the growth chamber. Seeds collected from kanamycin-resistant T_2 plants showed complete restoration of brown seed coat color. In the seedling assay, T_2 seedlings produced purple cotyledons when grown on a minimal medium (Figure 6). Spectrophotometric analysis of the extracts from 10-day-old Landsberg *erecta*- and *Zmf3′h1*-complemented *tt7* seedlings grown on minimal medium showed the characteristic absorbance of anthocyanidin pigments at a wavelength of 530 nm, while *tt7* mutant seedlings did not show this peak (Figure S3). Anthocyanin compounds present in wild-type, mutant, and *Zmf3′h1*-expressing seedlings were further characterized by HPLC. The peaks for different cyanidin glycosides were detected in both the wild-type and the *Zmf3′h1*-complemented *tt7* mutant and were absent in *tt7* mutant seedlings (Figure S3). In summary, these results demonstrate that the putative *Zmf3′h1* sequence encodes a functional F3′H enzyme capable of rescuing the *tt7* mutant phenotype in Arabidopsis.

Anthocyanin regulatory genes *c1* and *r1* are required for *pr1* expression in kernel aleurone pigmentation: To genetically place *pr1* in the C1/R1 regulatory network, crosses were made between *pr1* (*pr1/pr1*; *C1/C1*; *R1/R1*) and *c1* (*c1/c1*; *R1/R1*) and *pr1* and *r1* (*r1/r1*; *C1/C1*) mutants (see materials and methods). Two independent mutant alleles of each regulatory gene were used (*c1-MGS131036*, *c1-MGS14633*, *r1-MGS167054*, and *r1-MGS14638*). The F_1 ear phenotype indicated that both the *c1* and *r1* mutant stocks carry a functional *Pr1* allele. A representative F_2 ear from each cross is shown in (Figure S4). Purple and red kernels were due to *Pr1/−*; *C1/−*; *R1/−* and *pr1/pr1*; *C1/−*; *R1/−*, respectively. Colorless kernels indicated the presence of mutant alleles of *c1/c1* or *r1/r1*. If the regulation of *pr1* is downstream of both the *c1* and *r1* genes, we expect to see kernel phenotypes segregating as nine purple: three red: and four colorless. Chi-square analysis of F_2 ear phenotypes showed that purple, red, and colorless kernels segregated in a 9:3:4 ratio (Table 1). To confirm these results, we analyzed the segregation ratio of kernel aleurone phenotype on test-cross ears. A segregation ratio of 1:1 for purple and colorless kernels was observed on progeny ears from F_1 plants test-crossed to *c1* or *r1* mutant plants (see Table 2). Results from these genetic assays showed that the functional *c1* and *r1* alleles were required for *pr1* expression in the formation of purple aleurone pigment.

These genetic results were verified at the molecular level by studying the expression of *c1*, *r1*, and *pr1* genes in mutant as well as wild-type plants. Steady-state transcripts of *c1*, *r1*, and *pr1* were measured in the aleurones of a wild-type W22 line and two each of *c1* and *r1* mutant alleles using RT–PCR (Figure 7). W22 kernel aleurones showed steady-state transcripts of all three genes tested, while *c1* and *r1* mutants did not accumulate *c1* and *r1* transcripts, respectively. Interestingly, the *pr1* gene-specific transcripts could not be detected in colorless kernels of mutant *c1* and *r1* plants. Similarly, RT–PCR of two other anthocyanin pathway genes known to be regulated by *c1* and *r1*, *c2*, and *bz2*, showed steady-

TABLE 1

Regulation of *pr1* by *c1* and *r1* during purple anthocyanin synthesis in the kernel aleurone: number of kernels on F_2 progeny ears with specific aleurone color

c1 allele	Purple (9)	Red (3)	Colorless (4)	*P*-value χ^2	*r1* allele	Purple (9)	Red (3)	Colorless (4)	*P*-value χ^2
c1-MGS131036 (*c1 R1-g b1 pl1*)	465	185	216	0.13	*r1-MGS167054* (*r1-g C1 b1 pl1*)	688	265	336	0.10
c1-MGS14633	270	86	141	0.20	*r1-MGS14638*	614	232	298	0.20

χ^2 analysis was performed to detect significant deviation ($P < 0.01$) of observed phenotypic classes from expected.

state transcripts in W22 kernel aleurones, but these transcripts were absent in mutant *c1* or *r1* aleurones. These results demonstrated the requirement of functional *c1* and *r1* alleles for *pr1* transcript accumulation in the kernel aleurone. Furthermore, *in silico* analysis of the promoter sequence of the *pr1* gene identified a putative *cis*-regulatory sequence required for the binding of *c1* and *r1* encoded proteins. The sequence "AGGTGGTAGCTGGGA" lies 126 bp upstream of the putative transcription start site (TSS), which was previously shown to be required for binding of a C1 MYB protein and was designated as the C1-binding site (CBS) (MetInspector) (Sainz *et al.* 1997). In addition, a *cis*-sequence similar to an anthocyanin regulatory element (ARE), is present at −106 position (Tuerck and Fromm 1994). The ARE has been demonstrated to play an important role in the activation of anthocyanin genes (Tuerck and Fromm 1994; Lesnick and Chandler 1998). In conclusion, our results from genetic, molecular, and promoter sequence analyses show that *c1* and *r1* are required for *pr1* expression in the anthocyanin biosynthetic pathway and suggest a direct binding of C1 to the *Pr1* promoter region.

DISCUSSION

The *pr1* locus has been widely used as a phenotypic marker in maize; however, the molecular difference between *Pr1* and *pr1* plants has never been investigated. The results presented in this study show that transcripts for *Zmf3′h1* accumulate in aleurones of *Pr1*, but not in *pr1* aleurone tissue. Expression of *Zmf3′h1* in the silk and husk shows similar trend. The gene expression pattern of *Zmf3′h1* correlates with the accumulation of anthocyanidins in aleurone and dihydroflavonols in silk tissue. However, in their study on F3′H enzyme in maize, Larson *et al.* (1986) found some F3′H activity in seedlings of homozygous mutant *pr1* plants. They suggested that either the mutant *pr1* allele is hypomorphic *i.e.*, it has a reduced level of *pr1* gene expression or there is a duplicate *f3′h* gene present in maize. Despite these previous findings, we did not observe evidence of reduced levels of *pr1* gene expression or a second *f3′h* gene in any of the tissues studied. This discrepancy may be the result of sequence differences between the *Zmf3′h1* and the proposed second hydroxylase gene or it could be that they had detected the activity of a P450 hydroxylase other than F3′H in mutant *pr1* plants. Our results from the expression analysis confirm that the aleurone color difference between *Pr1* and *pr1* is due to the lack of *Zmf3′h1* transcripts in *pr1* and not due to any absence or change in the expression of other anthocyanin biosynthetic or regulatory genes.

We showed using TLC and HPLC analysis of kernel aleurone extracts that purple cyanidin glycosides and red pelargonidin glycosides accumulated in *Pr1* and *pr1* lines, respectively. This indicates a probable block in the formation of DHQ in mutant lines. DHQ can be formed from DHK by F3′H or from naringenin to eriodictyol via F3′H followed by F3H activity. Chromatographic analyses of the silk extracts from *Pr1* plants show the presence of DHQ and DHK as well, while the *pr1* plants have DHK only. The correlation of *Pr1* and *pr1* alleles to the types of flavonoid compounds produced is consistent with the role of *pr1* as F3′H encoding gene and is able to perform the 3′-hydroxylation reaction in the 3-hydroxyanthocyanin branch pathway. This was further confirmed by the presence of dominant peaks for

TABLE 2

Regulation of *pr1* by *c1* and *r1* during purple anthocyanin synthesis in the kernel aleurone: number of kernels on test-cross ears with specific aleurone color

c1 allele	Purple (1)	Colorless (1)	*P*-value χ^2	*r1* allele	Purple (1)	Colorless (1)	*P*-value χ^2
c1-MGS131036 (*c1 R1-g b1 pl1*)	446	475	0.34	*r1-MGS167054* (*r1-g C1 b1 pl1*)	466	447	0.53
c1-MGS14633 (*c1 R1-r B1 Pl1*)	430	450	0.50	*r1-MGS14638* (*r1-r C1 B1 Pl1*)	290	325	0.16

χ^2 analysis was performed to detect significant deviation ($P < 0.01$) of observed phenotypic classes from expected.

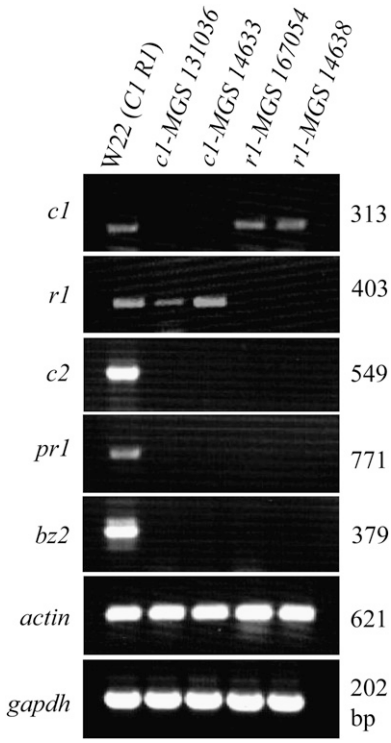

FIGURE 7.—Mutant *c1* and *r1* plants carrying *Pr1* do not accumulate *pr1* transcripts. Expression of *pr1* and other anthocyanin genes in aleurones were analyzed by RT–PCR in *c1-MGS131036*, *c1-MGS14633*, *r1-MGS167054*, and *r1-MGS14638* alleles. Purple aleurones from W22 were used as positive control for anthocyanin genes' expression, while actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as loading controls.

cyanidin and pelargonidin (which differ by one hydroxyl group attached at the 3′ position in cyanidin) in the methanolic extracts from purple and red aleurones, respectively.

Anthocyanin biosynthesis in maize is transcriptionally regulated by an interaction between two sets of transcription factors encoded by *c1/pl1* and *r1/b1*; *c1* and *r1* are required in the kernel aleurone, while *pl1* and *b1* function to activate these genes in the plant body (CHANDLER *et al.* 1989). Our genetic analysis revealed that the *pr1* gene plays a role in the *c1*- and *r1*-regulated anthocyanin biosynthetic pathway. RNA expression of *pr1* and other biosynthetic genes was not detected in the absence of functional alleles of *c1* and *r1*. R1 and C1 proteins physically interact; additionally, C1 binds directly to specific *cis*-regulating elements present in the promoters of biosynthetic genes (SAINZ *et al.* 1997). *In vitro* protein–DNA binding assays using point mutations, or *in vivo* transposon insertions in these *cis*-binding sites disrupt gene expression (GROTEWOLD *et al.* 1994; POOMA *et al.* 2002). Similar elements were found in the *pr1* promoter, indicating that *pr1* may be regulated by C1 and R1 in a fashion similar to other anthocyanin biosynthetic genes (SAINZ *et al.* 1997; LESNICK and CHANDLER 1998).

Our SNP-based genetic linkage analysis mapped the *Zmf3′h1* coding region to the *pr1* locus on chromosome 5L and this is in agreement with the B73 RefGen_v2 genome sequence. The proposed function of *Zmf3′h1* was verified by both the *in vitro* and *in vivo* complementation of the *pr1* phenotype. In transient assays, cotransformation of 35S::ZmF3′H1 and 35S::C1+R1 plasmid constructs causes the accumulation of purple pigments in *Zea mays* Black Mexican Sweet (BMS) cells as well as in kernel aleurones of MP708 (data not shown), both of which carry nonfunctional *pr1* alleles in their genetic background (GROTEWOLD *et al.* 1998). Our results from complementation of Arabidopsis *tt7* mutation by the *Zmf3′h1* explicitly confirm that the isolated *Zmf3′h1* gene encodes for a functional F3′H enzyme. Finally, the identification of a *Ds*-induced insertion reveals independently the role of *Zmf3′h1* in producing purple cyanidin glycosides from red pelargonidin glycosides.

The flavonoid biosynthetic pathway is present in both monocots and dicots, but the structural enzymes in this pathway are divergent in these two groups (DONG *et al.* 2001). One theory proposes that because of less stringent selective pressure on the enzymes of secondary metabolic pathways, these enzymes have diverged rapidly during evolution (PICHERSKY and GANG 2000). In contrast, mutations in Arabidopsis and petunia (both are dicots) reveal that flavonoid biosynthetic genes can be complemented by orthologs from *Z. mays*, a monocot (DONG *et al.* 2001). Similarly, we found that *Zmf3′h1*-transformed *tt7* seedlings produced cyanidin and its glycosylated derivatives. These results are surprising given that many maize genes, including *Zmf3′h1* (which shares 57% identity to Arabidopsis F3′H) have only moderate sequence identity to homologous genes in Arabidopsis. Additionally, the regulatory genes *c1* and *r1* from maize when expressed in transgenic Arabidopsis and tobacco plants resulted in increased anthocyanin pigmentation in both dicot species (LLOYD *et al.* 1992). These prior results along with the results of the current study demonstrate that many enzymes of the flavonoid biosynthetic pathway are conserved between monocots and dicots and that they can be transferred between these two divergent groups of flowering plants.

In this study, three spontaneous "loss-of-function" *pr1* alleles were genetically characterized to further confirm the function of the *pr1* gene. Two of these *pr1* alleles show an insertion of 24 TA dinucleotide repeats in the promoter region, while a 17-bp deletion was detected in the putative 5′-UTR of the third allele. Further, these deletion and insertion polymorphisms cosegregated with the red aleurone phenotype in a F_2 population segregating for *Pr1* and *pr1*. The variation of dinucleotide repeats (also known as tandem repeats) in the promoter region has been shown to interfere with expression levels of certain genes (MARTIN *et al.* 2005). In yeast, differences in promoter tandem repeat length affect transcription levels, which elevated with the

increase in the length of tandem repeats to a certain number and then diminished (Vinces *et al.* 2009). Tandem repeats can also reduce the promoter activity by changing the spacing between two binding sites or by inserting into a *cis*-element and making it unavailable for binding of an important *trans*-factor (Raijmakers *et al.* 2000). However, we were unable to demonstrate the role of the tandem repeats in limiting expression from the *pr1* locus.

In addition to elucidating the molecular identity of the *pr1* locus, this study provides a foundation to examine the role of DHK and DHQ as antifungal and insecticidal compounds (Hammerschmidt and Nicholson 1977; Hedin *et al.* 1983; Hipskind 1996; Karageorgou and Manetas 2006). F3′H enzyme activity is required for hydroxylation at the 3′ position on the B ring of flavonoid compounds and was observed to act on a wide range of substrates including flavonols, flavones, and flavanones (Larson and Bussard 1986). Interestingly, the hydroxylation pattern of the B ring is also the key determinant in the antioxidant property of anthocyanins; cyanidin, because of an additional hydroxyl group at its 3′ B-ring position, has a higher oxygen radical absorbing capacity as compared to pelargonidin and, therefore, has higher anticarcinogenic activity (Wang *et al.* 1997). It has been demonstrated that the *f3′h* gene in sorghum is induced in response to fungal infection, and its expression is correlated with the biosynthesis of flavonoid phytoalexins (Boddu *et al.* 2004). Moreover, *pr1* was detected as a major QTL for the synthesis of C-glycosyl flavones that have insecticidal activity against corn earworm (Lee *et al.* 1998; Cortes-Cruz *et al.* 2003). We are thus further interested in investigating the role of *pr1* in plant disease and insect resistance through the biosynthesis of different flavonoids that act as defense compounds.

We thank Catherine Svabek for her excellent technical assistance, Scott Harkcom, Jim Breining, John Shaffer, and the Penn State Agronomy Farm staff for their help with land preparation and tending of maize genetic nurseries. This work was supported in part by research support to S.C. under Hatch projects 4144 and 4154 of the College of Agricultural Sciences, Pennsylvania State University, and a United States Department of Agriculture–National Research Initiative-2007-35318-17795 award. This work was also supported by funding from the National Science Foundation to T.P.B. (D.B.I. 0501713). M.S. was supported by a doctoral assistantship from the Department of Crop and Soil Sciences, Pennsylvania State University.

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Communicating editor: C. S. Pikaard

GENETICS

Supporting Information
http://www.genetics.org/cgi/content/full/genetics.110.126136/DC1

Identification of the *Pr1* Gene Product Completes the Anthocyanin Biosynthesis Pathway of Maize

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DOI: 10.1534/genetics.110.126136

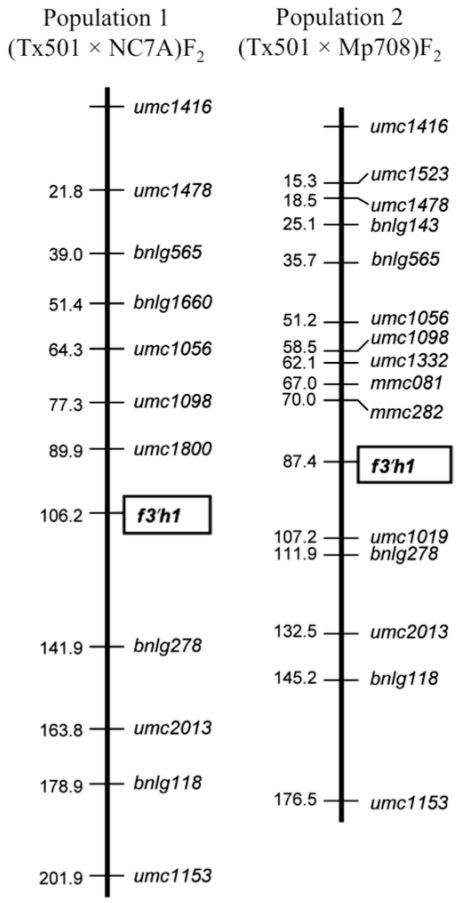

FIGURE S1.—*Zmf3′h1* maps to the *pr1* locus on chromosome 5L. Genetic linkage mapping of *Zmf3′h1* SNP marker on chromosome 5 for (Tx501 x NC7A) and (Tx501 x MP708) F_2 populations. The position of *Zmf3′h1* is indicated on both maps and corresponds to the *pr1* locus. Cumulative distances given in centimorgans are indicated to the left of the chromosome and marker loci are indicated to the right.

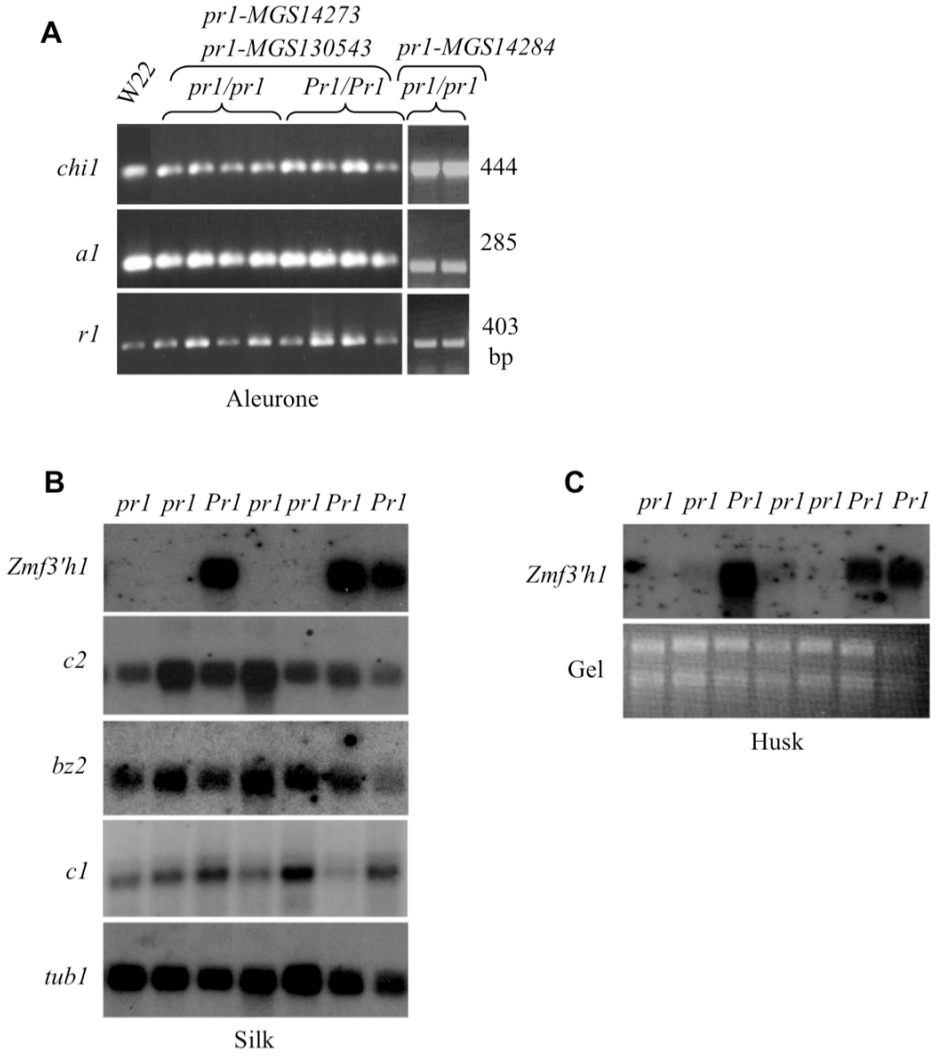

FIGURE S2.—RT-PCR and RNA hybridization analyses were performed on total RNA extracted from developing aleurones, young silks and young husk collected from plants segregating for *Pr1* and *pr1*. (A) RT-PCR for expression of anthocyanin genes *chi1*, *a1*, and transcription factor, *r1* in aleurone tissues of *pr1/pr1* and *Pr1/Pr1* plants. (B) Gel blot of RNA isolated from silk tissues was hybridized to *Zmf3'h1*, *c2*, *bz2*, *c1*, and *α-tubulin1* specific probes. (C) Husk RNA gel blot hybridized with *Zmf3'h1* probe. Ethidium bromide stained gel picture shows loading control for RNA in each lane.

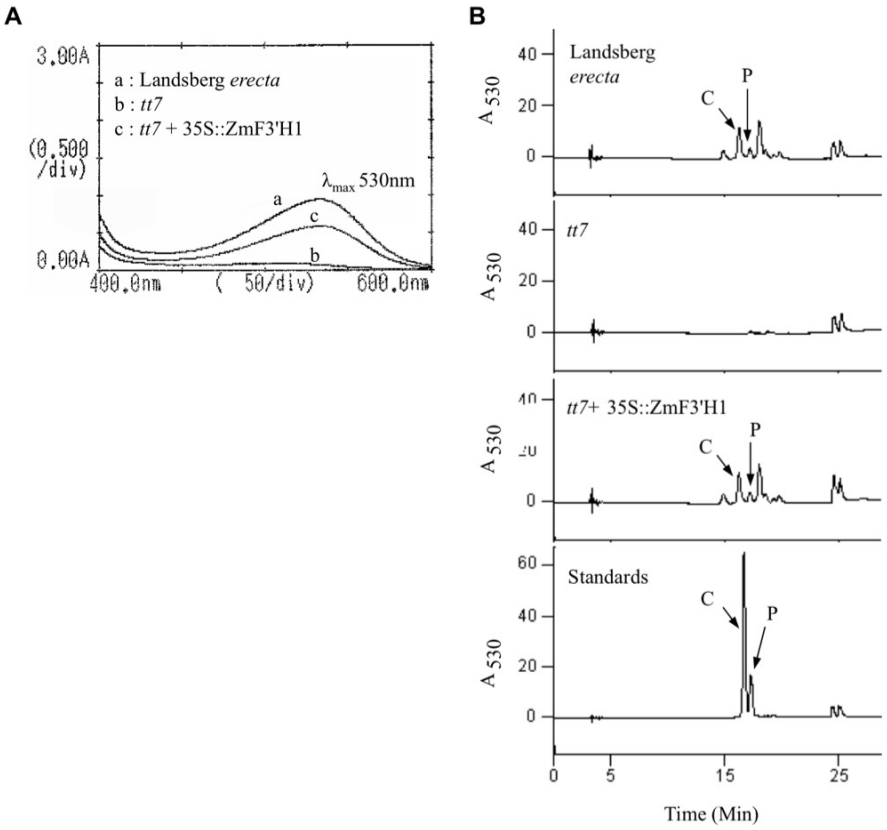

FIGURE S3.—Biochemical analysis of *Zmf3'h1* complemented Arabidopsis *tt7* mutant. (A) Spectrophotometric analysis of methanolic extracts from 10 day old seedlings grown on a minimal medium (a) Landsberg *erecta*, (b) 35S::ZmF3'H1 complemented *tt7*, (c) *tt7*. (B) HPLC analysis of anthocyanins present in hydrolyzed methanolic extracts of 10 day old seedlings from Landsberg *erecta*, *tt7*, and *Zmf3'h1* complemented *tt7* mutant. The standards for cyanidin and pelargonidin are also shown. C, cyanidin; P, pelargonidin; and CG, cyanidin glycosides.

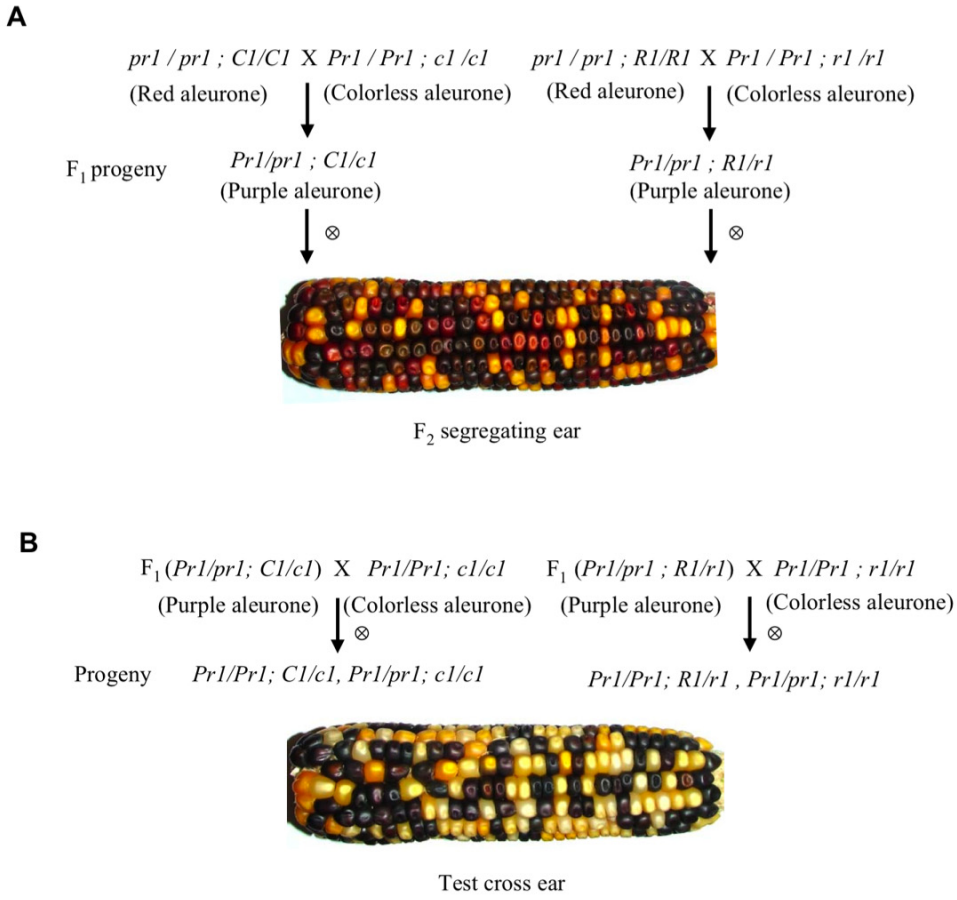

FIGURE S4.—Genetic assays to study regulation of *pr1* by the anthocyanin regulatory genes *c1* and *r1*. (A) Crossing scheme used to develop F_1 and F_2 progenies. Purple, red, and colorless kernel aleurone phenotypes were observed in F_2 segregating ears. (B) Crossing scheme used to develop the F_1 and test cross progenies. Purple and colorless kernel aleruone phenotypes were seen in representative test cross ears.

TABLE S1

PCR primers used in RT-PCR analysis

Gene	Primer combination		Product (bp)	Reference
pr1	5F3H-F2	5′-GAGCACGTGGCGTACAACTA-3′	771	This Study
	ZMR4	5′-AAACGTCTCCTTGATCACCGC-3′		
c2	CHSF	5′-TCGATCGGTCTCTCTGGTACAACGTA-3′	549	This Study
	CHSR	5′-TACATCATGAGGCGGTTCACGGA-3′		
chi1	CHIF	5′-GTGCGGAATTTAACATGGCGTGC-3′	444	This Study
	CHIR	5′-CGGCGCGAAAGTCTCTGGCTT-3′		
a1	A1	5′-CAATTCGTTGAACATGGAAGTAAG-3′	285	Piazza *et al.*, 2002
	A2	5′-CAATTCGTTGAACATGGAAGTAAG-3′		
bz2	BZ2F	5′-ATATGCGAGTCCGCAGTCATCGT-3′	379	This Study
	BZ2R	5′-TCGATGAGTGAGAGGCCGTGAA-3′		
c1	PL6	5′-TCGGACGACTGCAGCTCGGC-3′	313	Piazza *et al.*, 2002
	AC1	5′-CACCGTGCCTAATTTCCTGTCCGA-3′		
r1	OR31	5′-ATGGCTTCATGGGGCTTAGATAC-3′	403	Piazza *et al.*, 2002
	OR32	5′-GAATGCAACCAAACACCTTATGCC-3′		
actin	ActinF	5′-CCTTCGAATGCCCAGCAATG-3′	202	This Study
	ActinR	5′-GAGGATCTTCATTAGGTGGT-3′		
gapdh	GAP1	5′-AGGGTGGTGCCAAGAAGGTTG-3′	621	This Study
	GAP2	5′-GTAGCCCCACTCGTTGTCGTA-3′		
tubulin	Tub1	5′-AGGATCCACTTCATGCTTTCCTCC-3′	546	This Study
	Tub2	5′-CACCTTCCTCACCCTCATCAAACT-3′		

TABLE S2

Primers for PCR amplification

Primer combination	Primer sequences	Product (bp)
ZF3F2 ZF3R2	5′-AGTGCGAGGTGGACGGGTTC-3′ 5′-GCAGACGGCAGCAGTCTCCCCT-3′	387
OSF1 ZMR4	5′-CATACGGCCATGGACGTTGTGCCT-3′ 5′-AAACGTCTCCTTGATCACCGC-3′	1276
SBF12 SBR22	5′-CTTCTAGAACCGAG-3′ 5′-TTGGAT CCCCTACTCCGCTGCGTAT-3′	1788
P-1 P-2	5′-TGACTTGCACCTCCTTGTTCTGTC-3′ 5′-TTTAGTGCACAACCTTTAGGG-3′	441
P-3 P-4	5′-GTACGAAATTCCAGATCGCGGGTA-3′ 5′-ATAGCCACATGGTGTGGTGCGG-3′	349
MGR1 JSR01	5′-CACTGATACCCACGTACAACGCTT-3′ 5′-GTTCGAAATCGATCGGGATA-3′	1547
MGR2 JSR01	5′-TTGCGCTCGTACGGGAAAGGTA-3′ 5′-GTTCGAAATCGATCGGGATA-3′	670
MGF1 JSR05	5′-GTACGAAATTCCAGATCGCGGGTA-3′ 5′-CGTCCCGCAAGTTAAATATGA-3′	588