Hierarchical Patterns of Transgene Expression Indicate Involvement of Developmental Mechanisms in the Regulation of the Maize *P1-rr* Promoter

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ABSTRACT

The maize *P1-rr* gene encodes a Myb-homologous transcription factor that regulates the synthesis of red flavonoid pigments. Maize plants transformed with segments of the *P1-rr* promoter driving a *GUS* reporter gene exhibit significant variation in transgene expression, both between independent transformation events and among sibling plants derived from a single event. Interestingly, variability in spatial expression is not random; rather, transgene activity occurs predominantly in five patterns that fit a hierarchy: expression is most common in kernel pericarp, with sequential addition of expression in cob glumes, husk, silk, and tassel. The hierarchical expression pattern of P-rr::GUS transgenes suggests a possible model for developmental regulation of the *P1-rr* gene. Our results demonstrate that variability in transgene expression, a common occurrence in transgenic plant studies, can be informative if adequately analyzed to uncover underlying patterns of gene expression.

PLANT transformation is an important tool for basic research and is an essential component of biotechnology-based crop improvement. Transformation technologies have been used to design plants able to withstand insect attack, herbicide application, and viral and fungal diseases. In addition to improving agronomic value, plants are being engineered to produce modified plant products and recombinant proteins for pharmaceutical and industrial applications. The use of transgenic plants for analytical research and commercial production requires accurate and consistent transgene expression, *i.e.*, expression in the correct tissues, at the proper time, and at the appropriate levels. Unfortunately, variability in transgene expression is fairly ubiquitous and can include variation in expression levels, spatial distribution, stability (silencing), or heritability.

We have utilized plant transformation to analyze organ specificity and enhancer activity of regulatory elements present in the maize *P1-rr* gene promoter (Sidorenko *et al.* 2000; and data presented here). *P1-rr* is an allele of the maize *p1* gene that encodes a Myb-homologous transcription factor that regulates the synthesis of red flavonoid pigments, phlobaphenes, primarily in floral organs (Styles and Ceska 1989; Grotewold *et al.* 1991, 1994). Alleles of the *p1* gene confer distinct patterns of floral pigmentation, which are most conspicuous in the pericarp (outermost layer of the kernel derived from the ovary wall) and cob glumes (bracts subtending the kernel). Plants carrying a *P1-rr* allele have *r*ed pericarp and *r*ed cob glumes.

Maize plants were transformed with three constructs containing *P1-rr* upstream regulatory sequences fused to the maize *adhI* intron I and the bacterial *uidA* (GUS) gene, via particle gun bombardment of type-II callus derived from immature maize embryos (Sidorenko *et al.* 2000). These P-rr::GUS constructs differ in upstream regulatory sequences fused to a common *P1-rr* basal promoter plus 5′ untranslated leader sequence (−235 to +326; Figure 1). The P1.0b::GUS and P1.2b::GUS constructs contain a 1.0-kb proximal enhancer region (−1252 to −236), and a 1.2-kb distal enhancer region in reverse orientation (−6110 to −4841), respectively. These two regions were initially identified by transposon mutagenesis (Moreno *et al.* 1992) and were later functionally defined as transcriptional enhancers in transient expression assays (Sidorenko *et al.* 1999) and stable transgenic plants (Sidorenko *et al.* 2000). The third construct, P6.2b::GUS, incorporates the full-length *P1-rr* regulatory region (−6400 to −236) containing both enhancer regions. Sidorenko and co-workers showed that all three transgenic constructs, P1.0b::GUS, P1.2b::GUS, and P6.2b::GUS, directed GUS activity in the same organs pigmented by the endogenous *P1-rr* allele: pericarp, cob glumes, husk, silk, and tassel glumes. However, considerable variation in quantitative levels and spatial patterns of transgene expression was observed, both between independent transformation events and among plants within an event.

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In this article we present extensive analysis of the variation in spatial patterns among P-rr::GUS transgenic plants. Our data indicate that the variability in P-rr::GUS expression in various plant organs is nonrandom and forms a hierarchy. The hierarchical sequence of transgene expression is similar to the order in which these organs are initiated, suggesting a model for developmental regulation of the endogenous *P1-rr* allele.

MATERIALS AND METHODS

Production and analysis of transgenic maize: Transformation of maize with the P-rr::GUS constructs and histochemical analysis for GUS activity in floral and nonfloral organs of T_0, T_1, and T_2 plants was performed as described by Sidorenko *et al.* (2000). Briefly, transgenic maize plants were produced by particle bombardment of immature embryos of Hi-II germplasm and selection for resistance to the herbicide Bialaphos. The P-rr::GUS constructs were cobombarded together with plasmid DP3528, which contains the BAR gene (Deblock *et al.* 1987). Husk, silk, tassel glume, leaf blade, auricle, and sheath samples were collected for staining on the first or second day following silk emergence; pericarp and cob samples were collected 18–20 days after pollination. Early three-leaf stage T_1 seedlings were germinated in paper towel rolls and stained intact. T_0 plants were crossed with one of the following nontransgenic pollen donors of *P1-ww* genotype: Hi-II (Armstrong and Green 1985), inbred line 4Co63, or *P1-ww-1112* stock (Athma and Peterson 1991). T_1 plants were crossed exclusively with the inbred line *P-ww* 4Co63. Maize genomic DNA was prepared and analyzed by DNA gel blots as described by Sidorenko *et al.* (2000).

RESULTS

P-rr::GUS expression patterns resemble not only a *P1-rr* allele, but also *P1-rw* and *P1-wr* alleles in pericarp, cob glumes, and husk: Alleles of the *p1* gene have distinct patterns of pigmentation within maize floral organs and are classified by a two-letter suffix denoting the presence or absence of pigmentation in the pericarp and cob glumes, respectively. Common *p1* alleles include the following: *P1-rr*, red pericarp and red cob glumes; *P1-rw*, red pericarp and white cob glumes; *P1-wr*, white pericarp and red cob glumes; and *P1-ww*, white pericarp and white cob glumes. A similar two-letter code was adopted to score GUS activity in histochemically stained pericarp and cob glume samples from P-rr::GUS transformants. Visible blue staining of both the pericarp and cob glumes was classified as an RR pattern, since it resembles the pigmentation pattern of a *P1-rr* allele (Figure 2A). This was the predominant pattern observed among primary transformants of all three constructs (Table 1), occurring within 3 out of 3 P1.0b::GUS events, 4 out of 7 P1.2b::GUS events, and 13 out of 19 P6.2b::GUS events. Unexpectedly, some plants displayed patterns of GUS activity similar to the pigmentation patterns of *P1-rw* and *P1-wr* alleles (Figure 2, B and C; Table 1). Approximately one-third of the events for each construct contained plants with an RW pattern, while

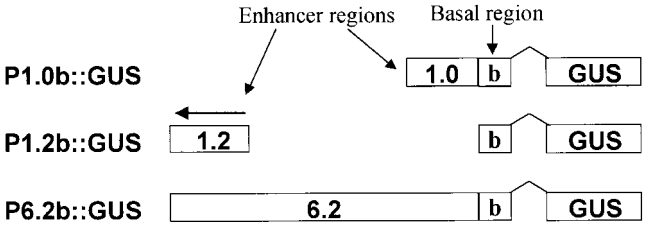

Figure 1.—Schematic of P-rr::GUS constructs. Boxes representing *P1-rr* upstream regulatory regions are labeled 1.0, 1.2 for the enhancer fragments, and 6.2 for the full-length promoter. Thick arrow indicates that the 1.2-kb fragment is in reverse orientation. Boxes b and GUS indicate the *P1-rr* basal promoter and β-glucuronidase coding region, respectively. Bent line represents intron I of the maize *adhI* gene.

the WR pattern (white pericarp and blue cob glumes) was observed in 5–43% of the events. In addition, over half of the events had plants with no GUS activity in pericarp and cob glumes (WW pattern; Figure 2D; Table 1). Husks, specialized leaves at the base of the ear, also exhibited distinct GUS staining patterns that resembled pigmentation patterns of endogenous *p1* alleles, *i.e.*, the uniform pigmentation of *P1-rr* husk and the margin pigmentation of *P1-wr* husk (Figure 2, E and F). Since P-rr::GUS transformants exhibit GUS expression patterns analogous to different *p1* alleles, this suggests that *P1-rr* regulatory sequences are capable of promoting not only the organ specificity of a *P1-rr* allele, but also those of other *p1* alleles.

Clonally derived plants have different patterns of expression: Interestingly, a single transformation event could give rise to plants with different expression patterns. A transformation event is defined as an insertion of single or multiple copies of the transgene construct at one chromosomal location within a single cell, from which multiple plants are clonally derived. Events that had more than one type of expression pattern in pericarp and cob glumes were considered heterogeneous events (example in Figure 2G); whereas homogeneous events consisted of plants with similar expression patterns. Out of 29 combined events of P6.2b::GUS, Pb1.0::GUS, and Pb1.2::GUS, 12 events were heterogeneous; 10 events were homogeneous; and 7 events had no expression in pericarp and cob glumes. Potentially, heterogeneous events could arise from the transformation of more than one cell in a callus piece, leading to the formation of chimeric callus lines from which genetically heterogeneous plants could be derived. To determine if the multiple expression patterns exhibited by heterogeneous events were caused by the production of chimeric callus lines, DNA blot analysis was performed on three to five T_1 progeny plants derived from each transformation event. For all Pb1.0::GUS and Pb1.2::GUS transgenic lines, sibling plants that had different patterns of expression displayed identical banding patterns—indicating that they were derived from

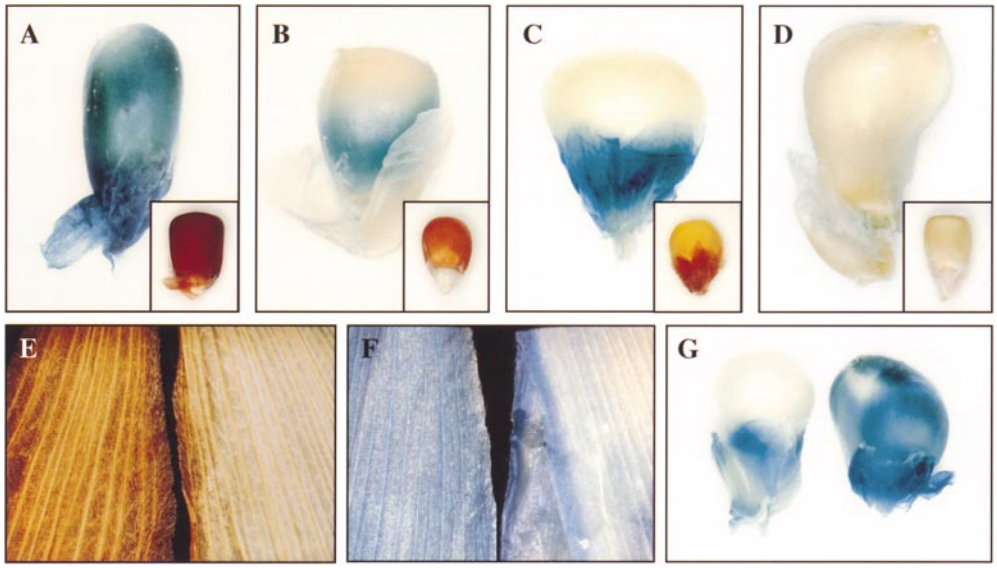

FIGURE 2.—P-rr::GUS expression patterns resemble pigmentation patterns of endogenous *p1* alleles. (A–D) Representative pericarp and cob glume staining patterns. (A) RR pattern; inset, *P1-rr* phenotype. (B) RW pattern; inset, *P1-rw* phenotype. (C) WR pattern; inset, *P1-wr* phenotype. (D) WW pattern; inset, *P1-ww* phenotype. (E) Husk pigmentation phenotypes of *P1-rr* (left) and *P1-wr* (right). (F) Representative P-rr::GUS expression patterns in husk. (G) WR (left) and RR (right) expression patterns in pericarp and cob glume of sibling plants carrying identical P6.2b::GUS transgenes.

the same transformation event (example in Figure 3). Even with the high transformation frequency of the P6.2b::GUS construct (28% of bombarded embryos yielded transgenic callus), only 2 out of 19 initial callus lines were chimeric for multiple independent events (not shown). One chimeric callus line contained 2 independent transformation events that were analyzed here as separate events; the other consisted of 3 independent events that were excluded from this analysis. Thus, the multiple expression patterns of P-rr::GUS heterogeneous events are produced by identical transgene insertions.

Expression pattern variation among sibling plants correlates with transgene methylation differences: Variability in expression patterns among plants carrying identical transgene alleles suggested that P-rr::GUS expression can be modulated by epigenetic mechanisms. Epigenetic alterations in transgene expression, such as the gradual decrease in transgene activity over successive generations (KILBY *et al.* 1992; GORDON-KAMM *et al.* 1999) and the spontaneous silencing of transgene expression from one generation to the next (reviewed in STAM *et al.* 1997), are frequently associated with changes in DNA methylation. To determine if differences in DNA methylation are associated with specific P-rr::GUS expression patterns, DNA from sibling plants with heterogeneous pericarp and cob expression patterns was digested with the methylation-sensitive enzyme *Hpa*II for DNA blot analysis. Plants derived from two heterogeneous events displayed a strict correlation between the pattern of GUS expression and the methylation status of the transgene. In one case, the transgenes of plants with a WR pattern were hypermethylated relative to those of sibling plants with an RR pattern (Figure 3). Similarly, in a line with both RR and WW expression patterns, the transgenes of plants with a WW pattern were consistently hypermethylated (not shown). In contrast, no difference in transgene methylation was detected by this method for three P-rr::GUS transgenic families that had RR/WR, RW/WW, and RR/WW expression pattern combinations. Since use of a single diagnostic restriction enzyme will not detect all potential methylated sites, this result does not exclude the possibility that DNA methylation differences may be associated with the pattern heterogeneity of these families.

TABLE 1

Patterns of P-rr::GUS expression in pericarp and cob glumes

Construct	RR[a]	RW[a]	WR[a]	WW[a]	Total[b]
P1.0b::GUS	3 (11)	1 (6)	1 (3)	2 (14)	3 (34)
P1.2b::GUS	4 (13)	1 (1)	3 (7)	5 (15)	7 (42)
P6.2b::GUS	13 (60)	5 (12)	1 (1)	9 (36)	19 (109)

T_0 generation.
[a] No. of events (no. of plants).
[b] Total number of events is less than the sum of events for each expression pattern because some transformation events contained plants with different expression patterns.

Hierarchical ordering of P6.2b::GUS transgene expression patterns: The uniform red pigmentation conditioned by *P1-rr* is most striking in pericarp and cob glumes, but *P1-rr* also confers color to other organs, including husk, silk, and tassel glumes. GUS activity in these five organs was scored from tissue samples of T_0 plants transformed with P6.2b::GUS, P1.0b::GUS, and P1.2b::GUS constructs. Although similar patterns of GUS activity were observed for all three constructs, the larger number of independent P6.2b::GUS transformation events allowed for statistical analysis of the expression patterns. Among these five organs, no single expression pattern was predominant for the majority of transformants; rather, there were five different prevalent patterns. One pattern was analogous to the pigmen-

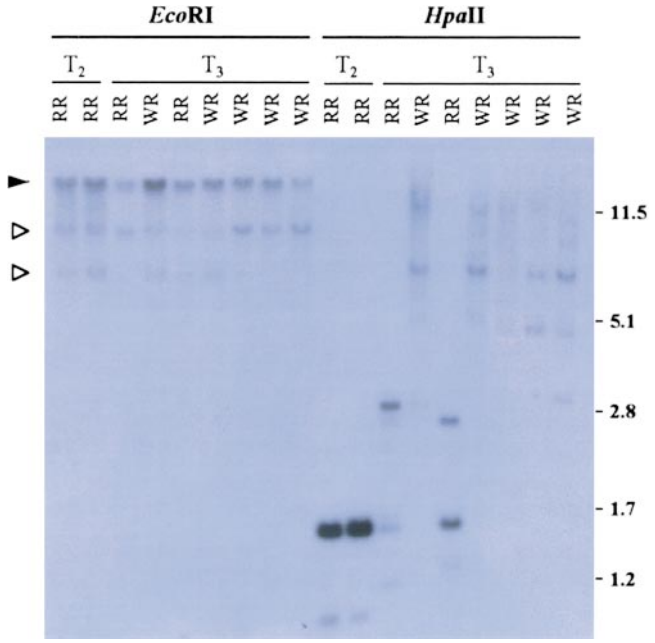

FIGURE 3.—DNA blot analysis of transgene structure and methylation state of a P1.2b::GUS transformation event having plants with RR and WR patterns. Genomic DNA was isolated from leaves of two T_2 plants and seven T_3 progeny of three sibling T_2 plants. Leaf DNA could be used because the methylation state of endogenous *p1* alleles is generally conserved among different tissues (DAS and MESSING 1994; CHOPRA *et al.* 1998). The DNA was cleaved with *Eco*RI or *Hpa*II, subjected to DNA blot analysis, and hybridized with *p1* genomic probe 15 (ATHMA and PETERSON 1991). Expression patterns of individual plants are listed above the lanes: RR (blue pericarp and cob) or WR (white pericarp and blue cob). In lanes digested with *Eco*RI, the solid arrowhead indicates the transgene band and open arrowheads indicate bands from the endogenous *P1-ww* alleles. In lanes digested with *Hpa*II, all hybridizing bands are from the transgene because fragments from the *P1-ww* alleles are not retained on the gel.

Expression Pattern					
Pericarp	Cob Glume	Husk	Silk	Tassel Glume	Events (Plants)
■	■	■	■	■	6 (19)
■	■	■	■		6 (15)
■	■	■			7 (11)
■	■				5 (12)
■					4 (9)
Subtotal					**14 (66)**
Deviant Patterns					6 (8)
No Expression					8 (35)
Total					**19 (109)**

FIGURE 4.—GUS expression patterns among five organs of P6.2b::GUS T_0 plants. The five predominant patterns among P6.2b::GUS transformants are listed in hierarchical order, with squares representing GUS activity in an organ. Plants with patterns inconsistent with the hierarchy or without expression in any organ are grouped as deviant patterns and no expression, respectively. The number of events containing plants with a given expression pattern is listed, followed in parenthesis by the number of plants with the same pattern. The total number of events and plants with hierarchical patterns is given as a subtotal. The sum of events is greater than the total number of events studied because a single event can contain plants belonging to more than one expression pattern category.

tation pattern conferred by a *P1-rr* allele, with blue staining of all five organs. The other four major patterns can be sequentially arranged, such that each subsequent pattern has expression in one less organ than the previous (Figure 4). The resulting hierarchical order, listed from most likely to least likely to have GUS activity, is: pericarp, cob glume, husk, silk, and tassel glume. All together, 66 plants from 14 events had expression patterns within the five categories of the hierarchy. In contrast, only 8 plants, derived from 6 events, exhibited seven different patterns that deviated from the hierarchical categories (Figure 4). Thus, the majority of P6.2b::GUS transgenic plants have hierarchical expression patterns, while plants with deviant expression patterns comprise a small minority of exceptional plants.

Most events contained plants with different types of expression patterns, although the expression patterns of each event usually belonged to adjacent categories within the hierarchy (Figure 5). This observation supports the idea that the hierarchy represents a continuum of gene expression patterns. A statistical test was developed to determine whether independent random expression in each organ could have produced the apparent hierarchy. Gene expression in any pair of tissues can be summarized as a 2×2 contingency table (*e.g.*, yes/no in pericarp $\times$ yes/no in cob). The hypothesis of independent expression in that pair of tissues can be tested by a one-sided version of a Chi-square test for paired data (SNEDECOR and COCHARN 1967). The 10 tests can be pooled into a single test for a hierarchy using the union-intersection test principle (MORRISON 1976), which concludes that there is evidence of a hierarchy only if every pairwise test is significant. The *P* values of the 10 pairwise tests ranged from <0.0001 to 0.012; the least significant pairwise test is that for husk and cob. Hence, there is strong statistical support for the conclusion that the patterns of P-rr::GUS transgene expression in pericarp, cob, husk, silk, and tassel follow a reproducible hierarchy. In addition, the same hierarchical expression pattern was observed in an independent study, in which P-rr::GUS constructs were used to address the effect of matrix attachment regions (MARs) on transgene expression. Among plants transformed with P1.0b::GUS and a MAR-containing selectable marker plasmid, 31 plants had expression patterns that fit within the hierarchy and only 5 plants gave deviant patterns, whereas, transformation with a different P-rr::GUS construct containing the 1.0 enhancer fragment plus an additional 1 kb of upstream flanking sequence pro-

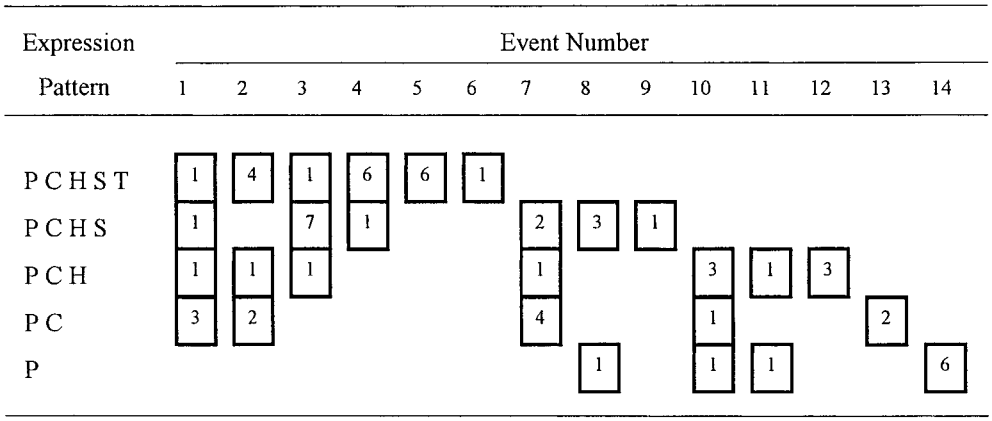

FIGURE 5.—Distribution of hierarchical expression patterns in 14 independent P6.2b::GUS transformation events. Numbers across the top line represent individual transformation events. The boxed numbers indicate the number of plants from each event with the expression pattern on the left. Expression patterns are indicated by single letter abbreviations for floral organ: P, pericarp; C, cob; H, husk; S, silk; T, tassel glume.

duced 12 plants with expression patterns that fit within the hierarchy and 6 plants with deviant patterns (L. V. SIDORENKO and S. M. COCCIOLONE, unpublished results).

Transgenic progeny exhibit hierarchical expression patterns: To determine the heritability and stability of the expression patterns produced by the P6.2b::GUS primary transformants, T_0 plants were outcrossed with nontransgenic *P1-ww* maize lines. Outcrossing maintains hemizygosity of the transgenes and avoids possible silencing effects associated with increasing transgene copy number through homozygosity (SCHEID *et al.* 1991; DE CARVALHO *et al.* 1992). Pericarp, cob, husk, silk, and tassel of the T_1 plants were histochemically stained for GUS activity. Sibling T_1 plants often displayed multiple expression patterns that again fit contiguous categories

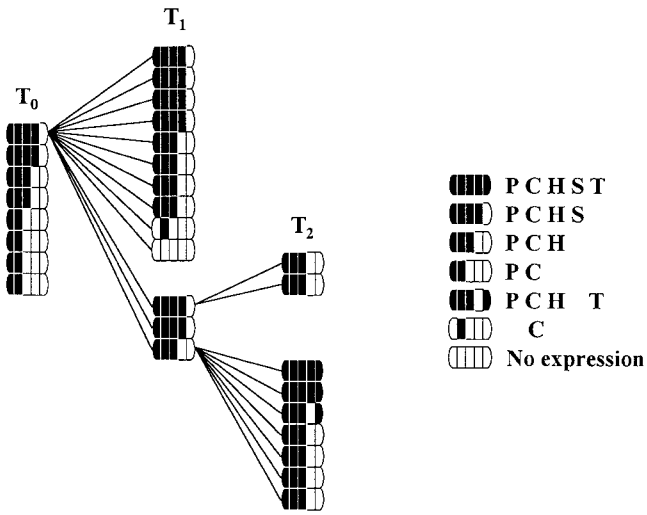

FIGURE 6.—Expression of a single P6.2b::GUS transgenic event in T_0, T_1, and T_2 generations. Oblongs represent individual plants, with lines connecting parent and progeny. For each plant, the solid regions of the oblongs indicate GUS activity in pericarp (P), cob glume (C), husk (H), silk (S), and tassel glume (T). All plants were crossed by a nontransgenic *P1-ww* line as a pollen parent. In the T_1 generation, plants from asingle progenitor plant were grown in the field (top group) or greenhouse (bottom group).

of the expression hierarchy (Figure 6). This trend was consistent irrespective of whether the plants were grown under field or greenhouse conditions, and it was again observed in a third generation (Figure 6). T_0 plants with expression patterns that deviated from the hierarchy produced progeny with either strictly hierarchical patterns or both deviant and hierarchical patterns (not shown). Overall, the transgene expression patterns, though variable, repeatedly fit the observed hierarchy for three successive generations.

P-rr::GUS expression in nonfloral organs: In general, expression of the *p1* gene is observed in tissues of the tassel and ear, although *p1*-regulated 3-deoxy flavonoids have been detected in coleoptiles (STYLES and CESKA 1981). Unexpectedly, 14 out of 27 T_1 P-rr::GUS transgenic lines sampled had GUS activity in at least one of the following: coleoptile, leaf, and root of seedlings (Figure 7); and sheath, auricle, and blade of adult leaves (not shown). The presence of P-rr::GUS in these vegetative organs varied among plants, and the intensity of GUS staining ranged from light to very dark in seedling organs and from light to moderate in adult leaves. No blue staining was observed in nontransformed plant material. GUS activity in the coleoptile was primarily localized to the two lateral vascular bundles that traverse the organ (Figure 7A). In the seedling roots, GUS activity was observed in the primary, lateral, and seminal adventitious roots. The most intense root staining was in the central cylinder composed of pith and vascular cells and at the site of lateral branch formation, although in seedlings with strong expression, GUS activity was observed in all tissues of the root (Figure 7B). Leaves of the primary regenerants grown in culture stained uniformly (Figure 7C), while leaves from greenhouse-grown seedlings stained unevenly—most likely because of poorer substrate penetration due to a thicker cuticle.

Within a vegetative organ, the same pattern of expression was observed for many independent transformation events, *e.g.*, GUS activity in coleoptiles was always localized to the lateral vascular bundles. This consistency across events implies that vegetative expression of the P-rr::GUS transgenes is not due to position effects, but

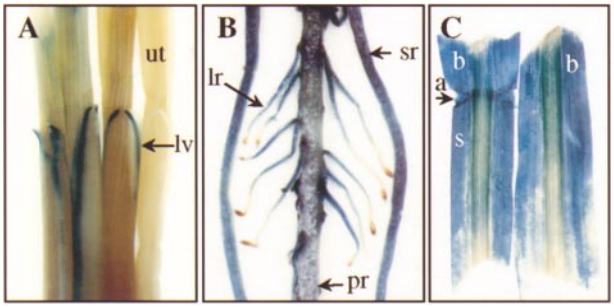

FIGURE 7.—P-rr::GUS expression in nonfloral organs. (A) Four T_1 seedlings segregating for a P6.2b::GUS transgene. GUS activity is in the lateral veins (lv) of the coleoptiles of the three transgenic seedlings on the left. No staining is observed in the nontransgenic seedling (ut) on the right. (B) Roots of a P6.2b::GUS T_1 seedling with GUS activity in lateral roots (lr), primary roots (pr), and seminal adventitious roots (sr). (C) Seedling leaves of a P1.0b::GUS T_0 plant with GUS activity in auricle (a), blade (b), and sheath (s).

instead reflects transcriptional activity directed by the transgene promoter.

DISCUSSION

P-rr::GUS transgenes exhibit extreme variation in expression patterns: The *P1-rr* allele is named for the striking red pericarp and red cob glume phenotype that it confers. We analyzed the expression patterns of three P-rr::GUS transgenes—one containing the full-length *P1-rr* promoter and two containing promoter deletions. Interestingly, all three transgenes were capable of conferring not only a *P1-rr*-like pattern in pericarp and cob glumes (blue staining pericarp and cob glumes), but also expression patterns characteristic of other *p1* alleles, *i.e.*, *P1-wr* (white pericarp and blue cob glumes) and *P1-rw* (blue pericarp and white cob glumes). Within the five organs that are pigmented by the endogenous *P1-rr* allele, there was a high level of variability in the spatial patterns of P-rr::GUS expression among transformants. The predominant patterns form a hierarchy of transgene expression, with each successive expression pattern having GUS activity in one additional organ, beginning with pericarp and followed sequentially by cob glume, husk, silk, and tassel glume. These different patterns of GUS activity were observed not only between independent transformation events, but also among sibling plants that carry the same transgene.

Variation in P-rr::GUS expression patterns is not inherent to plant transformation: While the endogenous *P1-rr* upstream regulatory region in its native genomic context confers stable expression, placement of the *P1-rr* upstream regulatory region into a foreign genomic environment, via transformation, appears to destabilize the promoter, causing enhanced sensitivity to epigenetic effects. These effects are nonrandom, as evidenced by the occurrence of an expression pattern hierarchy, and are considered epigenetic because different patterns of expression occur among sibling plants carrying identical transgene insertions. Variability in transgene expression is often attributed to position effect, the direct influence of genomic DNA sequences or chromosome structure near the site of transgene integration (FOBERT *et al.* 1991; GOLDSBROUGH and BEVAN 1991). However, position effect is an unlikely explanation for the spatial variation of P-rr::GUS transformants, since hierarchical expression patterns were observed across independent transformation events. In addition to position effect, the number of copies of the transgenic construct can influence transgene expression. Increased copy number has resulted in either enhancement (HOBBS *et al.* 1993; ELOMAA *et al.* 1995) or suppression of transgene expression (LINN *et al.* 1990; ASSAAD *et al.* 1993), although no correlation between copy number and transgene expression has also been reported (JONES *et al.* 1987; PEACH and VELTEN 1991). In our experiments, transgene copy number and levels of GUS activity did not correlate (SIDORENKO *et al.* 2000), nor was there a relationship between copy number and spatial patterns (S. M. COCCIOLONE, data not shown).

While quantitative variation in transgene expression is commonly observed, numerous transformation experiments exhibit little or no spatial pattern variation among independent transformation events (examples include BENFEY and CHUA 1989; LLOYD *et al.* 1992; DAI *et al.* 1996; GORDON-KAMM *et al.* 1999). As an example in maize, transformation with genomic sequences of *B-Peru*, a maize regulatory gene of the anthocyanin biosynthesis pathway, produced plants with identical patterns for all nine expressing events (SELINGER *et al.* 1998). Similarly, we observed one predominant expression pattern for plants transformed with the maize *a1* promoter fused to the GUS gene (S. M. COCCIOLONE, unpublished results). In contrast, the marked spatial pattern variability of the P-rr::GUS transgenes reported here strongly implies that the *P1-rr* promoter is inherently prone to spatial pattern variation.

The idea that some promoters are more prone to spatial pattern variation is supported by experiments in germ-line transformation of mice. PALMITER and BRINSTER (1986) noted that transgenes containing promoters that drive expression in one or a few cell types, if expressed at all, were expressed in the appropriate tissues regardless of having different chromosomal locations. In contrast, transgenes containing promoters that are activated in many cell types displayed variable expression among mice and among the tissues where the endogenous gene is expressed. The results of transformation experiments using the mouse metallothionein promoter, a promoter transcribed in many cell types, have striking similarities with our results. Transgenes containing the metallothionein promoter displayed variability in expression over several generations among offspring mice. Moreover, transgene expression did not

correlate with the number of transgenes being expressed, segregation of modifier genes, or DNA methylation changes. One explanation for variability offered by Palmiter and Brinster (1986) was that some gene promoters are very sensitive to nucleosome phasing changes and that during meiosis or early development the chromatin configuration might be altered.

Model for hierarchical transgene expression patterns: Our observations of P-rr::GUS transgene expression patterns can be explained by a model based on current knowledge of *p1* gene expression. Previous reports have established that the chromatin structure of the *P1-rr* allele differs between *p1*-expressing and nonexpressing tissues: DNaseI hypersensitive sites identified for *P1-rr* in pericarp, husk, and silk were absent or exhibited low sensitivity in leaf (Lund *et al.* 1995). Moreover, differences in DNaseI sensitivity were observed for an epiallele of *p1*, denoted *P-pr* for *p*atterned pericarp and *r*ed cob. The *P1-pr* epiallele is structurally indistinguishable from *P1-rr*, but it is hypermethylated and produces less *p1* transcript than the progenitor *P1-rr* allele (Das and Messing 1994). Hence, alterations in chromatin structure can alter the normal expression pattern of an endogenous *P1-rr* allele.

In our model, chromatin structure controls accessibility of a binding site in the *P1-rr* promoter to ubiquitous transcription factors (Figure 8A). This model proposes that transcription of the endogenous *P1-rr* gene is restricted by a closed chromatin conformation and that opening of the chromatin structure potentiates an increase in *p1* transcription. Subsequently, the chromatin structure of the *P1-rr* promoter remains in an open state through successive stages of ear development. We propose that the hierarchical patterns of P-rr::GUS expression result from variations in the temporal or spatial transition from a closed to an open chromatin state; *i.e.*, the timing of chromatin remodeling at the transgene locus may be advanced or delayed with respect to the normal development stages. Hence, an early transition in chromatin structure would result in transgene activation in additional vegetative organs and the full complement of floral organs. In contrast, late transitions would produce plants having transgene expression in particular subsets of floral organs. Support for this idea comes from the observation that the hierarchical ordering of P-rr::GUS expression (pericarp, cob glume, husk, silk, and tassel glume) is almost the reverse order of initiation of these organs: tassel initiation precedes that of the ear, within which development proceeds in the order of husk, cob glume, pericarp, and silk (Figure 8B; Cheng and Pareddy 1994). The exception is silk, which has a position in the hierarchy that is inconsistent with the order of organ initiation. Silk develops as an outgrowth of the pericarp, although it is more related in cell lineage to the cob glumes than to the majority of the pericarp (Dellaporta *et al.* 1991). An explanation for the placement of silk in the hierarchy is unclear at this time, but perhaps it may reflect a regulatory mechanism independent of the other floral organs. Further studies will be required to test our hypothesis that the nonrandom, hierarchical patterns of P-rr::GUS transformants are due to developmental variation in chromatin structure formation.

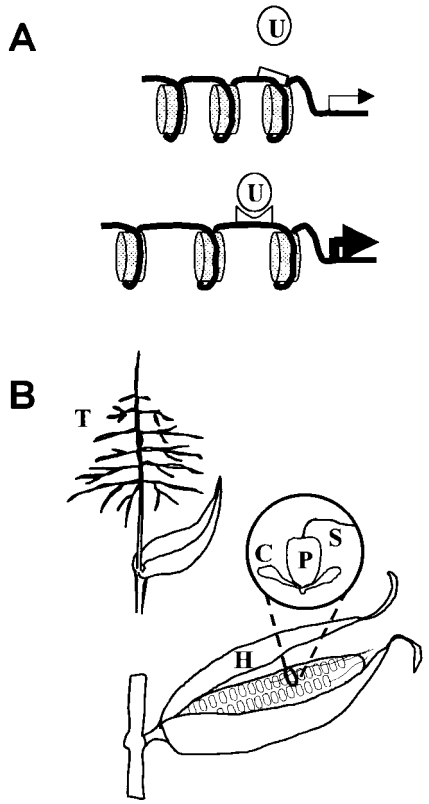

Figure 8.—Model for hierarchy of transgene patterns. (A) Chromatin-modulated control of a single *cis*-regulatory element. Organ-specific differences in chromatin structure allow (open state) or inhibit (closed state) binding of a ubiquitous *trans*-acting factor to a single *cis*-regulatory element in the *P1-rr* promoter. Cylinders represent nucleosomes. Thin arrow at transcription start site indicates low levels of transcription and thick arrow indicates elevated transcription levels. (B) Diagram depicting the order of floral organ initiation in maize. Organs: T, tassel; H, husk; C, cob; P, pericarp; and S, silk.

In summary: The variation in spatial patterns of transgene expression among P-rr::GUS transgenic plants was determined to have a unique hierarchical organization. Our study directly demonstrates that such variability can have a nonrandom basis, which we propose may reflect the action of regulatory mechanisms responsive to developmental signals and sensitive to epigenetic influences when associated with a transgenic locus.

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