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Identification and characterizations of *P1-wr* epialleles in maize that show a gain in pericarp function

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Homologous recombination mechanisms at *p1* have been proposed to be involved in the allelic diversity at *p1*. For example, the *P1-wr* allele has six tandemly-repeated gene copies, and is thought to be derived by gene duplication events from an ancestral allele. However, the *P1-wr* may be refractory to recombination since ears with pericarp pigmentation sectors are not present in *P1-rr/P1-wr* X *p1-ww* screens (Brink, Annu. Rev. Genet. 7:129-152, 1973; Chopra et al., Mol. Gen. Genet. 260:372-380, 1998). Toward this objective, a similar screen was performed to identify recombination events at *P1-wr* [W23]. In place of the wild type *P1-rr* allele, a loss of function mutant allele called *p1-ww-10:443-3* (Athma and Peterson, Genetics 128:163-173, 1991) was used. This approach allows for the easy identification of hypothetical *P1-wr/ p1-ww-10:443-3* recombinants exhibiting a gain of pericarp pigmentation.

However, we show by PCR and Southern analysis that recombination did not occur between these two alleles. Nevertheless, three heritable gains of pericarp pigmentation epialleles were identified and are characterized herein.

Genetic screen for gain of pericarp pigmentation. An intragenic transposition of *Ac* from *P1-ovov-1114* produced the *P1-vv*-83934* allele. The *P1-vv*-83934* has an 8-bp target duplication within the *p1* reading frame (Athma and Peterson, 1991). The excision of *Ac* from the *P1-vv*-83934* engendered a frame shift mutant allele called *p1-ww-10:443-3* (Grotewold et al., Proc. Natl. Acad. Sci. USA 88:4587-4591, 1991). The standard *p1* alleles used in this study are *p1-ww* [4co63], *P1-wr* [W23], and *P1-rr-4B2*. The *P1-rr-4B2* allele was introgressed into the W23 background by over six generations of backcrossing.

The *p1-ww-10:443-3* was crossed with *P1-wr* [W23] and the F1 progeny plants were then crossed with *p1-ww* [4co63] (Figure 1).

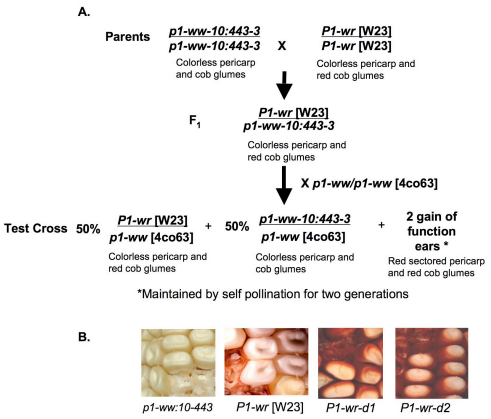


Figure 1. Gain of pericarp pigmentation crossing scheme used to generate new *P1-wr* epialleles. **A.** Diagram showing crosses performed for a gain of pericarp pigmentation screen. This screen generated two epialleles of *P1-wr* [W23] called *P1-wr-d1* and *P1-wr-d2* (see Figure 1D). **B.** Pericarp and cob glume pigmentation of *P1-wr-d1* and *P1-wr-d2* are shown. The phenotypes of the *P1-wr* [W23] and *p1-ww:10-443* parents that were used in this screen is also shown. (For full color, see p. 34.)

Approximately 80,000 test cross ears were open-pollinated and examined for a gain of pericarp pigmentation. Two parental ears produced progeny exhibiting kernel sectors of red pericarp pigmentation. F1 kernels from the red and colorless sectors were planted and the progeny was self-pollinated. The F2 progeny plants were self-pollinated and out-crossed to *p1-ww* [4co63]. Following this, there were two additional generations of self-pollination. During each generation, the pigmentation phenotype was examined on both the pericarp and cob for each allele.

DNA gel blot analysis. Seedling genomic DNA was prepared using a modified CTAB method (Saghai-Maroof et al., Proc.Natl. Acad. Sci. USA 81:8014-8018, 1984). PCR genotyping of the genomic DNA was done with standard conditions using primers listed in Table 1. Restriction digestion was achieved by using enzymes, reagents and protocols from Promega (Madison, WI). Restricted genomic DNA was fractionated on 0.8 % agarose gels and subsequently transferred to nylon membranes. Membranes were pre-hybridized for four hours and then hybridized for 15 h at 65°C in buffer containing NaCl (1 M), SDS (1%), Tris-HCl (10 mM) and 0.25 mg/ml salmon sperm DNA (Athma and Peterson, 1991). The *p1* probe fragments used include F8B, F8C, F13, and F15, and have previously been described (Chopra et al., 1998; Lechelt et al., Mol. Gen. Genet. 219:225-234, 1989; Sekhon et al., 2007) Blots were stripped of previous signal by boiling in 0.1% SDS before they were reused.

Table 1.

Primer Name	Sequence	Purpose	*P1* Alleles Amplified	Product Size
MRF	5'TGGAGCTCTTGCGTATCTAACGCT 3'	Genotyping	*P1-vv, P1-rr-4B2, p1-ww:10-443*	[a]481 bp
MRR	5' AGTGTGCACAGGGACACTTGAGTA 3'			
WRJ	5' CTGTCGGCTACTATCCCCTTGGTGA 3'	Genotyping	*P1-wr-d1, P1-wr-d2, P1-wr* [W23],	618 bp
WRK	5' GATCGCGAGCTGGAGGCGTTCGAGAC 3'			

[a]Another ~900 bp product was also amplified in all genotypes (*P1-vv, P1-rr-4B2, p1-ww:10-443, p1-ww* [4co63], *P1-wr* [W23], *P1-wr-d1,* and *P1-wr-d2*), but its sequence had only a limited homology with *p1* at its 5' end.

Pigmentation patterns of the gain-of-pericarp-function alleles. The two new *p1* alleles, called *P1-wr-d1* and *P1-wr-d2*, were discovered in a phenotypic screen as red kernel sectors on *P1-wr* [W23]*/ p1-ww-10:443-3* X *p1-ww* [4c063] pericarp (Figure 1). The red phlobaphene pigmentation on both alleles was limited to the silk attachment point and kernel gown. In subsequent generations, the pericarp pigmentation was no longer sectored; it instead encompassed the entire ear (Figures 1 and 2). However, there were some instances of ears with mosaic sectors on the pericarp gown (see second ear from left in Figure 2A). Variable pericarp pigmentation intensities and patterns were observed for both alleles. For example, some ears only had silk scar pigmentation, while other ears only had gown pigmentation (Figure 2). Furthermore, some ears had very low pigmentation levels that resembled *P1-wr.* Since the background of the stocks containing these alleles is heterogeneous, it might be that the variability in pigmentation levels depends on the genetic constituency of modifier alleles.

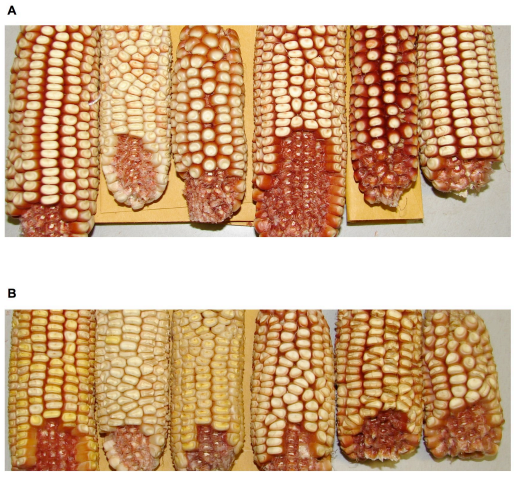
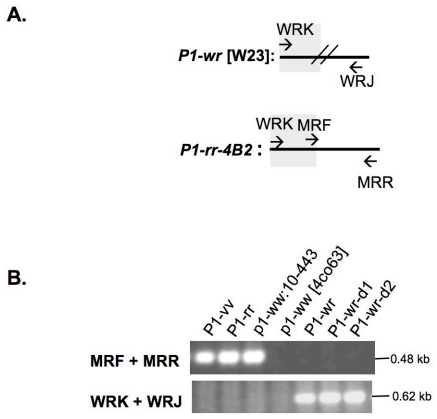


Figure 2. Pigmentation patterns and intensities in *P1-wr* [W23] epialleles. Ears show pericarp and cob glume pigmentation of **A.** *P1-wr-d1* and **B.** *P1-wr-d2.* Note that ectopic pericarp pigmentation can occur at the silk attachment point and/or kernel gown. (For full color, see p. 34.)

Gain-of-function *pericarp color1* alleles share an identical gene structure with *P1-wr* [W23]. Genomic DNA of the two gain of function alleles was compared by PCR genotyping with *p1-ww-10:443-3* and *P1-wr* [W23] (Figure 3). The RRF and RRR primer pair was used to amplify a 481 bp region in *p1-ww-10:443-3* (between 6349 and 6830 of accession Z11879) that is located in exon 3 and includes the 3'UTR and some downstream sequence (Figure 3A). The RRF and RRR primer pair does not amplify the 481 bp region in *p1-ww* [4co63] or *P1-wr* [W23], but does yield another ~900 bp product that is also present in *p1-ww-10:443-3.* The *P1-wr* genomic sequence of a *P1-wr* [W23] (accession EF165349) does not contain the 481 bp region, whereas the origin of the ~900 bp product has only a limited homology with *p1.* Conversely, the WRJ and WRK primer pair amplifies a 618 bp band in *P1-wr* [W23] that is located in the 3'UTR and downstream sequence (Figure 3A). The WRJ and WRK primer pair does not amplify *p1-ww-10:443-3* or *p1-ww* [4co63]. The *P1-wr-d1* and *P1-wr-d2* alleles had an amplification pattern identical to that of *P1-wr* [W23] (Figure 3B). Conversely, no evidence of the presence of sequence originating from *p1-ww-10:443-3* was found.



3. Molecular comparison of *P1-wr-d1* and *P1-wr-d2* with standard *p1* **A.** Diagram showing the 3' ends of *P1-wr* [W23] and *P1-rr-4B2.* The n of PCR primers used for genotyping is shown. The grey shaded represent the end of the gene sequences (i.e. exon 3). In the case of [W23], the sequence 3' of the shaded box is the distal promoter of the ream copy. The hash marks in *P1-wr* [W23] signify the junction n two repeated copies. Note that the MRF and MRR primers are not t in *P1-wr* [W23] and the WRJ primer is not contained in *P1-rr-4B2.* **B.** nalysis was done to compare *P1-wr-d1* and *P1-wr-d2* alleles with the parental *p1* alleles used in the gain of pericarp pigmentation screen (genetic screen is shown in Figure 1). Based on the amplification patterns, the *P1-wr-d1* and *P1-wr-d2* alleles could be classified as similar to *P1-wr* [W23].

To identify gene structural differences that may have led to the gain of function in pericarp tissue we assayed *P1-wr-d1* and *P1-wr-d2* by DNA gel blot analysis (Figure 4). Seedling genomic DNA was digested with ten restriction enzymes and hybridized with different *p1* homologous probes. This analysis revealed that *P1-wr-d1* and *P1-wr-d2* had an identical tandem-repeat gene structure with *P1-wr* [W23] (Figure 4A and B). Moreover, when the distal enhancer region of *p1* was examined, there were no *Sal*I fragments that resemble *P1-rr* (Figure 4C and D). Rather the bands (12.5kb and 1 kb) resemble the pattern expected for *P1-wr.* Additional results showed that there were no diagnostic *p1*-F15 *Sal*I fragments that resembled *P1-rr* (Figure 4C and D). Rather the bands (12.5kb and 1 kb) showed the pattern expected for *P1-wr.* Collectively, these results suggest that *p1-ww-10:443-3* has segregated from *P1-wr* and has not recombined with *P1-wr.* Hence, this data suggested that the gain of gown pigmentation in *P1-wr-d1* and *P1-wr-d2* may have arisen by spontaneous small nucleotide polymorphisms or epimutations in *P1-wr* [W23]

***P1-wr-d1* and *P1-wr-d2* are hypomethylated epialleles of *P1-wr* [W23].** Since *P1-wr-d1* and *P1-wr-d2* had no genetic differences from *P1-wr* [W23], we considered the possibility that epigenetic differences may have led to the gain of function in pericarp tissue. Hence, DNA gel blot analysis was used to construct a DNA methylation map comparing *P1-wr-d1, P1-wr-d2,* and *P1-wr* [W23] (Figures 5). We detected partial hypomethylation at the distal floral organ enhancer as evidenced by the diagnostic 500 bp F15-homolgous *Hpa*II band (Chopra et al., Genetics 163:1135-1146,

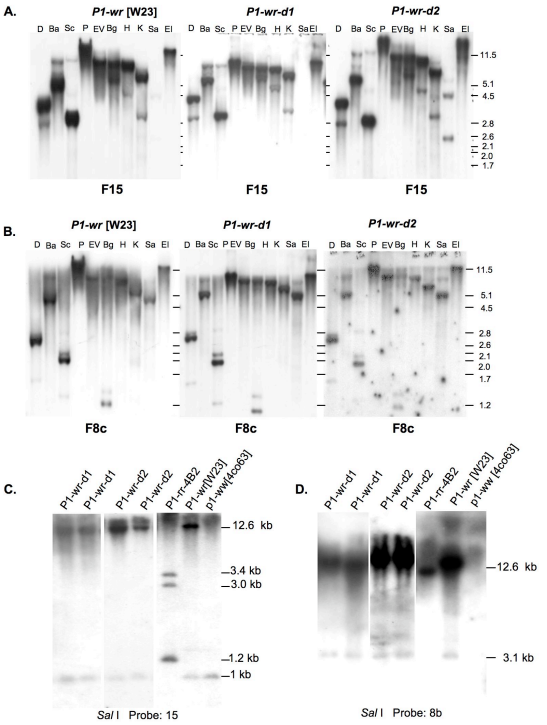
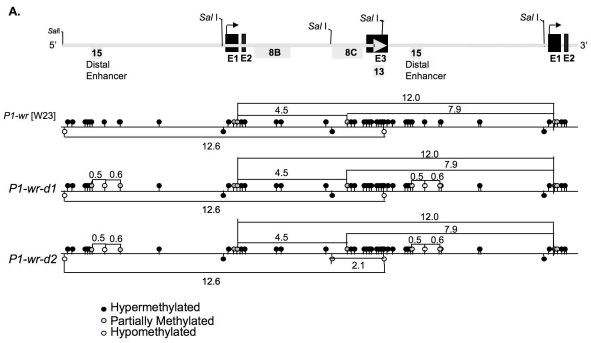


Figure 4. Structural comparison of *P1-wr-d1* and *P1-wr-d2* with *P1-wr* [W23] and *P1-rr-4B2*. Gene structure blots were made by digesting seedling leaf DNA with ten diagnostic restriction enzymes. Enzyme names are abbreviated as follows: D, *Dra*I; Ba, *Bam*HI; Sc, *Sca*I; P, *Pst*I; EV, *Eco*RV, Bg, *Bgl*II, H, HindIII; K, *Kpn*I; Sa, *Sac*I; EI, *Eco*RI. Blots were hybridized with *p1* probes corresponding with **A.** the distal floral organ enhancer (F15) and **B.** intron 2 (F8C). The blots in **C** and **D** show *Sal*I digested DNA that has been hybridized with distal enhancer probe F15 and intron 2 probe F8B, respectively. For a diagram showing the location of these probes in *P1-wr* [W23] refer to Figure 5. The sizes of molecular weight marker bands are indicated in kilobase pairs to the right of the blots.



B.

DNA methylation as compared with *P1-wr*			
	Regions of *P1-wr*		
Allele	Distal Enhancer	3' of Transcription Start Site	*Sal* site in intron 2
P1-wr-d1	Slightly hypomethylated	No difference	No difference
P1-wr-d2	Slightly hypomethylated	No difference	hypomethylated

Figure 5. *P1-wr-d1* and *P1-wr-d2* are hypomethylated epialleles of *P1-wr* [W23]. **A.** *P1-wr* [W23] was used as a template to construct the DNA methylation map for *P1-wr-d1* and *P1-wr-d2*. The intron/exon structure of *P1-wr* is provided on a line diagram above the methylation maps. The large grey

arrow on the line diagram represents the end of a copy in the tandem array. The bent arrows indicate the location of the transcription start sites. Exons are abbreviated as E1, E2, and E3. The placement of *p1* probes (grey shaded boxes) is shown immediately below the line diagram. DNA methylation maps are shown below the *P1-wr* [W23] gene structure. On the DNA methylation maps, black circles indicate hypermethylated sites; grey circles indicate partially-methylated sites; non shaded circles represent hypomethylated sites. **B.** Summary of the DNA methylation changes in *P1-wr-d1* and *P1-wr-d2* relative to that of *P1-wr* [W23].

2003) (Figure 6A). However, the presence of high molecular weight bands such as a 7.9 kb *Hpa*II band indicates that most copies remained hypermethylated at the distal enhancer region (Figure 5A).

At the intron 2 region of *p1,* we did not observe any differences using *Hpa*II digestion; however, differences were detected using *Sal*I (Figure 6B). Digestion with *Sal*I in *P1-wr* [W23] produces a 12.6 kb band that extends the entire length of the gene. This is because two of the three *Sal*I sites in *P1-wr* [W23] are hypermethylated. Interestingly, in *P1-wr-d2* we detected an additional 2.1 kb band which suggested that a *Sal*I in intron 2 (site 10,310 of *P1-wr* [W23] accession EF165349) was partially hypomethylated (Figure 5). This difference (2.1 kb band) was not observed in *P1-wr-d1* and therefore can be used to differentiate between the two epialleles (Figure 6B).

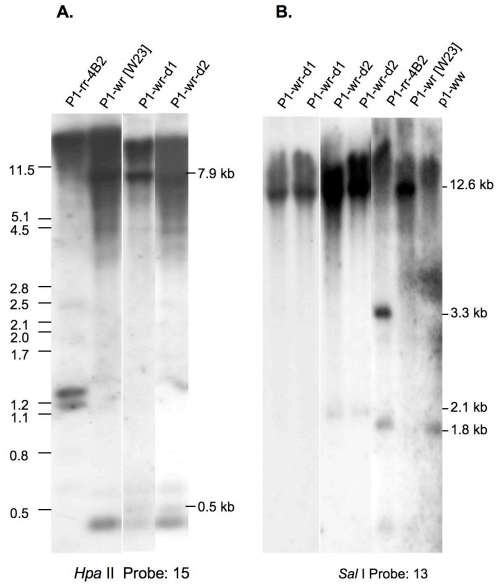


Figure 6. DNA gel blot showing methylation differences between *P1-wr* [W23] and its derived epialleles. **A.** Gel blots showing *Hpa*II digested genomic DNA hybridized with the *p1* distal enhancer probe 15. **B.** Gel blots showing *Sal*I digested genomic DNA hybridized with *p1* exon 3 probe 13. These and other blots were used to construct the DNA methylation maps for the *P1-wr-d1* and *P1-wr-d2* epialleles shown in Figure 5.

Notably, several *P1-wr* alleles from R. A. Brink's collection which have pericarp pigmentation confined to the kernel gown are also partially hypomethylated at this distal floral enhancer (Brink and Styles, MNL 40:149-160, 1966; Cocciolone et al., Plant J. 27:467-478, 2001). It was thus suggested that DNA hypomethylation was important for the gain of pericarp function (Cocciolone et al., 2001). However, Brink's *P1-wr* alleles were collected from numerous genetic sources and therefore would likely have subtle genetic differences such as SNPs. Herein, this gain of function screen employed *P1-wr* [W23], and hence, the resulting alleles are likely genetically identical to *P1-wr* [W23]. Therefore, these results help strengthen the theory that an epimutation in *P1-wr* can lead to the presence of silk scar and gown pigmentation.