Summary Statement: The ultimate aim of our work is to understand how a regulatory gene produces a specific pattern of gene expression during plant development. Our model is the P-wr gene of maize, which produces a distinctive pattern of pigmentation of maize floral organs. We are investigating this system using a combination of classical genetic and molecular approaches. Mechanisms of organ-specific gene expression are a subject of intense research interest, as it is the operation of these mechanisms during eukaryotic development which determine the characteristics of each organism. Allele-specific expression has been characterized in only a few other plant genes. In maize, organ-specific pigmentation regulated by the R, B, and Pl genes is achieved by differential transcription of functionally conserved protein coding sequences. Our studies point to a strikingly different mechanism of organ-specific gene expression, involving post-transcriptional regulation of the regulatory P gene. The novel pigmentation pattern of the P-wr allele is associated with differences in the encoded protein. Furthermore, the P-wr gene itself is present as a unique tandemly amplified structure, which may affect its transcriptional regulation.

Listed Scientific Contributions:
1. Determined the transcription profile of P-wr and its target genes C2, Chi, and A1 during pericarp and cob glume development. The target genes are not strongly activated in P-wr pericarps despite the presence of P-wr transcripts.
2. Demonstrated that P-rr and P-wr transcripts can be detected by in situ hybridization in all major cell types of the pericarp. This result shows that the colorless pericarp phenotype of P-wr is not caused by restricted localization of P-wr transcripts to cell types not competent to synthesize phlobaphene pigments.
3. Found that P-wr cDNA clones isolated from pericarp and cob glumes are identical. This result rules out the possibility that organ-specific pigmentation is achieved by cell-specific transcription or splicing.
4. Identified three coding sequence differences between P-rr and P-wr cDNAs. Two single nucleotide changes would produce single amino acid differences between P-rr and P-wr proteins. The third change is a major replacement of 3' end sequences, which would result in different COOH termini of P-rr and P-wr proteins. The unique P-wr COOH terminus contains a possible metal binding motif, which would be absent in the P-rr protein. Identification of these three differences between P-rr and P-wr enables the design of 6 domain-swap constructs (3 single mutant, and 3 double mutant) which can be tested to determine which, if any, protein differences are responsible for organ-specific activation of P-wr.
5. Determined that the P-wr locus contains a unique structure of tandem direct repeats of a 12.6 kb sequence. This novel gene structure was elucidated and confirmed by genomic Southern blotting, YAC clone studies, and genomic clone isolation and sequencing.

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Detailed Results, arranged according to the Specific Aims from original proposal:

1. Characterize the expression of *P-wr* in different organs of the plant, and test the ability of the *P-wr* pericarp and cob transcripts to transactivate the structural genes for pigment biosynthesis.


Northern blot results demonstrate that the *P* transcripts are present (22 days after pollination) in both pericarps and cobs of *P-wr* [W23] genotype, but the *C2* and *A1* genes required for flavonoid biosynthesis are not activated in *P-wr* pericarp, while *Chi*, the third structural gene in the pathway shows an unaltered basal level of transcription. We have also studied the kinetics of accumulation of *P-rr*, *P-wr*, *C2*, *CHI*, and *A1* transcripts in pericarps and cobs from plants of *P-wr* and *P-rr* genotype. Because pericarps and cobs show different levels of accumulation of flavonoid compounds at different stages of development (Styles and Ceska, 1989), these kinetic data provide more precise definition of the timing of transcription of both *P-wr* and *P-rr*, and their target genes. The three structural genes are highly transcribed in *P-rr* pericarps during 0 to 18 DAP period, while cob glumes show a poor rate of transcription during the same time. Compared to *P-rr*, the pericarps of *P-wr* show nearly negligible rate of transcription for the three structural genes during 5 to 18 DAP. For *P-rr* and *P-wr*, the results of the weak Northern blot signals were confirmed using RT-PCR. These RT-PCR experiments demonstrate that *P* transcript is present in *P-wr* pericarps and cob tissues throughout the developmental profile under study. In the early stages of ear development (0 to 5 DAP) the *P-wr* and *P-rr* messages have similar abundance, while during the latter stages of development (studied until 22 DAP), the *P-wr* pericarps and cobs show lower levels of *P* message as compared to *P-rr* pericarps and cobs. Thus, although *P-wr* transcripts are present during pericarp development, the structural genes for flavonoid biosynthesis are not highly activated as in *P-rr*.

B. in situ hybridization studies: The pericarp is composed of five different cell types. Although Northern blots show presence of *P-wr* mRNA in pericarp (see above), it is possible that *P-rr* and *P-wr* are expressed in different cell types within in the pericarp. Possibly, the *P-wr* mRNA could be expressed in a subset of pericarp cells in which the flavonoid biosynthetic genes cannot be activated. We tested this by in situ hybridization to compare the cellular distribution of *P-rr* and *P-wr* mRNAs. After optimization of protocols for preparation of maize kernel paraffin sections, in situ hybridizations were done with kernels 20 DAP (days after pollination). Single strand RNA probes from the 5' and 3' ends of the *P* message were used. Kernels of *P-rr* genotype showed strong hybridization to pericarp compared to background levels in endosperm and embryo. Hybridization to antisense RNA probes was detected at both the crown (top) and gown (sides) of kernel pericarp, and was distributed in epidermal through mesocarp cell layers. The *P-ww* allele (deletion of *P* gene) served as a negative control and had no signal in pericarp over background. *P-rr* pericarps also showed some hybridization to the sense probe; because this sense probe hybridization seems to be allele-specific (not observed with *P-ww* pericarps), we speculate that the *P-rr* gene may produce some antisense RNA. In situ hybridizations of kernels of *P-wr* genotype showed that no restriction of *P-wr* message to a subset of pericarp cell types. This result clearly disproves the hypothesis that *P-wr* is expressed in a subset of pericarp cells which are unable to produce phlobaphene pigmentation.

2. Use *P* antibodies to determine whether the *P-wr* messages are translated into proteins, and whether the protein products are post-translationally modified in an organ-specific manner.

Antibodies raised against *P-rr* recombinant protein (courtesy Erich Grotewold, Cold Spring Harbor labs) detect, on Western blots, a band of approximately 42 kD in *P-wr* pericarp and cob extracts; a less-abundant
band is visible in P-rr extracts (not shown). However, a band of similar size is present at high levels in extracts of the negative control, P-ww-1112, which has a deletion of the entire P gene (Atha and Peterson, 1991). One problem with these results is that the antibodies used are polyclonal antibodies against the P gene Myb-homologous domain, and it is possible that they cross-react with other maize Myb-homologs such as Pl. For this reason, and to distinguish P-wr proteins from P-rr proteins, we have recently raised antibodies against the unique C-terminal region of the P-wr protein (WR61). Characterization of P-encoded proteins is continuing.


We cloned P-wr-specific cDNAs using a PCR-based approach, beginning with the 3' end Rapid Amplification of cDNA Ends protocol (RACE; Frohman et al., 1988). Oligonucleotide EP5-2 (poly dt-adaptor) was first used to reverse transcribe 1 µg of polyA+ RNA followed by the second strand synthesis and PCR amplification using oligo couple EP5-4 (adapter) and EP5-16 (in the P-rr region homologous to P-wr). This reaction yielded the 3' end of P-wr cDNA from pericarps and cobs. Based on the sequence information from the 3' end sequence, oligonucleotide SC0-9 (unique to P-wr sequence) was synthesized and used along with oligo EP5-8 to amplify the middle and 5' end of the P-wr cDNAs. Primer extension followed by 5'RACE protocol has provided the 5' end from the start of transcription. PCR reactions were performed at least twice to confirm results. PCR products were subcloned in plasmid pBluescript II, and several independent clones were sequenced from each region.

To confirm our isolation of P-wr cDNA, we have also isolated genomic clones from the P-wr locus, which contains approximately 5-fold repetition of 12.6 kbp of sequences found in single copy in the P-rr gene. The entire 12.6 kbp P-wr repeat was sequenced from four recombinant phage clones containing overlapping P-wr segments. The P-wr repeat contains all of the P-rr coding sequences, except for a 700 bp fragment from the 3' end of P-rr (fragment 14) which is replaced by a new sequence of 210 bp (WR61) in the P-wr locus. In addition to repetition of the P-rr coding region, the untranscribed 5' and 3' P-rr sequences are also repeated in P-wr. The cDNA and genomic sequences are 100% identical emphasizing that a single type of gene sequence is present and transcribed in both pericarps and cob glumes. Finally, RFLP mapping of a segregating population shows that the P-wr phenotype is linked to the amplified structure of P-wr.

Several pieces of evidence allow us to conclude that our clones represent the P-wr gene and its cDNA. First, a DNA fragment from the P-wr cDNA 3' end (WR61) detects the same intense bands on a genomic Southern blot as seen with P-rr probes 13 and 15. Second, P-rr probe fragment 14 does not hybridize to the P-wr intense band on genomic Southern blots, nor is it present within the P-wr cDNA clone sequence. Third, the P-wr cDNA clone sequence is consistent with the P-wr restriction map derived from genomic Southern blots. And fourth, the P-wr genomic and cDNA nucleotide sequences are identical. Interestingly, the P-wr and P-rr cDNA sequences have certain differences. Both P-wr and P-rr cDNA have conserved cap addition site and complete sequence similarity in the 320 bp upstream to the start of translation. Likewise, the Myb and the putative transcriptional activation domains of P-wr and P-rr are identical. There are only two sequence differences at the 5' end: the fourth AA residue is changed from threonine in P-rr protein to alanine in P-wr protein, and the 264th residue is changed from alanine in P-rr to glutamate in P-wr. The most striking difference is at the 3' end, where P-wr has a shorter sequence relative to P-rr. The P-wr 3' end translation product has some homology to a zinc finger protein motif, which would be absent in the P-rr-encoded protein.
4. Use microprojectile bombardment to test the functionality of the \( P-wr \) pericarp and cob transcripts. We have initiated functional tests of \( P-wr \) cDNAs using microprojectile bombardment for transient assays of \( P \) function in pericarp and cob glume cells. For these tests, a reporter construct (\( A1::GUS \); the promoter of the \( A1 \) gene driving the GUS reporter gene) is co-bombarded together with an effector construct (\( 35S::P-rr, \ 35S::P-wr, \) and \( 35S::P-rrwr^* \)). The plasmid construct \( 35S::P-rrwr^* \) has the 3' end of \( P-rr \) replaced with the unique 3' end of \( P-wr \) using the common restriction enzyme site BstEI, thus keeping the rest of the \( P-rr \) sequence 5' to the unique \( P-wr \) region. We started with this construct because we were interested to see if this unique region functions differentially in pericarp and cob tissues of \( P-wr \) genotype W-23. Our transient expression experiments using different maize tissues have shown the following results:

<table>
<thead>
<tr>
<th>Plasmid/Tissue</th>
<th>( P-rr )</th>
<th>( P-wr )</th>
<th>( P-ww )</th>
<th>BMS cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1::GUS</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>A1::GUS // 35S::P-wr</td>
<td>+</td>
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<tr>
<td>A1::GUS // 35S::P-rr</td>
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<tr>
<td>A1::GUS // 35S::P-rrwr</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>35S::GUS</td>
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<td>+</td>
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</table>

'+' and '-' denotes presence and absence of GUS activity respectively as seen by histochemical staining for GUS enzyme. Pe = pericarp, Cb = cob glumes, // = co-bombarded with.

No GUS expression was observed when A1::GUS was shot alone, possibly because in these 15 DAP (days after pollination) kernels the endogenous \( P-rr \) or \( P-wr \) proteins are present at levels too low to allow activation of the \( A1 \) promoter sequences bombarded into the cells. Bombardment of the \( A1::GUS \) reporter construct into 0 to 5 DAP kernels of does give \( P \)-dependent transactivation of the \( A1::GUS \) construct. Interestingly, the qualitative results obtained with co-bombardment of A1::GUS with either of the three effector plasmids are similar. The function (transactivation of A1 promoter) has been observed in BMS cell suspensions, and pericarp and cob tissues of \( P-rr \), \( P-wr \) and \( P-ww \) genotypes. This shows that (1) the isolated \( P-wr \) cDNA is functional, (2) a chimeric protein containing the N terminus of \( P-rr \) and the C terminus of \( P-wr \) is functional.

5. Analyze, by genetic and molecular methods, four \( P-ww \) mutants derived from \( P-wr \) to characterize the defect in each mutant. We studied several \( P-ww \) mutants derived from \( P-wr \) lines after tissue culture (in collaboration with Shawn Kaeppler, U. Nebraska). Two of the \( P-ww \) mutants (one derived from B73, and the other from A634) have lost most or all of the amplified \( P-wr \) structure present in the parent line. A third tissue-culture derived \( P-ww \) mutant has retained the \( P-wr \) amplified region, but it appears to be hypermethylated (Shawn Kaeppler, personal communication). Finally, the recombinant inbred line RI 53 (from Ben Burr's T232 x CM37 population) is phenotypically \( P-ww \), but it has the intense Southern blot bands characteristic of \( P-wr \). However, digestion with methylation-sensitive restriction enzymes indicates that the \( P-wr \) locus is hypermethylated in this mutant.