The Purple Cauliflower Arises from Activation of a MYB Transcription Factor[1[W][OA]]

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Anthocyanins are responsible for the color of many flowers, fruits, and vegetables. An interesting and unique *Purple* (*Pr*) gene mutation in cauliflower (*Brassica oleracea* var *botrytis*) confers an abnormal pattern of anthocyanin accumulation, giving the striking mutant phenotype of intense purple color in curds and a few other tissues. To unravel the nature of the *Pr* mutation in cauliflower, we isolated the *Pr* gene via a combination of candidate gene analysis and fine mapping. *Pr* encoded a R2R3 MYB transcription factor that exhibited tissue-specific expression, consistent with an abnormal anthocyanin accumulation pattern in the mutant. Transgenic Arabidopsis (*Arabidopsis thaliana*) and cauliflower plants expressing the *Pr-D* allele recapitulated the mutant phenotype, confirming the isolation of the *Pr* gene. Up-regulation of *Pr* specifically activated a basic helix-loop-helix transcription factor and a subset of anthocyanin structural genes encoding flavonoid 3′-hydroxylase, dihydroflavonol 4-reductase, and leucoanthocyanidin dioxygenase to confer ectopic accumulation of pigments in the purple cauliflower. Our results indicate that the genetic variation including a Harbinger DNA transposon insertion in the upstream regulatory region of the *Pr-D* allele is responsible for the up-regulation of the *Pr* gene in inducing phenotypic change in the plant. The successful isolation of *Pr* provides important information on the regulatory control of anthocyanin biosynthesis in *Brassica* vegetables, and offers a genetic resource for development of new varieties with enhanced health-promoting properties and visual appeal.

Vegetables and fruits are fundamental components of human diets. They not only are important sources of essential vitamins and minerals, but also contain a wide variety of secondary metabolites important to human health. Colored vegetables and fruits have gained an increasing interest as functional foods, owing to their high levels of plant pigments with potent nutritional and health-promoting effects. Among them, purple cauliflower (*Brassica oleracea* var *botrytis*) is a very eye-catching vegetable and available commercially. The purple coloration is due to the accumulation of anthocyanins.

Anthocyanins are a group of flavonoid compounds that fulfill important biological functions in protecting plants against various biotic and abiotic stresses. All of the anthocyanin biosynthetic pathway genes and numerous regulatory factors have been identified from studies of Arabidopsis (*Arabidopsis thaliana*), maize (*Zea mays*), petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*), and other plant species (Broun, 2005; Dixon et al., 2005; Koes et al., 2005; Grotewold, 2006). Transcriptional regulation of structural genes appears to be a major mechanism by which anthocyanin biosynthesis is regulated in plants. R2R3 MYB and basic helix-loop-helix (bHLH) transcription factors as well as WD40 proteins represent the three major families of anthocyanin regulatory proteins (Paz-Ares et al., 1987; Chandler et al., 1989; Ludwig and Wessler 1990; de Vetten et al., 1997; Quattrocchio et al., 1999). They form regulatory complexes to activate expression of anthocyanin structural genes (Goff et al., 1992; Grotewold et al., 2000). In Arabidopsis, several MYB proteins, including PAP1, PAP2, MYB113, MYB114, and MYBL2 (Borevitz et al., 2000; Dubos et al., 2008; Gonzalez et al., 2008; Matsui et al., 2008), three bHLH proteins of TT8, GL3, and EGL3 (Nesi et al., 2000; Payne et al., 2000; Zhang et al., 2003), and a WD40 repeat protein of TTG1 (Walker et al., 1999) are involved in anthocyanin biosynthesis. While the R2R3 MYB proteins of PAP1, PAP2, MYB113, and MYB114 cause tissue-specific anthocyanin accumulation in Arabidopsis (Borevitz et al., 2000; Gonzalez et al., 2008), the R3-MYB protein, MYBL2, acts as an inhibitor of anthocyanin biosynthesis (Dubos et al., 2008; Matsui et al., 2008). TT8 is required for the full transcriptional activation of late anthocyanin pathway genes (Nesi et al., 2000), and is partially functionally redundant with its closest ho-

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mologs, GL3 and EGL3 (Zhang et al., 2003). The WD40 protein, TTG1, is known to physically interact with the MYB and bHLH transcription factors in controlling anthocyanin biosynthesis (Zhang et al., 2003).

R2R3 MYB transcription factors have been implicated to play an important role for color difference in plant species. Activation of an R2R3 MYB transcription factor confers anthocyanin production in a number of anthocyanin-accumulating plants (Borevitz et al., 2000; Mathews et al., 2003). In contrast, loss of function of a R2R3 MYB results in color loss in the normal anthocyanin-accumulating tissues. For example, a retrotransposon insertion in the promoter of Vv*MYBA1* and mutations in the adjacent Vv*MYBA2* inactivate their expression and convert red-skinned grape (*Vitis vinifera*) into white-skinned one (Walker et al., 2007). In *Antirrhinum majus*, alteration of *MYB*-related gene expression controls floral pigmentation intensity and pattern (Schwinn et al., 2006).

Similarly, bHLH transcription factors have also been found to be responsible for color difference in plant species. Alteration of a bHLH transcription factor causes an increase in red pigment production in the aleurone of maize (Burr et al., 1996). Knockout of a bHLH function by a frame shift changes seed pericarp color from red into white in rice (*Oryza sativa*; Sweeney et al., 2006). An insertion of a DNA transposon into a bHLH regulatory gene alters flower color of the common morning glory (*Ipomoea tricolor*) into pale pigmented flowers (Park et al., 2007).

Mutant analyses have facilitated gene discovery and elucidation of the regulatory control of anthocyanin biosynthesis. Although there are large numbers of researches on the underlying mechanisms controlling anthocyanin production in flowers, fruits, and model plants, only few have focused on vegetables. Purple cauliflower mutant represents an interesting mutation that confers profound anthocyanin production in the otherwise low-pigmented curds and seeds—the two tissues of agricultural importance. Thus, this mutant provides an excellent opportunity to reveal regulatory control of anthocyanin biosynthesis in vegetable crops.

The cauliflower purple mutation was found to be controlled by a single, semidominant gene. We designate the symbol *Pr-D* for the *Purple* allele, and *pr* for its wild-type counterpart. Through a combination of candidate gene analysis and fine mapping, we isolated the *Pr* gene and found that it encoded a R2R3 MYB transcription factor. Comparison of *Pr* sequences from wild type and the mutant allele revealed allelic variation including a Harbinger DNA transposon insertion in the upstream regulatory region of the *Pr-D* allele. Such an alteration caused an increased *Pr* gene transcription, which in turn up-regulated anthocyanin structural gene expression to produce the striking purple phenotype. The activation of the *Pr* gene, probably by introduction of new regulatory motifs in the promoter region, provides a way that anthocyanin transcriptional regulation can be switched on differently in plants.

RESULTS

Phenotypic Characterization of the Purple Cauliflower Mutant

The cauliflower purple mutant plants grew and developed normally when compared to white cauliflower under normal growth conditions in field and in greenhouse. The mutant exhibited a tissue-specific pattern of anthocyanin accumulation. Intense purple coloration was observed in curd and a few other tissues of the plant (Fig. 1, A–D, F, and G), including young seedlings, very young leaves, and very young flower buds and siliques, as well as the endosperm of seeds, a tissue that is rarely reported to accumulate pigments in anthocyanin-accumulating mutants, e.g. in red cabbage (*Brassica oleracea* var *capitata*; Yuan et al., 2009). The purple color was not observed in older leaves, stems, flower petals, and older siliques (Fig. 1, B, E, and F). The purple phenotype appears to be associated primarily with very young tissues, curds, and seeds, rather than in flower petals, the most common anthocyanin-accumulating tissue. Under the same growth conditions, the wild-type cauliflower plants exhibited no purple hue in these tissues (Fig. 1, A–G).

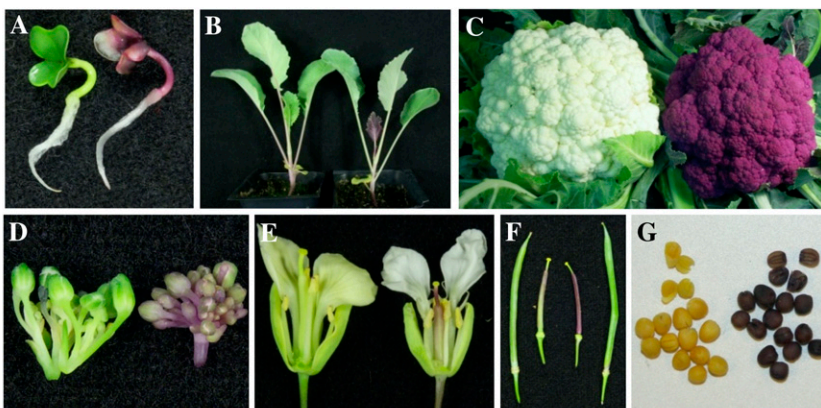


Figure 1. Phenotypic comparison between wild type and the cauliflower *Pr-D* mutant. A, Young seedlings of 5-d-old plants. B, Young plants of 3-week-old. C, Curds of cauliflower plants grown in field. D, Young flower buds. E, Flowers. F, Young (inner) and old siliques of wild type (left) and mutant (right). G, Seed endosperms.

Purple Mutant Predominantly Accumulates Cyanidin Glucosides

To examine the composition and content of anthocyanins accumulated in young leaves, curds, and seeds of the purple mutant, we performed HPLC analysis and found high levels of anthocyanins in the purple tissues (Fig. 2A). The curds accumulated approximately 3.75 mg cyanidin diglucoside equivalent g^{-1} fresh weight, a level that was comparable with that found in blueberries (*Vaccinium myrtillus*; Gao and Mazza, 1994). In contrast, the samples of the wild-type control plants contained undetectable amounts of anthocyanins (data not shown), indicating that the anthocyanin pathway was biochemically quiescent under normal growth conditions.

Several different groups of anthocyanins (e.g. delphinidin, pelargonidin, and cyanidin) exist in the plant kingdom (Tanaka et al., 2008). To identify anthocyanin composition in the mutant, anthocyanins in curd tissue were separated and analyzed using HPLC-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS; Wu and Prior, 2005b). The purple mutant contained one major peak (Fig. 2B), which was identified as cyanidin 3-(coumaryl-caffeyl) glucoside-5-(malonyl)glucoside (Fig. 2C). The other minor peaks were also identified as different forms of cyanidin glycosides based on the dominant ion pairs observed in the mass spectrometry data and anthocyanin structures (Mazza and Miniati, 1993; Wu and Prior, 2005a).

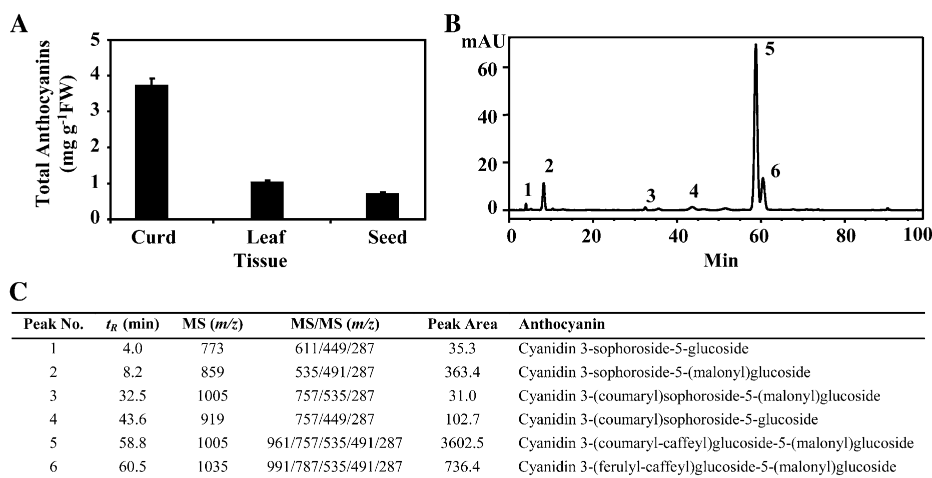
The Purple Mutation Is Controlled by a Single Semidominant Gene

To examine whether the purple mutant was controlled by one or multiple genes, we crossed the purple mutant Graffiti to an inbred white cultivar Stovepipe, selfed the heterozygous F1 plants, and generated a large F2 population. A subpopulation of 102 individuals was germinated in a greenhouse. Genotyping of these F2 individuals was completed by visually examining the absence and presence of light or dark purple color in very young leaves and curds of the progeny, followed by further confirmation of the individual genotype in some cases by visual examination of the color with 16 F3 individuals. The segregation ratio of these F2 plants for white:light purple:dark purple was 31:53:18. χ^2 test showed a good fit with a 1:2:1 ratio ($P > 0.05$), which was consistent with the ratio expected for the progeny derived from selfing a parent heterozygous at one locus. This result suggests that the purple phenotype is controlled by a single, semidominant gene, *Pr*.

Expression of Anthocyanin Biosynthetic and Regulatory Genes

To investigate whether *Pr* represented one of the biosynthetic genes that was significantly up-regulated in the *Pr-D* mutant, we examined the expression of anthocyanin pathway genes in curds and leaves of wild type and the *Pr-D* mutant by northern-blot analysis. The cDNAs coding *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), *flavonoid 3′-hydroxylase* (*F3′H*), *dihydroflavonol 4-reductase* (*DFR*), *leucoanthocyanidin dioxygenase* (*LDOX*), and *UDP-glucosyltransferase* (*UGT*) were used as probes. While the early pathway genes were expressed at similar levels between wild type and the *Pr-D* mutant in both leaves and curds, the late pathway genes, *BoF3′H*, *BoDFR*, and *BoLDOX*, were dramatically up-regulated in the mutant (Fig. 3A). The co-up-regulation of three structural genes suggests that *Pr* is unlikely a mutation of one specific anthocyanin pathway gene.

Many regulatory genes that control anthocyanin biosynthesis have been isolated from plant species (Paz-Ares et al., 1987; Chandler et al., 1989; Ludwig and Wessler 1990; de Vetten et al., 1997; Quattrocchio et al., 1999). Since cauliflower genes typically share high coding sequence identity with Arabidopsis genes, the available sequences of anthocyanin regulatory genes from Arabidopsis and the significant



Peak No.	t_R (min)	MS (*m/z*)	MS/MS (*m/z*)	Peak Area	Anthocyanin
1	4.0	773	611/449/287	35.3	Cyanidin 3-sophoroside-5-glucoside
2	8.2	859	535/491/287	363.4	Cyanidin 3-sophoroside-5-(malonyl)glucoside
3	32.5	1005	757/535/287	31.0	Cyanidin 3-(coumaryl)sophoroside-5-(malonyl)glucoside
4	43.6	919	757/449/287	102.7	Cyanidin 3-(coumaryl)sophoroside-5-glucoside
5	58.8	1005	961/757/535/491/287	3602.5	Cyanidin 3-(coumaryl-caffeyl)glucoside-5-(malonyl)glucoside
6	60.5	1035	991/787/535/491/287	736.4	Cyanidin 3-(ferulyl-caffeyl)glucoside-5-(malonyl)glucoside

Figure 2. Analysis of anthocyanin content and composition in the *Pr-D* mutant. A, Total anthocyanin levels as cyanidin diglucoside equivalent in curds, leaves, and seeds. FW, Fresh weight. B, HPLC elution profile of anthocyanins accumulated in curds. The absorbance was monitored at A_{520} nm. C, Major anthocyanins identified by HPLC-ESI-MS/MS in curds.

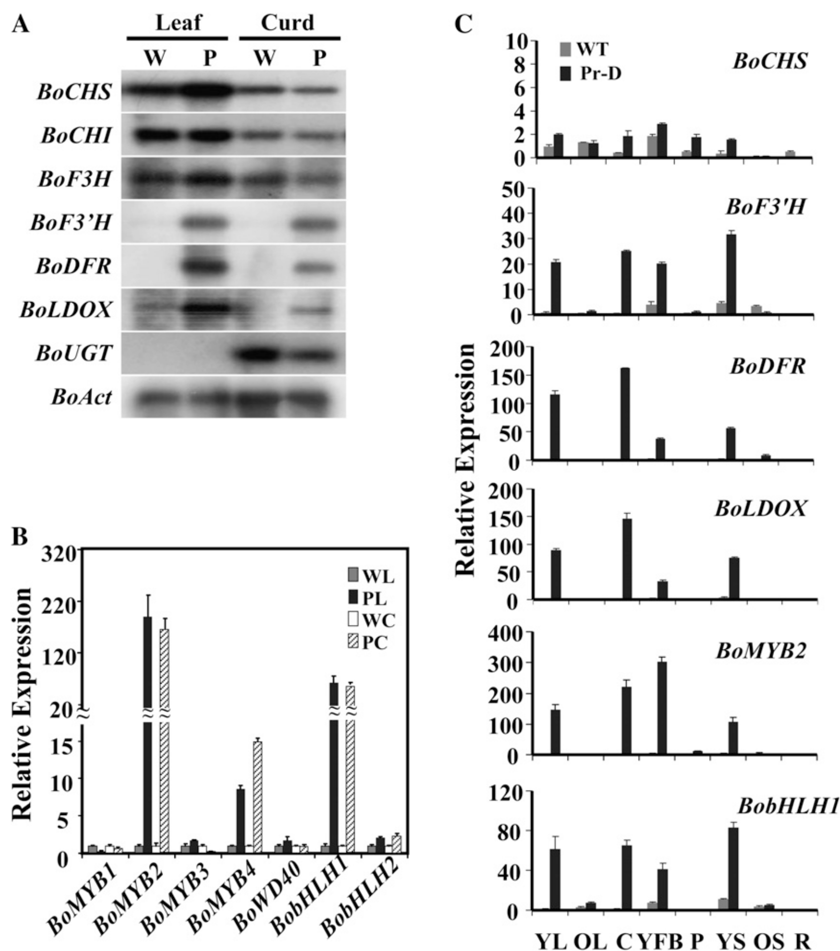


Figure 3. Expression of anthocyanin structural and regulatory genes in wild type and the *Pr-D* mutant. A, northern-blot analysis of transcript levels of anthocyanin pathway genes in curds and leaves. *BoAct* serves as equal loading control. P, *Pr-D* mutant; W, wild-type control. B, qRT-PCR analysis of anthocyanin regulatory genes in curds and leaves. The identities of the amplified products were confirmed by sequencing. Expression of genes in wild-type leaves and curds were set to 1. WL, Wild-type leaves; PL, *Pr-D* leaves; WC, wild-type curds; PC, *Pr-D* curds. BoWD40, BobHLH1, and BobHLH2 are cauliflower homologs of Arabidopsis *TTG1*, *TT8*, and *EGL3*, respectively. C, qRT-PCR analysis of transcript levels of *BoCHS* and the differentially expressed genes during different developmental stages. Expression of genes in wild-type (WT) young leaves was set to 1. YL, Young leaves; OL, old leaves; C, curds; YFB, young flower buds; P, petals; YS, young siliques; OS, old siliques; R, roots. qRT-PCR results represent mean values + SD from three biological replicates with three technical replicates for each.

amounts of *Brassica* sequence information in the public domains provide direct resources for designing the gene-specific primers for some cauliflower homologs. In Arabidopsis, the R2R3 MYB family proteins of PAP1, PAP2, MYB113, and MYB114, bHLH proteins of TT8 and EGL3, and WD40 protein of TTG1 are known to regulate anthocyanin biosynthesis. To investigate whether the *Pr* gene represented a mutation of one of the known regulatory genes, transcript levels of these homologous genes in wild type and the *Pr-D* mutant were examined by quantitative reverse transcription (qRT)-PCR using gene-specific primers (Yuan et al., 2009; Supplemental Table S1). As shown in Figure 3B, *PAP*-like MYB family genes, i.e. *BoMYB2* and *BoMYB4*, as well as *BobHLH1*, a homologous gene of Arabidopsis *TT8* (Nesi et al., 2000) exhibited differential expression in both leaves and curds between wild type and the *Pr-D* mutant.

Anthocyanins in the *Pr-D* mutant exhibited tissue- and development-specific accumulation (Fig. 1). To examine whether the specific anthocyanin accumulation pattern was correlated with gene expression pattern, we analyzed transcript levels of *BoCHS* and the differentially expressed genes in different tissues of wild-type and the *Pr-D* plants. Similar level of *BoCHS* transcript was observed in tissues between wild type and the *Pr-D* mutant. In contrast, the transcripts of *BoF3'H*, *BoDFR*, and *BoLDOX*, as well as *BoMYB2* and *BobHLH1* accumulated highly in very young leaves, curds, very young flower buds, and young siliques in the mutant. The expression patterns of these genes were consistent with the tissue-specific anthocyanin accumulation pattern in the *Pr-D* mutant.

Identification of a MYB Gene That Cosegregates with *Pr-D*

To test whether *BobHLH1* or one of the *PAP*-like MYB family genes represented *Pr*, association mapping was carried out to investigate whether any of them cosegregated with the *Pr-D* locus. To develop markers for association mapping, full genomic fragments of *BobHLH1* and four *BoMYB* genes including promoter sequences were isolated through genomic DNA walking from both wild type and the *Pr-D* mutant. Comparison of the DNA sequences between wild type and mutant allele revealed that there were no polymorphisms for *BobHLH1* and *BoMYB1*, suggesting that they were unlikely the gene representing *Pr*. Based on different insertion/deletion or single

nucleotide polymorphisms in *BoMYB2*, *BoMYB3*, and *BoMYB4*, PCR-based markers were developed (Supplemental Table S1). These three *MYB* genes were found to cosegregate with the *Pr-D* locus in a mapping population of 102 F2 plants.

To define the gene that represented *Pr*, the three *BoMYB* genes were mapped in a large mapping population of 1,898 F2 individuals. Two and seven recombinant events were detected for *BoMYB3* and *BoMYB4* marker, respectively. In contrast, no recombinant event was observed for *BoMYB2* marker, indicating that *BoMYB2* cosegregated with the *Pr-D* locus and most likely the *Pr* gene. A high-resolution genetic map for this region is shown in Figure 4A.

Structural Analysis of *Pr*

The *Pr* gene from both wild-type and mutant plants contained an open reading frame of 744 bp that encoded a protein of 247 amino acids with an estimated molecular mass of 27.99 kD. *Pr* encoded BoMYB2 that was predicted to be a nuclear protein (WoLF PSORT, wolfpsort.org) and contained R2 and R3 MYB repeat domains with the signature motif of KPRPR[S/T]F, specific for MYB proteins that activate anthocyanin biosynthesis (Stracke et al., 2001; Fig. 4B). Noticeably, the KPRPR[S/T]F motif overlaps with a *TAS4*-siR81 target site identified in Arabidopsis *PAP1* and *PAP2* (Rajagopalan et al., 2006), suggesting a possible addi-

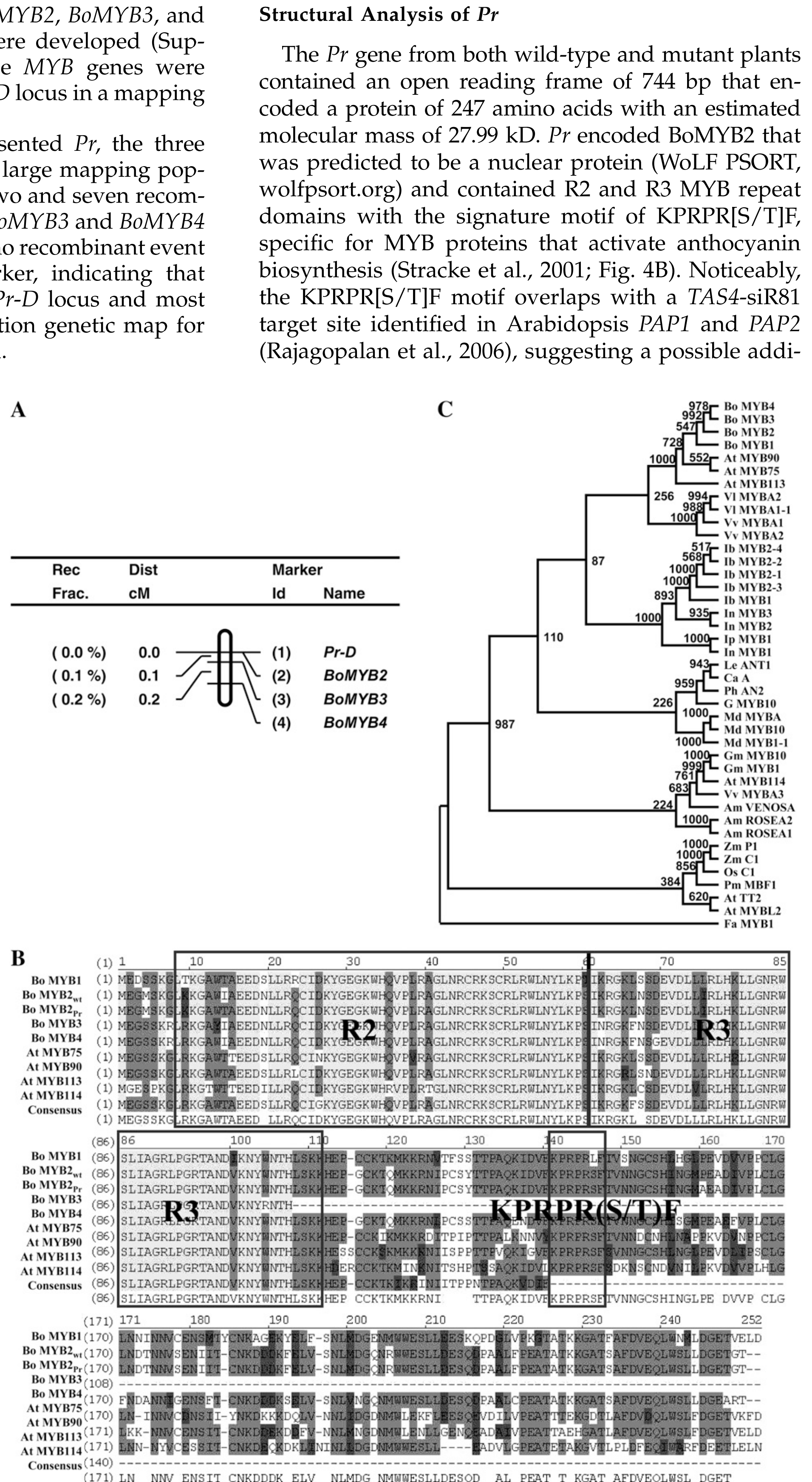


Figure 4. High-resolution genetic map of the *Pr* region, MYB sequence alignment, and phylogenetic tree of BoMYB homologs. A, Linkage map of *BoMYB2*, *BoMYB3*, *BoMYB4*, and the *Pr-D* locus in a mapping population of 1,898 F2 individuals. B, Sequence alignment of cauliflower and Arabidopsis R2R3 MYB proteins. R2 and R3 repeat domains as well as the conserved KPRPR [S/T]F motif are indicated. C, Phylogenetic tree of BoMYBs and R2R3 MYBs from other plant species. Numbers along branches indicate bootstrap support determined from 1,000 trials. The length of the branch lines indicates the extent of divergence. The GenBank accession numbers of these proteins are provided in Supplemental Table S2.

tion level of *BoMYB2* regulation. BoMYB2 shared 79.8% to 89.9% amino acid sequence identity to the other BoMYB proteins, and 69.1% to 86.3% to the Arabidopsis PAP-like proteins (Fig. 4B). Alignment of the coding sequences of wild type and the mutant gene showed that there were only two single nucleotide differences, which resulted in two amino acid changes from Ile to Thr and Pro to Ala at position 14 and 159, respectively (Fig. 4B). Phylogenetic analysis indicates that BoMYBs are most closely related to Arabidopsis PAP-like proteins and clustered with R2R3 MYB transcription factors involved in regulating anthocyanin biosynthesis from other plant species (Fig. 4C).

Functional Complementation of the Purple Mutant Phenotype in Arabidopsis and Cauliflower

To confirm the function of *Pr*, genomic fragments of the *Pr* gene from both wild-type and mutant plants were introduced into wild-type Arabidopsis and cauliflower. Over 40 independent transgenic Arabidopsis lines were generated for each construct. Like the vector-only control (Fig. 5A, 1–6), the transgenic Arabidopsis expressing the wild-type *pr* allele under the control of endogenous promoter (pr_{pro}:*pr*) exhibited no or very low levels of pigmentation (Fig. 5C, 1–6). In contrast, the *Pr-D* (Pr_{pro}:*Pr-D*) transformants showed tissue-specific anthocyanin accumulation (Fig. 5B, 1–6). Purple pigments accumulated mainly in young tissues of Arabidopsis, such as young leaves and young flower buds, and in seeds, but not in old leaves, flower petals, or mature siliques. A similar pattern of anthocyanin accumulation was also observed in transgenic cauliflower plants expressing the *Pr-D* allele (Fig. 5G, 1–6). Ectopic expression of the *Pr-D* allele under the control of endogenous promoter in both transgenic Arabidopsis and cauliflower induced tissue-specific anthocyanin accumulation, which showed the same anthocyanin accumulation pattern as in the *Pr-D* mutant. Thus, these results further confirm the isolation of the *Pr* gene.

Both wild-type and mutant *Pr* genes under the control of cauliflower mosaic virus (CaMV) 35S ($35S_{pro}$:*pr* and $35S_{pro}$:*Pr-D*) were also introduced into wild-type Arabidopsis and cauliflower. Overexpression of either wild-type or mutant gene resulted in the production of dark-purple transgenic plants with high levels of anthocyanin accumulation in Arabidopsis (Fig. 5, D and E, 1–6). Pigments accumulated in the entire transgenic plants that included leaves, roots, flower buds, flower petals, siliques, and seeds. Similarly, when these gene constructs were introduced into wild-type cauliflower plants, anthocyanins appeared in the entire transgenic plants (Fig. 5I, 1–6 and J, 1 and 2). Interestingly, the young top curd tissues of some overexpression lines were white (Fig. 5, I and J, 2) with the below curd tissues purple (Fig. 5I, 4). These results suggest that both wild-type and mutant genes encode functional proteins.

Figure 5. Complementation of the purple phenotype in wild-type Arabidopsis and cauliflower. Top images: Arabidopsis transgenic lines containing empty vector (VC), the *Pr-D* allele with endogenous promoter (Pr_{pro}:*Pr-D*), the wild-type *pr* allele with endogenous promoter (pr_{pro}:*pr*), the *Pr-D* allele with CaMV 35S promoter ($35S_{pro}$:*Pr-D*), and the wild-type *pr* allele with CaMV 35S promoter ($35S_{pro}$:*pr*). The sections from top represent 7-d-old seedlings, 3-week-old plants, young flower buds, flowers, older siliques, and seeds. Scale bars for seed images: 0.1 mm. Bottom images: Cauliflower transgenic lines containing the same five constructs as top images. The different sections from top represent T0 young plants, young curds, flowers, part of more mature curds, siliques, and seeds.

Pr Specifically Regulates a bHLH Transcription Factor and a Subset of Genes Involved in Anthocyanin Production

A number of late anthocyanin pathway genes and a transcription factor *BobHLH1* along with *Pr* were expressed highly in the *Pr-D* mutant (Fig. 3). To investigate whether expression of *Pr* in transgenic cauliflower caused such specific activation of gene expression, transcript levels of anthocyanin biosyn-

thetic genes and a number of regulatory genes in various transgenic lines were analyzed. The late pathway genes, *BoF3'H*, *BoDFR*, *BoLDOX*, and the transcription factor *BobHLH1* were expressed highly in the Pr_{pro}:*Pr-D* transformants as well as in $35S_{pro}$:*Pr-D* and $35S_{pro}$:*pr* transgenic lines in comparison with vector-only control and wild-type pr_{pro}:*pr* lines (Fig. 6). The early pathway genes showed no dramatic up-regulation. These results clearly demonstrate that *Pr* specifically activates *BobHLH1* and the late pathway genes in controlling anthocyanin biosynthesis in cauliflower.

Allelic Variation at Upstream Regulatory Region of the *Pr-D* Allele Enhances Promoter Activity

Pr was expressed highly in the mutant and shared 99.2% nucleotide sequence identity in the coding region with the wild-type gene. Thus, we hypothesized that the mutation in the promoter region controlled *Pr* expression in regulating anthocyanin accumulation. Analysis of the promoter sequences between the wild-type and *Pr-D* alleles revealed that the first −370-bp sequences were nearly identical except five nucleotide differences and one extra TATA box at the wild-type promoter (Supplemental Fig. S1). A Harbinger DNA transposon insertion (giri: www.girinst.org) was found at −373 bp of the *Pr-D* mutant allele. To test whether the sequence rearrangement due to the mutation altered promoter activity, comparable lengths of promoter sequences from wild-type and *Pr-D* allele were fused to GUS gene (Fig. 7A) and transformed into Arabidopsis. Interestingly, although the promoter sequences between the −378/−373 regions of *Pr-D* and *pr* were nearly identical, the mutant $Pr\text{-}D_{-373}$:*GUS* transformants exhibited significantly higher GUS activity than the wild-type pr_{-378}:*GUS* transformants

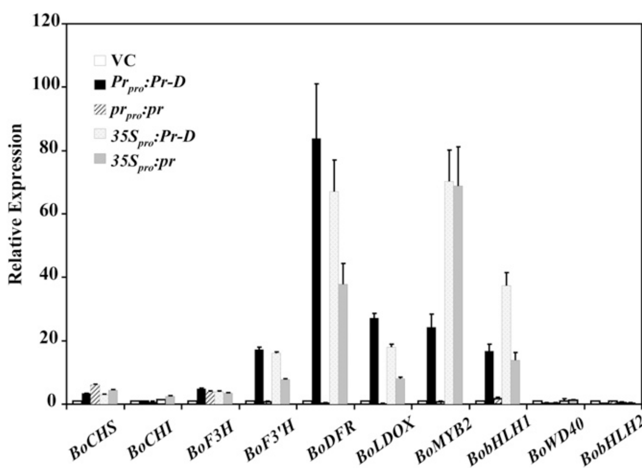


Figure 6. Expression of anthocyanin structural and regulatory genes in transgenic cauliflower. qRT-PCR analysis of transcript levels of selected genes in young leaves of cauliflower transformants containing different *Pr* gene constructs. The transcript levels of genes in vector control lines were set to 1. Results reported represent mean values ± SD from three biological replicate lines with three technical replicates for each.

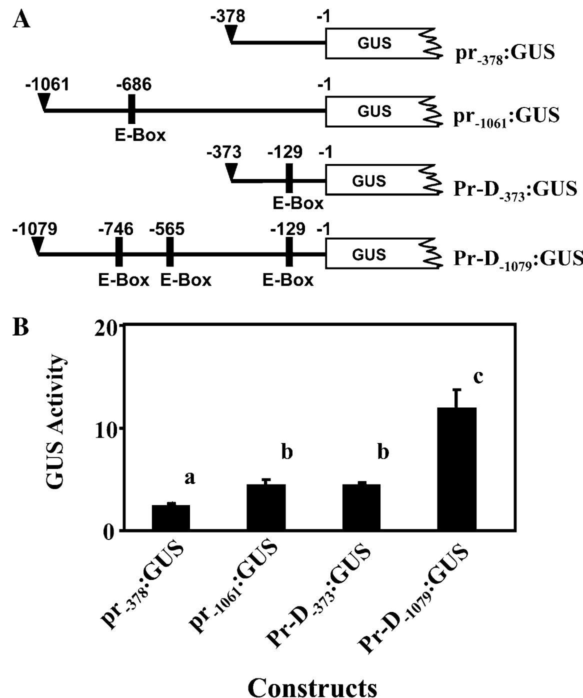


Figure 7. Analysis of promoter activity of the *Pr* gene. A, GUS constructs containing different lengths of promoter sequences. The promoter regions were chosen based on transposon insertion site at −373 bp and the transposon transposase site at −1,079 bp. B, GUS activity of 4-week-old stable Arabidopsis transgenic lines containing different GUS constructs. GUS activity was measured from at least four independent transgenic lines with three repeats and expressed as μmol 4-methylumbelliferone min^{-1} mg^{-1} protein. Error bars indicate SD ($n >$ 4 plants). The letters above the bars indicate significant differences ($P <$ 0.01).

($P < 0.01$). Similarly, transformants expressing $Pr\text{-}D_{-1079}$:*GUS* that included part of the transposon sequence also yielded significantly higher GUS activity than transgenic lines containing wild-type pr_{-1061}:*GUS* construct (Fig. 7B). These results indicate that the allelic alteration at the upstream regulatory region of the mutant allele enhances the promoter activity of the *Pr* gene in activating its expression.

DISCUSSION

The *Pr* gene of cauliflower confers anthocyanin production in otherwise anthocyanin nonaccumulating tissues, such as curds and seed endosperms, turning them purple. Here we report the successful isolation of the *Pr* gene via a combination of candidate gene analysis and fine mapping. The *Pr* gene was found to encode a R2R3 MYB protein. R2R3 MYB transcription factors belong to a large protein family (Stracke et al., 2001; Allan et al., 2008). In Arabidopsis, MYB transcription factor subgroup that activates anthocyanin biosynthesis includes four genes, *PAP1*, *MYB113*, *MYB114*, and *PAP2*, with the last three localized in tandem on chromosome one, an arrangement also seen in grape (Walker et al., 2007). A total of four

different *MYB*-like genes, *BoMYB1*, *BoMYB2*, *BoMYB3*, and *BoMYB4*, were isolated from the cauliflower genome. Three of them, i.e. *BoMYB2*, *BoMYB3*, and *BoMYB4*, appear to be located next to each other. High-resolution mapping and functional complementation in both cauliflower and Arabidopsis clearly confirm the identification of *BoMYB2* as the *Pr* gene. *BoMYB2* appears to be an important MYB transcription factor in regulating anthocyanin biosynthesis in *Brassica* genomes. A previous study of red cabbage varieties also reveals that the constitutive anthocyanin production is associated with an increased expression of only *BoMYB2* among the four *BoMYBs* (Yuan et al., 2009). *BoMYB2* from cauliflower shares 99.6% nucleotide sequence identity with that from cabbage.

Pr and *BobHLH1* as well as a number of late pathway genes including *BoF3′H*, *BoDFR*, and *BoLDOX*, were expressed highly in the *Pr-D* mutant. Introduction of the *Pr-D* allele into wild-type cauliflower specifically increased the transcript levels of *BobHLH1* and the same set late structural genes in the transformants. The up-regulation of *BobHLH1* in both the *Pr-D* mutant and the *Pr-D* transformants strongly suggests that *Pr* regulates the expression of *BobHLH1*. Although expression of *MdMYB10* does not elevate a bHLH transcription factor expression in apple (*Malus domestica*; Espley et al., 2007), *PAP1* has been shown to regulate the expression of *TT8* in Arabidopsis (Baudry et al., 2006). Similarly, *AN2* and *AN4*, the genes encoding MYB transcription factors, activate the bHLH transcription factor of *AN1* in petunia (Spelt et al., 2000). *Pr* and *BobHLH1* likely work together to coordinately regulate several transcripts of anthocyanin late pathway genes in conferring anthocyanin accumulation in the *Pr-D* mutant and transformants.

Despite the fact that MYB and bHLH transcription factors share similar functions among plants, they exhibit species-specific differences in activating part or the entire set of anthocyanin pathway genes. For example, in maize the C1/P1 family of MYB proteins and the R/B family of bHLH proteins activate the entire set of anthocyanin structural genes (Chandler et al., 1989; Ludwig and Wessler 1990; Cone et al., 1993; Grotewold et al., 1994). In petunia and Arabidopsis, these two families of proteins control a subset of structural genes (Quattrocchio et al., 1993; Spelt et al., 2000; Gonzalez et al., 2008). Like the later cases, BoMYB2 and BobHLH1 regulated a subset of anthocyanin structural genes in inducing tissue-specific expression in the *Pr-D* mutant. However, how *Pr* exhibits its unique tissue-specific regulation of anthocyanin accumulation remains to be determined.

The activity of *MYB*-like genes has been suggested to be the primary cause of natural variation in anthocyanin pigmentation in plants (Quattrocchio et al., 1999; Schwinn et al., 2006). Increased expression of R2R3 MYB transcription factors was found to be responsible for anthocyanin production in a number of anthocyanin-accumulating mutants. For example, the constitutive up-regulation of *PAP1*, *ANT1*, and *MdMYB10* causes anthocyanin accumulation throughout the plant in *pap1-D* Arabidopsis (Borevitz et al., 2000), *antl* tomato (*Solanum lycopersicum*; Mathews et al., 2003), and red-fleshed apple (Espley et al., 2007). While overexpression of *PAP1* and *ANT1 MYB* transcription factors is due to activation-tagged insertions in their promoter sequences (Borevitz et al., 2000; Mathews et al., 2003), the high transcript level of *MdMYB10* was recently found to be due to the formation of a minisatellite-like structure comprising multiple repeats of a promoter segment that generates a novel autoregulatory motif (Espley et al., 2009). Unlike these reported anthocyanin-accumulating mutants, the *Pr-D* mutant appears to display a different mechanism in activating *Pr* expression.

The coding regions of *Pr-D* and *pr* shared 99.2% sequence identity and both of them encoded functional proteins. Thus, the sequence variation in the promoter region of *Pr* is likely responsible for the activation of *Pr* in controlling anthocyanin biosynthesis. Indeed, the promoter from wild type and the *Pr-D* alleles showed different capacity in activating GUS expression. For example, although the promoter of the −378/−373 regions of *Pr-D* and *pr* shared nearly identical sequences, significantly higher GUS activity was observed in the transformants expressing the mutant $Pr\text{-}D_{-373}$:*GUS* than the wild-type pr_{-373}:*GUS*. Detailed examination of the $Pr\text{-}D_{-373}/pr_{-373}$ sequences revealed that an insertion of two nucleotides in $Pr\text{-}D_{-373}$ range generated a new regulatory motif, the E-box (5′-CANNTG-3′), which is a cis-acting element with binding consensus site for bHLH proteins (Toledo-Ortiz et al., 2003; Supplemental Fig. S1). Further, both $Pr\text{-}D_{-373}$ and wild-type pr_{-1061} sequences contained one E-box and the transformants expressing these constructs exhibited similar promoter activity. The $Pr\text{-}D_{-1079}$ sequence harbored two additional E-boxes and gave significantly higher promoter activity than wild-type pr_{-1061} sequence (Fig. 7). The transposon insertion in the *Pr-D* allele introduced additional E-box cis-acting elements, which likely provide more binding sites for bHLH transcription factors to activate *Pr* expression, as suggested in a recent study of the DNA transposon *mPing* in rice (Naito et al., 2009). E-boxes are identified in the promoter region of anthocyanin structural genes and have been shown to be required for bHLH transcription factor binding in enhancing gene expression (Shirley et al., 1992; Hartmann et al., 2005). Future experiments will help clarify the involvement of E-box amplification in activating *Pr* expression. Nevertheless, our results reported here clearly indicate that the mutation at the upstream promoter region in the *Pr-D* allele resulted in activating *Pr* gene transcription to confer the phenotypic change in cauliflower. *Pr* exerts profound effect on anthocyanin production in plants. The successful cloning of *Pr* and the discovery of it as primary determinant of color in cauliflower have implications for breeding vegetable crops with enhanced health-promoting properties and visual appeal.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Purple cauliflower (*Brassica oleracea* var *botrytis*) arose from a spontaneous mutation found in a cauliflower field about 20 years ago. A commercial purple cauliflower cultivar Graffiti (Harris Seeds) and a white cultivar Stovepipe as wild-type control were used in this study. Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia was used for genetic transformation. Cauliflower plants were grown either in a greenhouse under 14-h-light/10-h-dark photoperiod at 23°C or in a field. Arabidopsis plants were grown in a growth room under 14-h-light/10-h-dark photoperiod at 23°C.

HPLC-ESI-MS/MS Analysis of Anthocyanins

Anthocyanins in Graffiti was extracted and analyzed following the method as described by Wu and Prior (2005b). Freeze-dried curd sample (0.5 g) was ground into powder and extracted twice in total 25 mL methanol/water/acetic acid (85:15:0.5). The extract was diluted 2-fold. Aliquot (10 μL) was injected into a Zorbax stablebond analytical SB-C18 column (4.6 × 250 mm, 5 μm, Agilent Technologies) and separated using 5% formic acid (A) and 100% methanol (B) as mobile phases on an HP 1100 series HPLC. Low-resolution electrospray mass spectrometry as described (Wu and Prior, 2005b) was performed to identify the major anthocyanin peaks in the purple curd sample. Quantification was carried out based on peak areas and a calibration curve generated with a commercial standard of cyanidin 3,5-diglucoside chloride (INDOFINE Chemical Company).

Nucleic Acid Analysis

Genomic DNA was isolated as described previously (Lu et al., 2006). Total RNA from purple and white cauliflower tissues was extracted using Trizol reagent following the manufacturer's instruction (Invitrogen). mRNA was isolated from total RNA using PolyATtract mRNA isolation system IV (Promega).

For northern-blot analysis, mRNA (2 μg) samples were used. Prehybridization and hybridization with P^{32}-labeled probes were performed as described previously (Li et al., 2001). Probes used included *CHS* (U21762), *CHI* (U20894), *F3H* (U14735), and *LDOX* (YAY780) from Arabidopsis, as well as *F3'H*, *DFR*, *UGT*, and *Actin* amplified from white cauliflower DNA using primers as listed in Supplemental Table S1.

qRT-PCR analysis was conducted according to the requirements and guidelines described by Udvardi et al. (2008). The cDNAs were synthesized from DNase I treated total RNA (5 μg) using Superscript III reverse transcriptase (Invitrogen). qRT-PCR was carried out using SYBR Green PCR master mix following the manufacturer's instruction (Applied Biosystems) in an Applied Biosystems 7900HT fast real-time PCR system. Gene-specific primers, which were confirmed to produce specific gene products by sequencing (Yuan et al., 2009), are listed in Supplemental Table S1. The relative transcript levels were calculated as described previously (Lyi et al., 2007).

PCR walking on genomic DNA was performed using the Universal GenomeWalker kit following the manufacturer's instruction as described previously (Li and Garvin, 2003). Wild-type and mutant cauliflower genomic DNA (2.5 μg) were digested with *Hin*cII, *Rsa*I, *Sma*I, *Stu*I, *Swa*I, *Dra*I, *Eco*RV, *Pvu*II, *Sca*I, and *Ssp*I, respectively, and ligated to the GenomeWalker adaptors to produce Genome walking libraries. PCR products of secondary nested reactions were cloned into pCR2.1 vector (Invitrogen) and sequenced.

Association Mapping of the Candidate Genes

DNA of the parents and 1,898 F2 individuals of the mapping population were extracted (Lu et al., 2006). To design PCR-based markers, the candidate genes from both mutant and wild-type plants were isolated. Based on insertions and deletions or single nucleotide polymorphisms in the two alleles of these genes, primer sets for *BoMYB2*, *BoMYB3*, and *BoMYB4* markers were developed (Supplemental Table S1). $BoMYB2_m$ and $BoMYB4_m$ were codominant markers and $BoMYB3_m$ was dominant marker. Genetic linkage map was generated using MapMaker (www.broadinstitute.org/ftp/distribution/software/ mapmaker3).

Sequence Analysis

DNA and protein sequences were analyzed using the program of DNAStar (Lasergene). Multiple sequence alignments were produced by Vector NTI (Invitrogen) or CLC sequence viewer using default setting. The phylogenic tree was calculated by DNAStar and visualized by Treeview version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/ treeview.html).

Plasmid Construction and Plant Transformation

To create the constructs for phenotypic complementation, genomic DNA of *Pr* including 1,755 or 1,061 bp of promoter sequences for the mutant and wild-type allele, respectively, were amplified. Despite of extensive efforts, the upstream above 1,061 bp promoter sequence for wild-type allele could not be obtained due to highly repeated sequence and was not found from public sequence databases. The amplified products were cloned into pCAMBIA 1300 vector (CAMBIA) with a nopaline synthase terminator to produce pr_{pro}:*pr* and Pr_{pro}:*Pr-D* constructs. To generate the overexpression constructs, comparable genomic DNA from start to stop codon of both *Pr* alleles were amplified and inserted into pCAMBIA 1300S containing CaMV 35S promoter (Zhou et al., 2009) to produce $35S_{pro}$:*pr* and $35S_{pro}$:*Pr-D*. To create the GUS constructs, region of wild-type *pr* promoter (−378 and −1061 bp), and *Pr-D* promoter (−373 and −1,079) were amplified and inserted into pSG506 vector (kindly provided by Dr. Susheng Gan, Cornell University). The promoter fragments were selected based on transposable element insertion site. The fragments were then subcloned into pCAMBIA 1300 to produce various promoter-GUS constructs. All constructs were verified by sequencing. The constructs and vector-only controls were electroporated into *Agrobacterium tumefaciens* strain GV3101, and transformed into wild-type Arabidopsis using a flower-dipping method (Clough and Bent, 1998), and into cauliflower using *A. tumefaciens*-mediated tissue culture method (Lu et al., 2006).

GUS Activity Analysis

Quantitative analysis of GUS activity in transformants expressing different GUS constructs was carried out using fluorometric assay (Blazquez, 2007) with slight modification. Four-week-old Arabidopsis leaf tissues (25 mg) from at least four independent transgenic lines for each construct were extracted independently in 100 μL GUS extraction buffer. Total proteins in the crude extract were quantified using the RC DC protein assay kit (Bio-Rad). Extract (5 μL) was then added to 450 μL GUS extraction buffer containing 1 mM 4-methylumbelliferyl β-D-glucuronide and incubated at 37°C. Aliquot of the reaction mixture (20 μL) was added into 180 μL stop solution (1 M sodium carbonate) every 10 min. Fluorescence was then measured using fluorometer Fluorolite 1000 (DYNEX technologies) at excitation wavelength of 365 nm and emission wavelength of 450 nm. The analysis was repeated three times. The mean values for each construct were compared.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers GU219986 ($BoMYB_{Pr-D}$), GU219987 ($BoMYB2_{pr}$), GU219985 (*BoMYB1*), GU219988 (*BoMYB3*), GU219989 (*BoMYB4*), and GU219990 (*BobHLH1*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Nucleotide sequence comparison of the promoter region at −378/−373 bp of the wild type and mutant *Pr* gene.

Supplemental Table S1. List of primers used in this study.

Supplemental Table S2. List of the GenBank accession numbers of the proteins used in the phylogenic tree of Figure 4C.

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