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A Regulatory Gene as a Novel Visible Marker for Maize Transformation

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The temporal and spatial patterns of anthocyanin pigmentation in the maize plant are determined by the presence or absence of the *R* protein product, a presumed transcriptional activator. At least 50 unique patterns of pigmentation, conditioned by members of the *R* gene family, have been described. In this study, microprojectiles were used to introduce into maize cells a vector containing the transcription unit from one of these genes (*Lc*) fused to a constitutive promoter. This chimeric gene induces cell autonomous pigmentation in tissues that are not normally pigmented by the *Lc* gene. As a reporter for gene expression studies in maize, *R* is unique because it can be quantified in living tissue simply by counting the number of pigmented cells following bombardment. *R* may also be useful as a visible marker for selecting stably transformed cell lineages that can give rise to transgenic plants.

MOST MAIZE CELL TYPES HAVE THE capacity to accumulate anthocyanin pigments (*1*). Pigmentation within the plant is controlled by at least ten genes that encode regulatory or structural proteins of the anthocyanin biosynthetic pathway (*2*). The *R* gene family determines the location, timing, and amount of anthocyanin expression in the maize plant and seed (*3*). The overall pigmentation pattern in the plant represents the additive effect of each family member.

The *R-Navajo* (*R-nj*) gene, which pigments the crown of the kernel, was cloned by tagging with the transposable element *Ac* (*4*) and was found to be homologous with three other *R* genes, *P*, *S*, and *Lc*, which display different pigmentation patterns (*4*). Homology between *R-nj* and *Lc* facilitated the cloning of a full-length *Lc* cDNA. The protein encoded by this cDNA has features characteristic of transcriptional activators and DNA binding proteins (*5*). From these data and previous genetic analyses (*6*), we proposed that the diverse patterns of anthocyanin pigmentation conditioned by different *R* genes reflect differences in the *R* gene promoters rather than their gene products (*5*).

To test this hypothesis, we used microprojectile bombardment (*7, 8*) in an in vivo assay to analyze *R* gene expression. We bombarded maize kernels with microprojectiles (*9*) coated with the vector pPHI443, which contains the entire *Lc* protein coding region fused to a constitutive promoter from cauliflower mosaic virus (*10*). Pigmented cells were detected in the aleurone layer 14 to 36 hours after bombardment (Fig. 1A). In the absence of pPHI443 DNA, aleurone cells were unpigmented because they lacked a functional *R* gene. Since the *Lc* gene does not normally pigment aleurone cells, this experiment supports our contention that *R* genes are functionally equivalent and that pigmentation can be induced in a novel tissue by changing the *R* promoter.

To examine the versatility of this marker, we bombarded a variety of tissues with pPHI443. After the bombardment of germinating seedlings, pigmented cells were seen in the epidermal cell layers of the coleoptile (Fig. 1B), primary root (Fig. 1C), mesocotyl, scutellar node, and coleorhiza (Fig. 1D). Pigmented cells were also observed in the surface cell layers of other immature tissues such as pericarp (both inner and outer layers), culm, husk, scutellum, and anther locules. In young leaves pigmentation was observed in both epidermal cells and trichomes (Fig. 1E).

To determine whether the introduced *R*-encoded protein is constrained by factors similar to those observed for endogenous *R* gene products, we cotransformed a variety of maize tissues with pPHI443 and an analogous vector, pPHI459, encoding β-D-glucuronidase (GUS) (*10*). Expression of both markers can be seen simultaneously in most tissues (Fig. 1F), because red cells retain their pigment when treated with GUS histochemical stain. However, in cells of the inner endosperm, pigmentation was never

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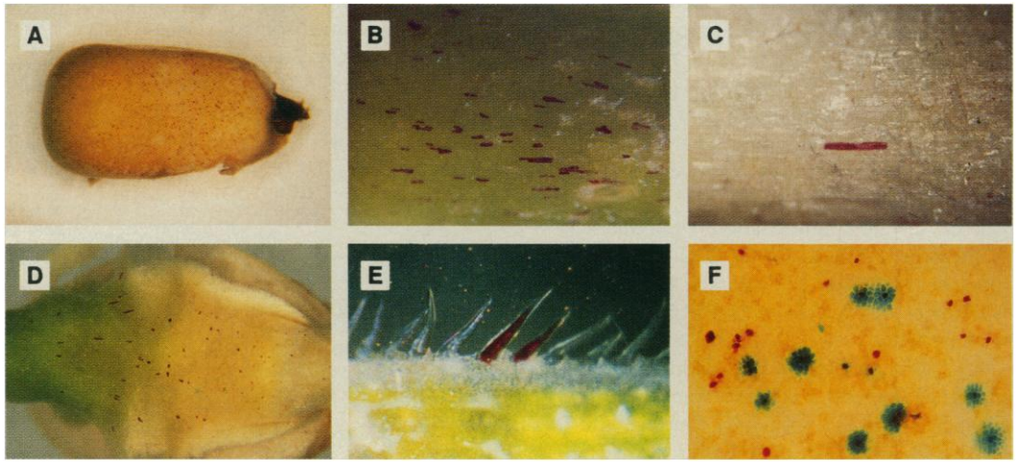

Fig. 1. Expression of an *R* gene introduced into different tissues. Aleurone (**A**), coleoptile (**B**), root (**C**), mesocotyl, scutellar node, coleorhiza (**D**), and marginal leaf hair (**E**) were bombarded with gold particles coated with pPHI443 DNA as described (*9*) and photographed approximately 48 hours later. Aleurone cells (**F**) cotransformed with pPHI443 and pPHI459 were examined after histochemical staining for GUS (*13*).

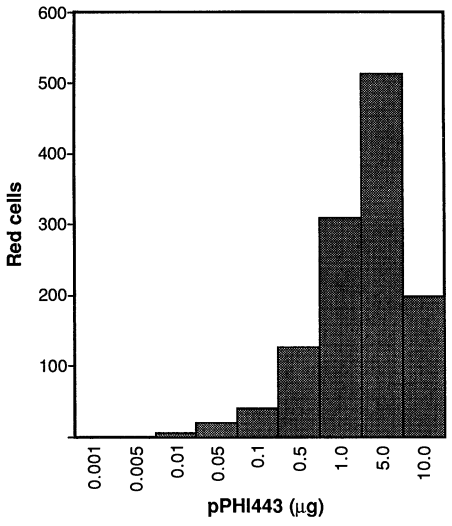

Fig. 2. Effect of pPHI443 concentration on the number of red cells induced in germinating seedlings. Embryos were isolated as described (*9*), incubated on Murashige and Skoog media containing 0.5% Gel-Rite and bombarded about 36 hours later. Mixtures of pPHI459 and the amount of pPHI443 DNA indicated (10 μg total) were precipitated onto 4.375-mg gold beads (*9*), and the volume was adjusted to 30 μl. Data show total numbers of red cells seen in eight seedlings bombarded separately with 1-μl portions of each bead preparation.

observed after bombardment with pPHI443 and pPHI459, although GUS was actively expressed. This result, together with the fact that there are no maize lines known with pigmented endosperms (*2*), suggests that the anthocyanin pathway may be suppressed in endosperm cells by a factor (or factors) other than the absence of the *R*-encoded protein.

Cotransformation of pPHI443 and pPHI459 constructs into different genetic backgrounds was also used to demonstrate that the induction of pigmentation was influenced by the genetic constitution of the *Pl* locus and by light. In lines containing recessive *pl*, light is required for the induction of pigment synthesis in the seedling and plant but not the aleurone (*2*). Pigmentation is independent of light in all tissues of *Pl* lines. Bombardment of *pl* and *Pl* tissues incubated in the light or darkness (*11*) demonstrated that the introduced gene responded to the genetic background as an endogenous gene would. That is, aleurone pigmentation was independent of *Pl* or light, whereas seedling pigmentation required one or the other of these factors. Expression of the cotransformed GUS marker was independent of light and *Pl* in both aleurone and seedling tissues.

All cells pigmented by the introduction of pPHI443 showed a cell-autonomous phenotype (Fig. 1, A to F). In contrast, the pattern of histochemical staining for GUS was not cell-autonomous (Fig. 1F). One benefit of the cell-autonomous phenotype is that the number of pigmented cells can be unambiguously counted. Thus, the expression of *Lc*-containing constructs can be quantified in living tissue by counting the number of red cells. For example, the number of red cells observed in germinating seedlings is dependent on the amount of pPHI443 delivered (Fig. 2) (*12*).

The ability of constructs containing *Lc* cDNA to induce pigmentation provides a relatively simple assay for *R* constructs mutagenized in vitro. The putative *Lc* protein contains several sequence motifs found in other eukaryotic regulatory proteins. These include regions presumed to be necessary for DNA binding (the *myc* homology region) and transcriptional activation (the acidic domain) (*5*). Questions regarding the contributions of these regions to *R* function can now be addressed by introducing constructs expressing mutant *R* proteins into maize tissues.

The *R* gene has many potential applications for studying the developmental biology and molecular genetics of maize. Tissue-specific promoters from maize and possibly other monocots can be analyzed for their cis-acting control regions by fusion with the *Lc* cDNA. For this purpose, *R* has advantages over other commonly used reporter genes because visualization of *R* expression does not require a complicated biochemical assay or expensive histochemical staining. Pigmented cells are visible 14 hours after bombardment and many cell types retain pigment for several weeks. In addition, *R* expression from different promoters can be normalized against a common standard because both GUS and *R* expression can be visually monitored in the same tissue.

Perhaps the most novel feature of this marker is that cell-autonomous expression can be visualized in almost all tissues of maize without disturbing the integrity of the plant. Introduction of this marker into meristematic cells may permit the identification of stably transformed somatic lineages, which can be observed as pigmented sectors in the developing plant. Stably transformed lineages that give rise to germinal tissues should yield transformed maize plants in the following generation.

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9. Mature dry seeds of W22 *A1 A2 Bz1 Bz2 C1 C2 r-g*: Stadler *B-b pl* (obtained from M. Albertsen, Pioneer Hi-Bred) were surface-sterilized and soaked in distilled water overnight at room temperature. After the pericarp and the embryo were removed, the remainder of the kernel was split into halves and incubated with the exposed aleurone layer uppermost on Murashige and Skoog media containing 0.25*M* sorbitol and 0.5% Gel-Rite. Embryos were dissected out and germinated on media lacking sorbitol. All tissues were incubated at 30°C under a regime of 18 hours of light and 6 hours of darkness. Half-kernels were bombarded the following day, whereas germinating seedlings were bombarded 36 to 48 hours after plating, with a 1-μl portion of a 30-μl mixture containing 10 μg of DNA precipitated onto 4.375 mg of gold particles (Engelhard A1570 Flakeless) essentially as described (*7*), except that spermine was used instead of spermidine.
10. The plasmid pPHI443 contains an enhanced promoter spanning nucleotides −421 to +2 of CaMV 35*S* with the region from −421 to −90 duplicated in tandem [R. C. Gardner *et al.*, *Nucleic Acids Res.* **9**, 2871 (1981)], a 79-bp Hind III–Sal I fragment from pJI101 spanning the 5′ leader sequence of tobacco mosaic virus [D. R. Gallie, D. E. Sleat, J. W. Watts, P. C. Turner, T. M. A. Wilson, *Nucleic Acids Res.* **15**, 3257 (1987)], a 579-bp fragment spanning the first intron from maize Adh1-S [E. S. Dennis *et al.*, *ibid.* **12**, 3983 (1984)], a 2415-bp Xba I fragment spanning the *Lc* cDNA (*5*), and a 281-bp fragment containing a polyadenylation site from the *Agrobacterium tumefaciens* nopaline synthase gene [M. Bevan, W. M. Barnes, M.-D. Chilton, *ibid.* **11**, 369 (1983)] in pUC18 [C. Yanisch-Perron, J. Vieira, J. Messing, *Gene* **33**, 103 (1985)]. The control plasmid pPHI459 contains similar expression signals, but an 1870-bp fragment from pRAJ275 spanning the GUS coding sequence [R. Jefferson, S. Burgess, D. Hirsh, *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8447 (1986)] was inserted in place of the *Lc* cDNA.
11. Seeds of W22 *A1 A2 Bz1 Bz2 C1 C2 r-g*:Stadler *B-b pl* and W22 *A1 A2 Bz1 Bz2 C1 C2 r-g*:Stadler *B-b Pl* [obtained from M. Albertsen (Pioneer Hi-Bred) and J. Kermicle (University of Wisconsin), respectively] were used. Germinating seedlings and half-kernels (*9*) were incubated in the dark or in a regime of 18 hours of light and 6 hours of darkness both before and after bombardment with a 1:1 mixture of pPHI443 and pPHI459.
12. In aleurone cells, the optimal dose of pPHI443 is lower than in seedling tissues. Using a 1-μl portion of a 30-μl mixture containing 0.5 μg of pPHI443 and 4.375 mg of particles, between 100 and 500 red cells were obtained per half-kernel. With higher doses of pPHI443 the number of red cells decreased.
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