

# Engineering Secondary Metabolism in Maize Cells by Ectopic Expression of Transcription Factors

Erich Grotewold,<sup>a,1</sup> Mark Chamberlin,<sup>b</sup> Maurice Snook,<sup>c</sup> Bupe Siame,<sup>d</sup> Larry Butler,<sup>d,2</sup> Jan Swenson,<sup>b</sup> Sheila Maddock,<sup>b</sup> Grace St. Clair,<sup>b</sup> and Ben Bowen<sup>b,3</sup>

<sup>a</sup>Cold Spring Harbor Laboratory, 1 Bungtown Road, P.O. Box 100, Cold Spring Harbor, New York 11724

<sup>b</sup>Pioneer Hi-Bred International, Inc., 7300 N.W. 62nd Avenue, P.O. Box 1004, Johnston, Iowa 50131

<sup>c</sup>U.S. Department of Agriculture–Agriculture Research Service, Russell Research Center, P.O. Box 5677, Athens, Georgia 30604

<sup>d</sup>Department of Biochemistry, Purdue University, West Lafayette, Indiana 47907

**Manipulation of plant natural product biosynthesis through genetic engineering is an attractive but technically challenging goal. Here, we demonstrate that different secondary metabolites can be produced in cultured maize cells by ectopic expression of the appropriate regulatory genes. Cell lines engineered to express the maize transcriptional activators C1 and R accumulate two cyanidin derivatives, which are similar to the predominant anthocyanin found in differentiated plant tissues. In contrast, cell lines that express P accumulate various 3-deoxy flavonoids. Unexpectedly, P-expressing cells in culture also accumulate phenylpropanoids and green fluorescent compounds that are targeted to different subcellular compartments. Two endogenous biosynthetic genes (*c2* and *a1*, encoding chalcone synthase and flavanone/dihydroflavonol reductase, respectively) are independently activated by ectopic expression of either P or C1/R, and there is a dose–response relationship between the transcript level of P and the degree to which *c2* or *a1* is expressed. Our results support a simple model showing how the gene encoding P may act as a quantitative trait locus controlling insecticidal C-glycosyl flavone level in maize silks, and they suggest how *p1* might confer a selective advantage against insect predation in maize.**

## INTRODUCTION

Many widely used natural products are derived from plant secondary metabolism. Examples include drugs, such as aspirin, morphine, digoxin, or taxol, and a large number of food colors and fragrances (Alfermann and Petersen, 1995; Dörnenburg and Knorr, 1996). However, only a small number of compounds sold commercially today are produced from plant cell culture, primarily because yields are often low or unreliable (Alfermann and Petersen, 1995). New approaches for optimizing natural product yields in plant cell cultures are clearly needed (Yeoman and Yeoman, 1996). One attractive strategy for manipulation of secondary metabolism would involve ectopic expression of genes that regulate specific biosynthetic pathways. Flavonoid biosynthesis provides an ideal plant-specific secondary metabolic pathway to investigate the feasibility of this approach, because most of our knowledge about the mechanisms used

by plants to regulate secondary metabolism has come from the study of this pathway (reviewed in Koes et al., 1994; Shirley, 1996).

Flavonoids are ubiquitous plant secondary metabolites that play a role in many fundamental aspects of plant biology, including pigmentation, pollen viability, plant–microbe interactions, and protection from UV light (reviewed in Forkmann, 1991; Van der Meer et al., 1993). Their importance as pharmaceutical and nutritional components of our diet is becoming increasingly apparent as more is understood about their medicinal and pharmacological properties (Middleton, 1996). In maize and other cereals, at least two flavonoid biosynthetic pathways have been characterized that are regulated independently. One pathway results in the commonly occurring 3-hydroxy flavonoids, such as anthocyanins, whereas the other pathway produces 3-deoxy flavonoids, some of which have been associated with insecticidal or antifungal activity (Styles and Ceska, 1975; Waiss et al., 1979; Elliger et al., 1980; Snyder and Nicholson, 1990; Reid et al., 1992; Byrne et al., 1996a, 1996b).

Anthocyanin biosynthesis is controlled by genes encoding homologs of two transcriptional regulators, R and C1

<sup>1</sup>Current address: Department of Plant Biology, Ohio State University, Columbus, OH 43210.

<sup>2</sup>Deceased.

<sup>3</sup>To whom correspondence should be addressed. E-mail bowenb@phibred.com; fax 515-270-3367.

(reviewed in Koes et al., 1994). Introduction of these genes into many cell types of maize and other plants is often sufficient to induce pigmentation caused by anthocyanin accumulation (Goff et al., 1990; Ludwig et al., 1990; Bowen, 1992; Lloyd et al., 1992; Quattrocchio et al., 1993). Production of 3-deoxy flavonoids, such as *C*-glycosyl flavones, 3-deoxyanthocyanins, flavan-4-ols, and phlobaphenes, requires *P*, a Myb-related transcriptional regulator that acts independently of *R* and *C1* in maize floral organs (Styles and Ceska, 1989; Grotewold et al., 1991a, 1994). Previously, we demonstrated that *P* can bind directly to and transactivate the *a1* gene, which is involved in both 3-deoxy and 3-hydroxy flavonoid biosynthesis (Grotewold et al., 1994). However, it has never been clear whether *P* by itself is sufficient to induce in their entirety any of the pathways that lead to accumulation of *C*-glycosyl flavones or other 3-deoxy flavonoids.

Here, we demonstrate that two separate but overlapping pathways of flavonoid metabolism can be engineered in cultured maize cells by expression of just one or two transgenes encoding regulatory transcription factors. The predominant compounds induced by expressing *P* in cultured cells are *C*-glycosyl flavones closely related to those that are responsible for insecticidal activity in maize silks. Using transgenic maize cell lines that express *P* either constitutively or under inducible control, we also provide evidence for specialized vesicle trafficking pathways that can sort different secondary metabolites into separate subcellular compartments. Finally, we show how allelic variation in the expression level of *P* would be sufficient to account for the action of *p1* (the gene encoding *P*) as an additive quantitative trait locus (QTL) controlling silk maysin content (Byrne et al., 1996b). Thus, we have not only demonstrated the feasibility of engineering production of specific secondary metabolites by ectopic expression of transcriptional activators, but we have gained further insights into maize flavonoid biosynthesis, intracellular trafficking of secondary metabolites, and the molecular basis of quantitative variation in secondary metabolite levels.

## RESULTS

### Induction of Specific Flavonoids and Phenylpropanoids by Ectopic Expression of *C1/R* or *P* in Black Mexican Sweet Cells

As shown in Figure 1A, Black Mexican Sweet (BMS) suspension cells of maize do not accumulate pigmented flavonoids, because they do not express *R*, *C1*, or *P* (data not shown). Using particle gun bombardment, we were able to generate transgenic BMS cell lines containing either 35S::P (pPHP1962) or 35S::C1 and 35S::R (pPHP687) by cotransformation with a 35S::BAR herbicide resistance marker. Figures 1B and 1C show that transformants expressing *C1* and *R* (*C1/R*) are

easily distinguished by their conspicuous reddish purple anthocyanin pigmentation, which increased in intensity when the cultures were grown under illumination. To determine the identity of the predominant anthocyanins accumulating in the *C1/R*-expressing transformants, acidic methanol extracts were analyzed by both thin-layer chromatography and HPLC (see Methods). Two compounds not seen in extracts from untransformed BMS cells were detected. Both compounds were hydrolyzed to cyanidin by mineral acid at rates consistent with that expected for flavonoid *O*-glycosides. In contrast to cyanidin derivatives normally found in maize plants (reviewed in Stafford, 1990), both derivatives in BMS cells contain arabinose rather than glucose (see Methods). The number and positioning of the *O*-arabinosyl groups in each of the two cyanidin derivatives were not elucidated further.

Cell lines transformed with 35S::P looked phenotypically similar to untransformed cells when maintained in low light. No visible pigmentation caused by phlobaphenes or 3-deoxyanthocyanins characteristic of maize floral organs expressing *p1* was seen initially in any of the cell lines (data not shown). To investigate whether 3-deoxy flavonoids accumulated in cell lines transformed with 35S::P, we employed a colorimetric test for the presence of flavan-4-ols or their derivatives. Acidic butanol extracts of *P*-expressing transformants turned bright pink within 15 to 30 min at room temperature, indicating that flavan-4-ols were present (Watterson and Butler, 1983), whereas extracts from untransformed cells were colorless. Further analysis indicated that the main flavan-4-ol accumulating in *P*-expressing cells was luteoforol (see Methods). Figures 2A and 2B show that extracts of *P*-expressing BMS cells and immature *P-rr* pericarps, which are known to accumulate this flavan-4-ol (Styles and Ceska, 1989), had very similar absorption spectra. Figure 2 also shows that the absorption properties of extracts from *P*-expressing BMS and immature *P-rr* pericarps were affected similarly by boiling (Figures 2D and 2E) and differed from *C1/R*-expressing BMS cell extracts (Figures 2A, 2C, and 2F; see Methods).

After several days of exposure to light, cultures expressing *P* became speckled on their surface with small clusters of brown cells (Figures 1D and 1E) that exhibited an intense green autofluorescence when viewed with epifluorescence and a fluorescein isothiocyanate filter set (Figure 1F). As is commonly found in plant cell cultures that accumulate secondary metabolites (Alfermann and Petersen, 1995; Dörnenburg and Knorr, 1996; Yeoman and Yeoman, 1996), cells expressing either *P* or *C1/R* constitutively were slower growing than were untransformed BMS cells, and high-producing cell lines either sometimes lost their pigmentation altogether or showed wide fluctuations in pigmentation over time. Nevertheless, with repeated visual selection, we were able to derive homogeneously brown cell lines expressing *P* constitutively that retained the phenotypic features described above, even when grown in the dark. Cell lines selected in this way were analyzed further to identify the secondary metabolites that were induced.

Reverse-phase HPLC profiles of methanolic extracts of P-expressing cells and untransformed BMS cells are compared in Figures 3A and 3B. Five major phenolic compounds absorbing at 340 nm (peaks a to e) and a large number of less prevalent species were detected in the transformed cell extracts (Figure 3B). Induction of these compounds in callus by ectopic expression of P indicates that factors specific to floral tissues where P is normally expressed are not necessary for their biosynthesis.

Peaks b to d were not observed at any appreciable level in untransformed BMS (Figure 3A) and appeared to be C-glycosyl flavones from their UV absorption spectra. Further fractionation and characterization by fast atom bombardment mass spectrometry (FAB-MS; see Methods) indicated that b, c, and d were isoorientin, isovitexin, and isoscoparin, respectively. Small quantities of the 2''-O-rhamnosyl derivatives of isoorientin and isoscoparin were also present (Figure 4).

The phenolic compound a found in methanolic extracts of the P-expressing lines (Figure 3B) was identified as ferulic acid by comparing its retention time and UV absorption spectrum with an authentic standard. P-expressing cells accumulated 70% more of this phenylpropanoid than did untransformed BMS cells (cf. Figures 3A and 3B). The UV absorption spectrum of peak e was indistinguishable from a related phenylpropanoid, chlorogenic acid (data not shown). These results indicate that P expression can affect the levels of specific phenylpropanoids as well as 3-deoxy flavonoids.

None of the major peaks with strong absorption at 340 nm exhibited green fluorescence analogous to that seen in situ in P-expressing cells (Figures 1F to 1H). Further characterization of fractions that could be responsible for the green autofluorescence induced by P will be reported elsewhere.

### Differential Trafficking of Secondary Metabolites

In the cytoplasm of BMS cells transiently expressing C1/R, anthocyanins first accumulate within vesicles known as anthocyanoplasts (Peckett and Small, 1980; Nozzolillo and Ishikura, 1988; Figure 1C) that ultimately coalesce into a single vacuole filling most of the cell. Figure 5 shows electron microscopy of BMS cells ectopically expressing C1 and R. Clearly revealed are the anthocyanoplasts that were derived from the smooth endoplasmic reticulum (SER), as has been concluded from previous studies in other plants (Stafford, 1990; Hrazdina and Jensen, 1992). The SER became dilated and filled with granular material (Figure 5A) that appeared to condense into discrete foci at the membrane (Figures 5B and 5D) and later as discrete spherical deposits (Figures 5C and 5D). Eventually, this electron-dense material filled the entire lumen of the SER (Figures 5E and 5F). No such structures were seen in untransformed BMS cells (Figure 5H).

In BMS cells transformed with 35S::P, two types of fluorescent bodies were distinguishable by epifluorescence microscopy: large, diffuse olive-green bodies filling most of the cell lumen, which photobleached rapidly (Figure 1G), and

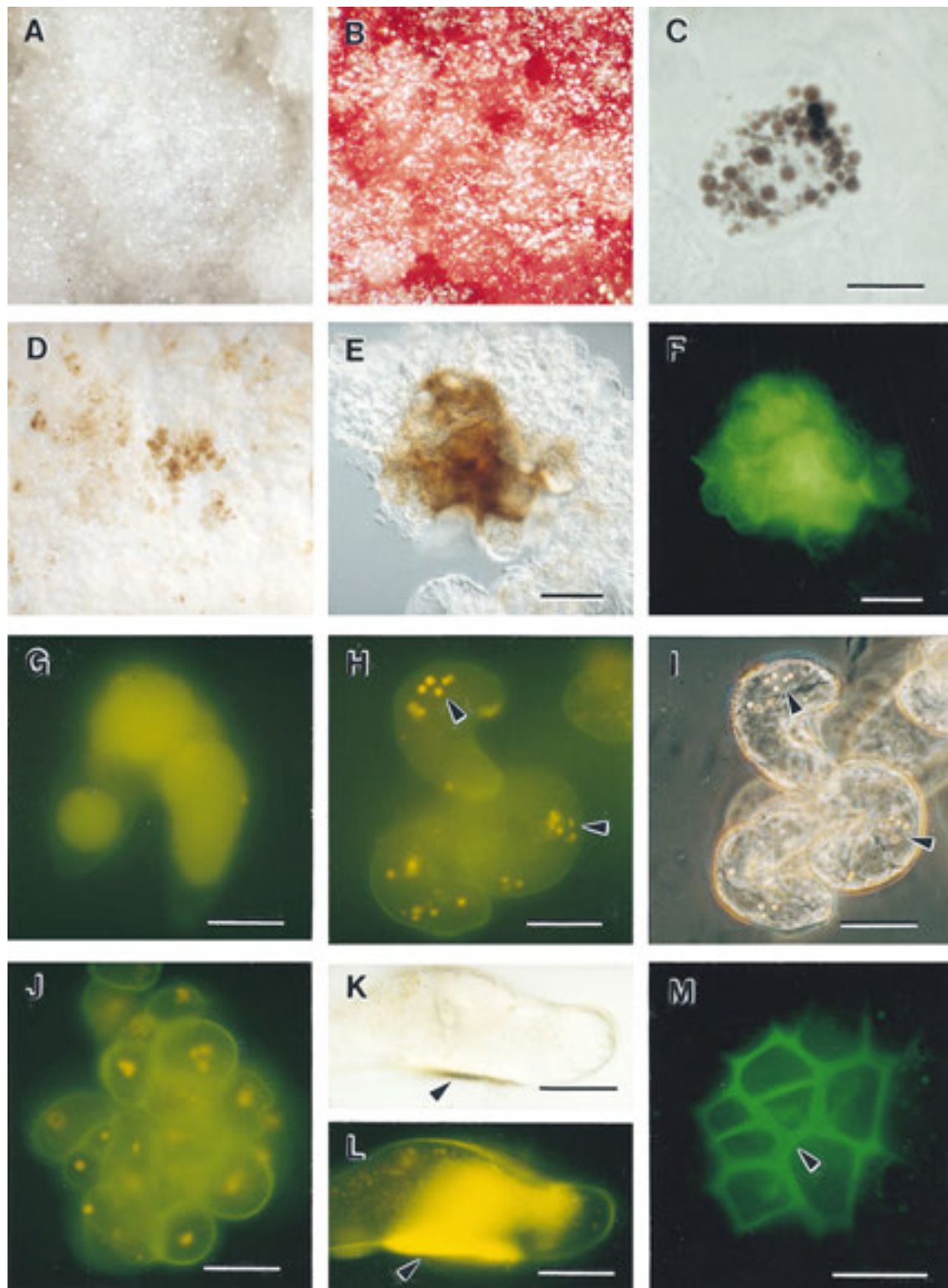
small, spherical, intensely fluorescent, nonphotobleaching greenish orange vesicles (Figures 1H and 1J) that could also be visualized with phase contrast optics (Figure 1I). In cells expressing P under the control of an estradiol-inducible promoter (B.A. Roth, C. Brouwer, C. Brooke, B. Bowen, and P. Solan, manuscript in preparation), the large diffusely fluorescent bodies appeared to be induced earlier than the smaller vesicles. Once fully induced, the majority of cells appeared similar to those expressing P constitutively from the cauliflower mosaic virus 35S promoter, that is, they contained one or both types of cellular bodies and had fluorescent walls (Figure 1J). Some cells exhibited patches of fluorescence that looked as if they had been generated recently by fusion of the small vesicles with the plasmalemma (Figures 1K and 1L). Fluorescent cell walls were also observed when P was transiently expressed from a 35S promoter in scutellar cells (Figure 1M), indicating that induction of fluorescent compounds by P is not limited to BMS cells in culture.

At the ultrastructural level, electron-dense spherical bodies were also found in dilated SER of P-expressing callus cells, similar to those observed in C1/R-expressing cells (cf. Figure 5C with Figures 6A and 6B). These inclusions were clearly induced by P expression (Figure 6H). In subsequent stages of development, some osmiophilic bodies were noted in the central vacuole (Figure 6C), whereas others were fused with the plasmalemma, and their contents appeared to be deposited within the cell wall space (Figures 6D to 6F). This confirmed our observations by light microscopy of autofluorescent vesicles (Figures 1G to 1J), their fusion with the plasmalemma (Figures 1K and 1L), and the emptying of their contents in the space between the plasmalemma and the cell wall (Figure 1M). Thus, in P-expressing cells, secondary metabolites enter one of two intracellular trafficking pathways that deliver materials into either the vacuole or the cell wall. In addition, because each compartment can be distinguished by its autofluorescence properties, some or all of the metabolites sorted by each pathway must be different from one another. The differential compartmentalization in P-expressing cells of coordinately regulated metabolites that may be closely related adds another level of complexity to regulation by P that was not previously recognized from classical genetic analysis and biochemical studies.

In addition to electron-dense vesicles, multilamellar bodies lacking osmiophilic material were seen at high frequency in BMS cells expressing either P or C1/R (Figures 5G and 6G) but not in untransformed cells (Figures 5H and 6H). Given the high activity of membrane trafficking in these cells, these structures are probably intermediates involved in endosomal membrane recycling (Herman and Lamb, 1992).

### Regulatory Specificity of P and C1/R in Transgenic BMS Cells

The accumulation of different flavonoid compounds in BMS confirmed that P and C1/R must regulate different sets of



**Figure 1.** Ectopic Expression of C1/R or P in Maize Cells.  
All cells are BMS. Bars = 50  $\mu$ m, unless noted otherwise.

biosynthetic genes. Previously, it was demonstrated that transcription of the anthocyanin biosynthetic genes *c2*, *chi1*, *f3h*, *a1*, *a2*, *bz1*, and *bz2* in various plant tissues can be induced by R and C1 (Dooner et al., 1991; Bodeau and Walbot, 1992; Deboo et al., 1995). In pericarp tissues expressing *p1*, however, only *c2*, *chi1*, and *a1* are expressed (Grotewold et al., 1994). To investigate which genes were induced by ectopic expression of P or C1/R in BMS, we analyzed RNA from several transgenic cell lines and compared expression patterns with those found in *P-rr* pericarps and untransformed BMS.

Figure 7A shows that transcripts for *f3h*, *a2*, *bz1*, and *bz2* were detected at high levels only in the anthocyanin-accumulating BMS line expressing C1 and R (35S::C1 and 35S::R). In each of the independent P-expressing cell lines analyzed and in *P-rr* pericarps, these transcripts were absent. However, both *a1* and *c2* were expressed in the cell lines transformed with 35S::P. This confirms the transient transactivation of *A1*::luciferase by P in cultured cells observed previously (Grotewold et al., 1994; Tuerck and Fromm, 1994) and demonstrates that ectopic expression of P in BMS is also sufficient to induce *c2* in vivo. Thus, P activates only a subset of the anthocyanin biosynthetic genes regulated by C1 and R.

Previously, we showed that the *chi1* transcript accumulates in *P-rr* but not in *P-ww* pericarps, in which the *p1* gene is deleted (Grotewold and Peterson, 1994; Figure 7A). In addition, a transcript homologous to *chi1* is found in plant tissues expressing C1/R or their homologs Pl/B (Grotewold and Peterson, 1994; Deboo et al., 1995). Strikingly, no transcript for *chi1* was detected in P- or C1/R-expressing cell lines (Figure 7A). Although BMS cells appear to harbor genomic sequences characteristic of *chi1* and other *chi* genes found in maize (Grotewold and Peterson, 1994; data not shown), it is possible that our BMS cell line may lack *chi1* per se or have a *cis*-acting mutation that prevents its activation by P or C1/R. Alternatively, our transformants may not express P or C1/R at sufficient levels to activate *chi1*, or else

BMS might lack a *trans*-acting factor that is required in addition to either P or C1/R for *chi1* expression. This latter possibility is consistent with the observation that in contrast to *A1*::luciferase (Grotewold et al., 1994), we have been unable to detect transactivation of a *CHI1*::luciferase reporter by P or C1/R in maize suspension cells (data not shown). Furthermore, the available sequence of the *chi1* promoter has no consensus P binding sites (Grotewold and Peterson, 1994; Grotewold et al., 1994), and no high-affinity binding sites for P in the *chi1* promoter have been identified in vitro (data not shown). Thus, *chi1* may not be regulated in the same way as other flavonoid biosynthetic genes in maize, and additional cellular factors may be required.

In addition to the absence of *chi1* transcripts, we were unable to detect any chalcone isomerase enzyme activity in P- or C1/R-expressing callus. Control experiments indicated the presence of chalcone isomerase activity in *P-rr* but not in *P-ww* pericarps (data not shown). Thus, in BMS cells, chalcone isomerase appears to be unnecessary for flux through either of the flavonoid pathways regulated by P or C1/R. This indicates that isomerization of chalcone to flavanone can occur in vivo without an enzymatic catalyst in maize.

As we showed previously (Grotewold et al., 1991a), two alternatively spliced P transcripts (2.0 and 1.0 kb) were detected in *P-rr* pericarps (Figure 7A). No transcript was seen in *P-ww* pericarps or untransformed BMS cells. Three of the four cell lines transformed with 35S::P, which contains the functional coding sequence from P-cDNA1 (Grotewold et al., 1991a, 1994) fused to the 35S promoter, accumulated a single P-homologous transcript somewhat shorter than the 2.0-kb transcript present in *P-rr* pericarps. This was expected, because pPHP1962 lacks ~150 bp from the 5' untranslated region of P-cDNA1 (Grotewold et al., 1994). The remaining 35S::P transformant (13/1-9) shown in Figure 7A accumulated a shorter transcript that was insufficient in size to encode the entire P protein. This transcript may be derived from a rearranged transgene, although this was not

**Figure 1.** (continued).

**(A)** Nontransformed callus.

**(B)** Pigmented callus expressing C1/R.

**(C)** Anthocyanoplasts in a single cell transiently expressing C1/R. Bar = 20  $\mu$ m.

**(D)** Brown cell clusters typical of P expression after several days of exposure to light.

**(E)** and **(F)** Cluster of P-expressing cells viewed under phase contrast **(E)** and epifluorescence **(F)** optics. Bars = 100  $\mu$ m.

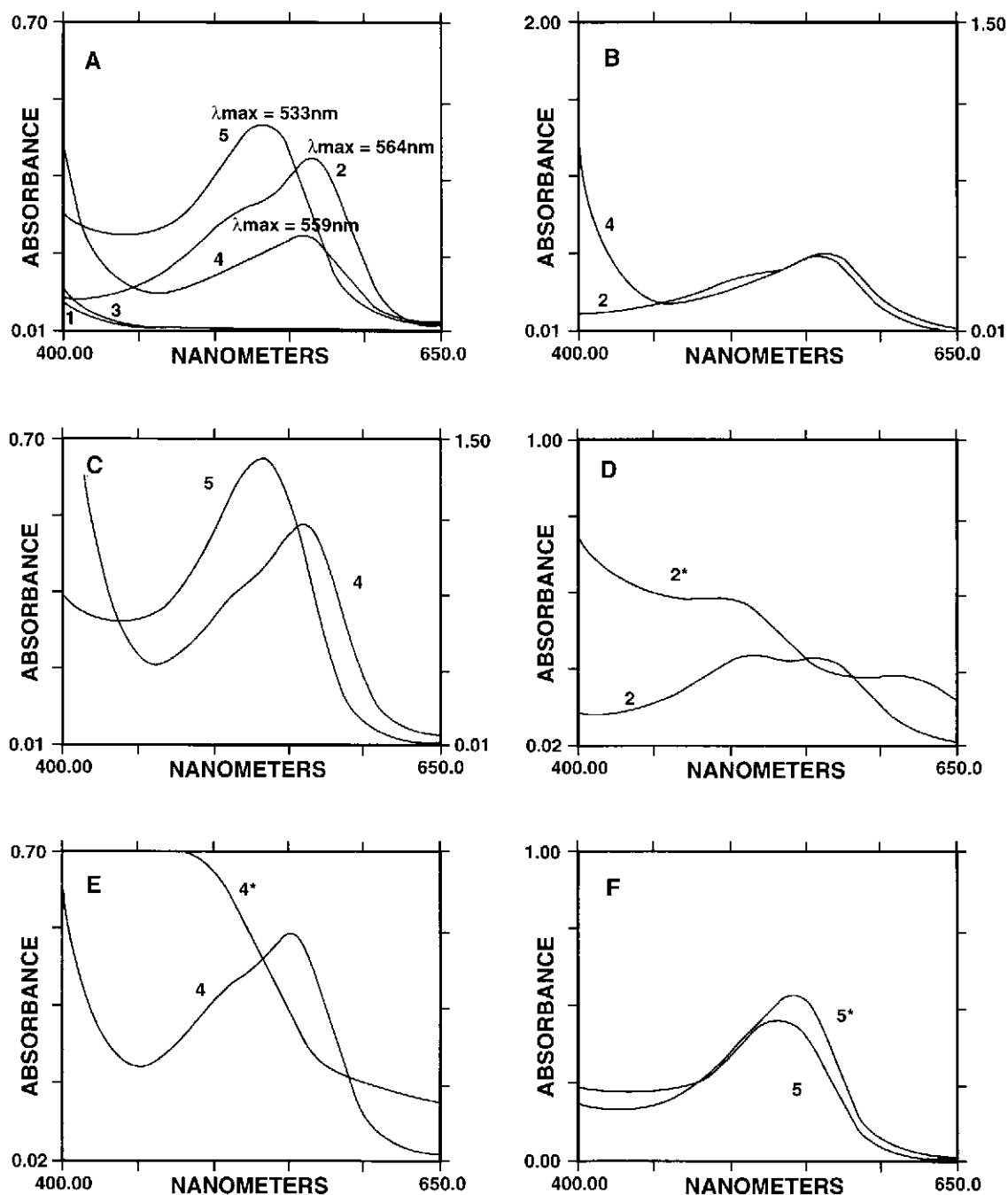
**(G)** Large olive-colored autofluorescent bodies nearly fill the lumens of cells expressing P under estradiol-inducible control after 4 days of induction.

**(H)** and **(I)** Small autofluorescent vesicles (arrowheads) in cells transformed with estradiol-inducible P at 4 days after induction, as seen under epifluorescence **(H)** and phase contrast **(I)** optics.

**(J)** Autofluorescent vesicles and walls in cells expressing P under estradiol-inducible control after 4 days of induction.

**(K)** and **(L)** P-expressing cell showing accumulation of autofluorescent material **(L)**, arrowhead) coincident with and emanating from a site of browning **(K)**, arrowhead) at the cell surface.

**(M)** Accumulation of autofluorescent material between the cell wall and plasmalemma (arrowhead) of scutellar cells from a 12-days-after-pollination maize embryo 48 hr after bombardment with a P-expressing plasmid (pPHP1962).



**Figure 2.** Comparison of Acidic-Butanol Extracts from P- or C1/R-Expressing BMS Cells.

(A) Absorption spectra of *P-ww* (curve 1) or *P-rr* (curve 2) pericarps, untransformed BMS callus cells (curve 3), P-expressing BMS line 14/2-15 (curve 4), or C1/R-expressing BMS (curve 5).  $\lambda_{max}$  corresponds to the absorption maximum (in nanometers) for each extract.

(B) Absorption spectra of *P-rr* pericarp extracts (curve 2) and P-expressing BMS cells (curve 4).

(C) Absorption spectra of extracts from BMS cells expressing P (curve 4) or C1/R (curve 5).

(D) Absorption spectra of *P-rr* pericarp extracts before (curve 2) or after (curve 2\*) boiling for 10 min.

(E) Absorption spectra of P-expressing BMS cell extracts before (curve 4) or after (curve 4\*) boiling for 10 min.

(F) Absorption spectra of extracts from C1/R-expressing BMS cells before (curve 5) or after (curve 5\*) boiling for 10 min.

rigorously investigated. However, the truncated P protein expressed in this cell line must be able to function as a transactivator, because both *c2* and *a1* were induced.

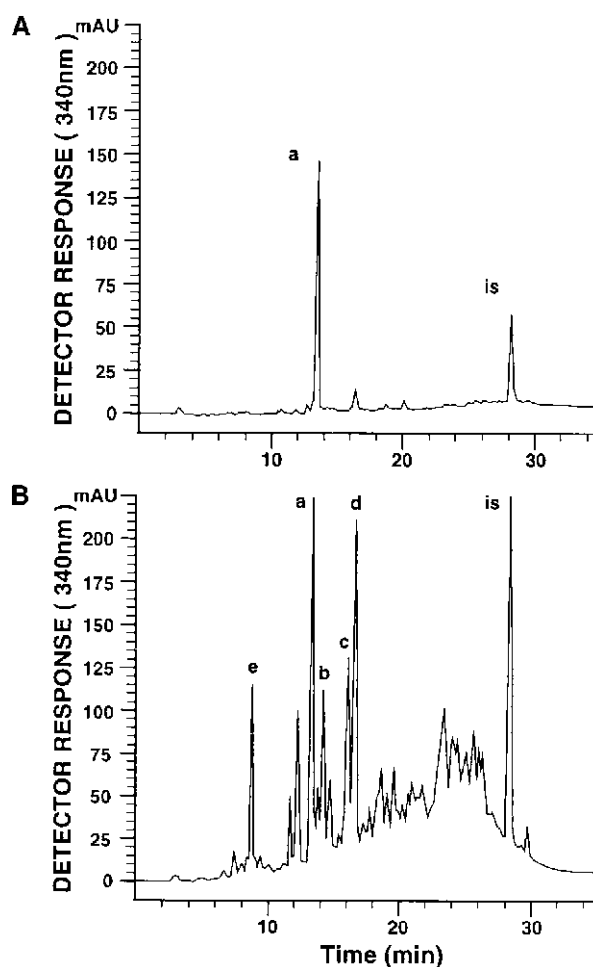
The observation that cell lines transformed with 35S::P accumulate different levels of *p1* mRNA (Figure 7A) provided us with a unique opportunity to examine the degree to which expression of different *p1*-regulated genes is dependent on *p1* expression levels. To investigate this further, RNA gel blot hybridization signals for *p1*, *c2*, and *a1* in five additional P-expressing cell lines (images not shown) were quantified with a PhosphorImager and normalized to the level of ubiquitin (*ubi*) transcript found in each sample. Figure 7B shows the relationship between the levels of *p1* transcript found in each cell line and the accumulation of *a1* and *c2* mRNA. The data were best modeled by a quadratic curve, but linear regression provided nearly as good a fit ( $r^2 = 0.887$  for *a1*;  $r^2 = 0.961$  for *c2*). Thus, the genetic effect of increasing P levels on *c2* and *a1* expression can most simply be described as additive. In *P-rr* pericarps, the normalized values for *p1*, *c2*, and *a1* transcripts were four to eight times higher than in any of the transgenic cell lines analyzed in this experiment. This could be due to an increase in *p1* mRNA on a per cell basis, a reduction in the relative level of *ubi* mRNA, or a combination of both.

## DISCUSSION

In this study, we demonstrated that ectopic expression of appropriate regulatory genes constitutes a viable strategy for engineering production of alternative secondary metabolites in plant cell cultures. P expression is sufficient to induce accumulation of flavonoids different from those regulated by C1/R and appears to have additional effects on phenylpropanoid biosynthesis. Two of the genes shared by anthocyanin and 3-deoxy flavonoid biosynthesis were shown to be independently activated by either P or R/C1. In addition, we observed that closely related secondary metabolites in P- or C1/R-expressing cells are targeted to different subcellular compartments by specific intracellular trafficking pathways. Perhaps surprisingly, chalcone isomerase is not required for flux through any of the flavonoid pathways regulated by P or C1/R. Finally, molecular analysis of our transgenic cell lines corroborates the recent suggestion that the gene encoding P can act as a major quantitative trait locus controlling silk maysin levels in maize (Byrne et al., 1996b).

### Metabolites Induced by P and C1/R in BMS Cells and in Plant Tissues

Figure 4 summarizes the compounds induced by P or C1/R expression in BMS that we identified in this work, and Figure 8 illustrates our current understanding of the biosynthesis of flavonoids and related compounds in maize. The grid shown



**Figure 3.** Accumulation of Ferulic Acid and C-Glycosyl Flavones in P-Expressing BMS Cells.

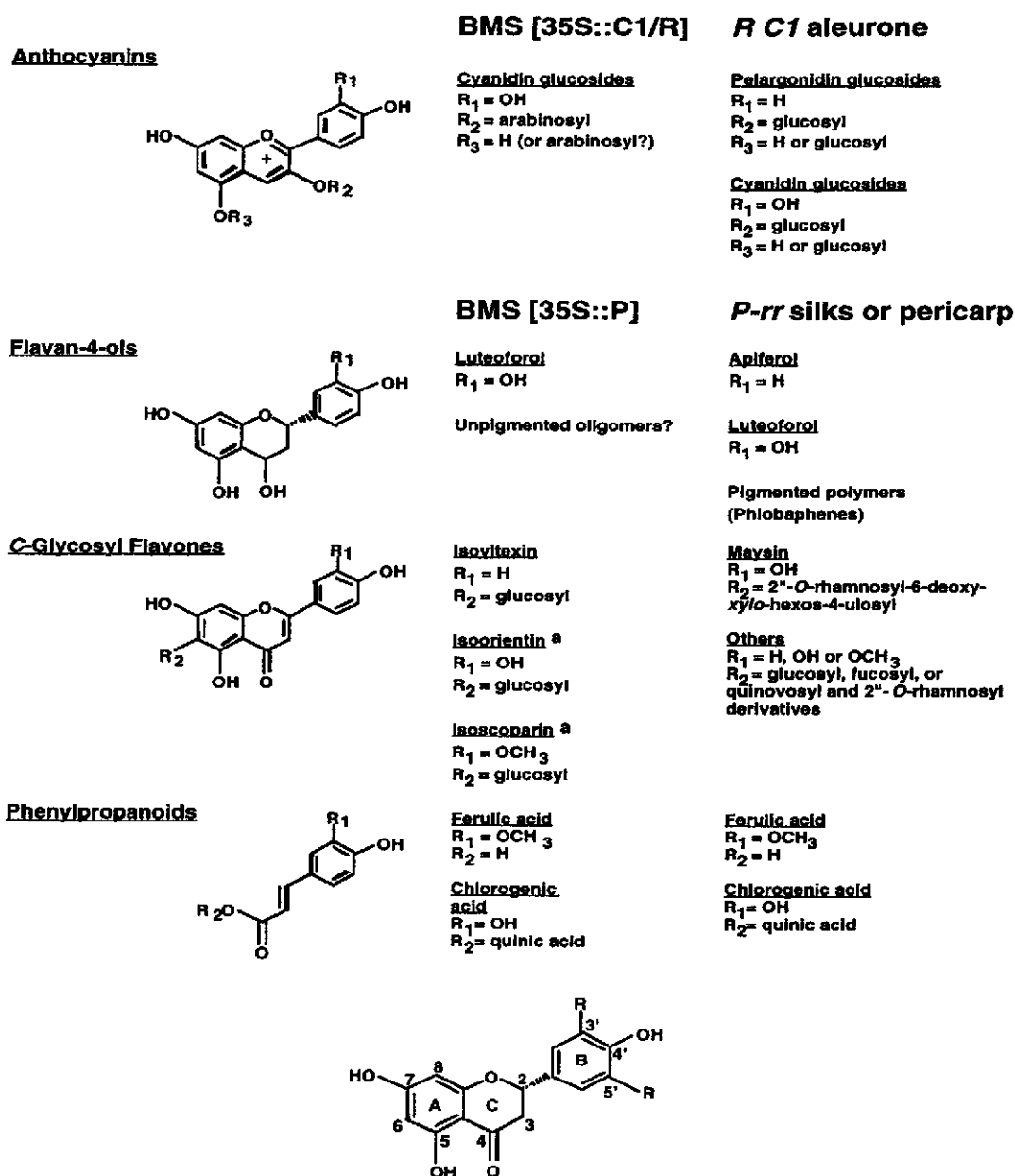
**(A)** Reverse-phase HPLC profiles of extracts from untransformed BMS cells.

**(B)** Reverse-phase HPLC profiles of extracts from BMS cell line 14/2-15 expressing P.

Major peaks absorbing at 340 nm are labeled as follows: a, ferulic acid; b, isoorientin; c, isovitexin; d, isoscoparin; e, probably chlorogenic acid; and is, chrysin, an internal reference standard that was loaded at threefold higher concentration in **(B)** than in **(A)**. mAU, multi-absorption units.

in Figure 8 is consistent with the substrate specificities reported for flavonoid 3'-hydroxylase and two flavonoid 3'-O-methyltransferases in maize, and with the observation that oxidation and methylation generally precede glycosylation during flavonoid biosynthesis (Larson et al., 1986; Larson, 1989; Tobias and Larson, 1991).

To produce C-glycosyl flavones (e.g., isovitexin, isoorientin, or isoscoparin, labeled IV, IO, and IS, respectively, in Figure 8)



**Figure 4.** Comparison of Compounds Accumulating in Transgenic BMS Cells and in Maize Plant Tissues.

Anthocyanins found in C1/R-expressing BMS cells in culture or in *R C1* aleurone are compared in the upper panel. Flavan-4-ols, *C*-glycosyl flavones, and major phenylpropanoids found in P-expressing BMS cells in culture or in *P-rr* silks or pericarp are shown in the middle panel; <sup>a</sup> indicates that low quantities of the 2''-*O*-rhamnosyl derivatives were also detected. At the bottom, a generic flavonoid structure is shown; rings are labeled A to C, and carbon atoms at which substitutions can occur are numbered 2 to 8 and 3' to 5'.

from flavanones, enzymes are required for 2,3-*cis*-desaturation and 6-*C*-glucosylation. The only flavonoid *C*-glucosyl transferase described from plants utilizes 2-hydroxyflavanones but not flavones or flavanones as substrates (Kerscher and Franz, 1987, 1988). 2-Hydroxyflavanones have also been

postulated as intermediates in 2,3-*cis*-desaturation of flavanones by the two forms of flavone synthase that have been described in the literature. However, an alternative mechanism for 2,3-*cis*-desaturation of flavanones has also been proposed (Britsch, 1990), so the mechanism by which



2-hydroxyflavanones are generated for C-glycosyl flavone biosynthesis is still unclear. Given that some 2-hydroxyflavanones have been reported as natural products in several plants (Harborne, 1988), it is possible that an as yet uncharacterized enzyme (i.e., flavanone 2-hydroxylase labeled as F2H in Figure 8) catalyzes their synthesis.

An alternative possibility is that C-glycosylation in maize proceeds via a different mechanism than that described in the dicot *Fagopyrum esculentum* (Kerscher and Franz, 1987, 1988). The *F. esculentum* enzyme produces a mixture of 8-C- and 6-C-glucosyl flavones, through tautomerization of the C-glycosylated 2-hydroxyflavanone intermediates. In the P-expressing BMS cells analyzed here and in silks of maize (Elliger et al., 1980; Snook et al., 1995), only 6-C-glycosyl flavones have been described, suggesting that a similar tautomerization may not occur. Likewise, spontaneous cyclization of naringenin chalcone that might occur in the absence of chalcone isomerase should produce (+) isomers of naringenin and its derivatives, but these have not been reported in maize previously. The presence or absence of (+) flavone and flavanone isomers in the engineered cell lines reported here was not investigated rigorously. If, as seems likely, (+) isomers are not present, O-glycosylation of naringenin chalcone at the free C-ring hydroxyl (Figure 4) and further enzyme-catalyzed rearrangement might generate the (–) C-glycosyl flavone isomers in the absence of chalcone isomerase. Resolution of these issues may be facilitated by the cell lines in which activation of this pathway by P can be induced by estradiol. Following the elegant approach of Eberhardt et al. (1993), it may be possible to use nuclear magnetic resonance techniques to elucidate the structure of intermediates labeled at high specific activity with carbon-13 in vivo after induction.

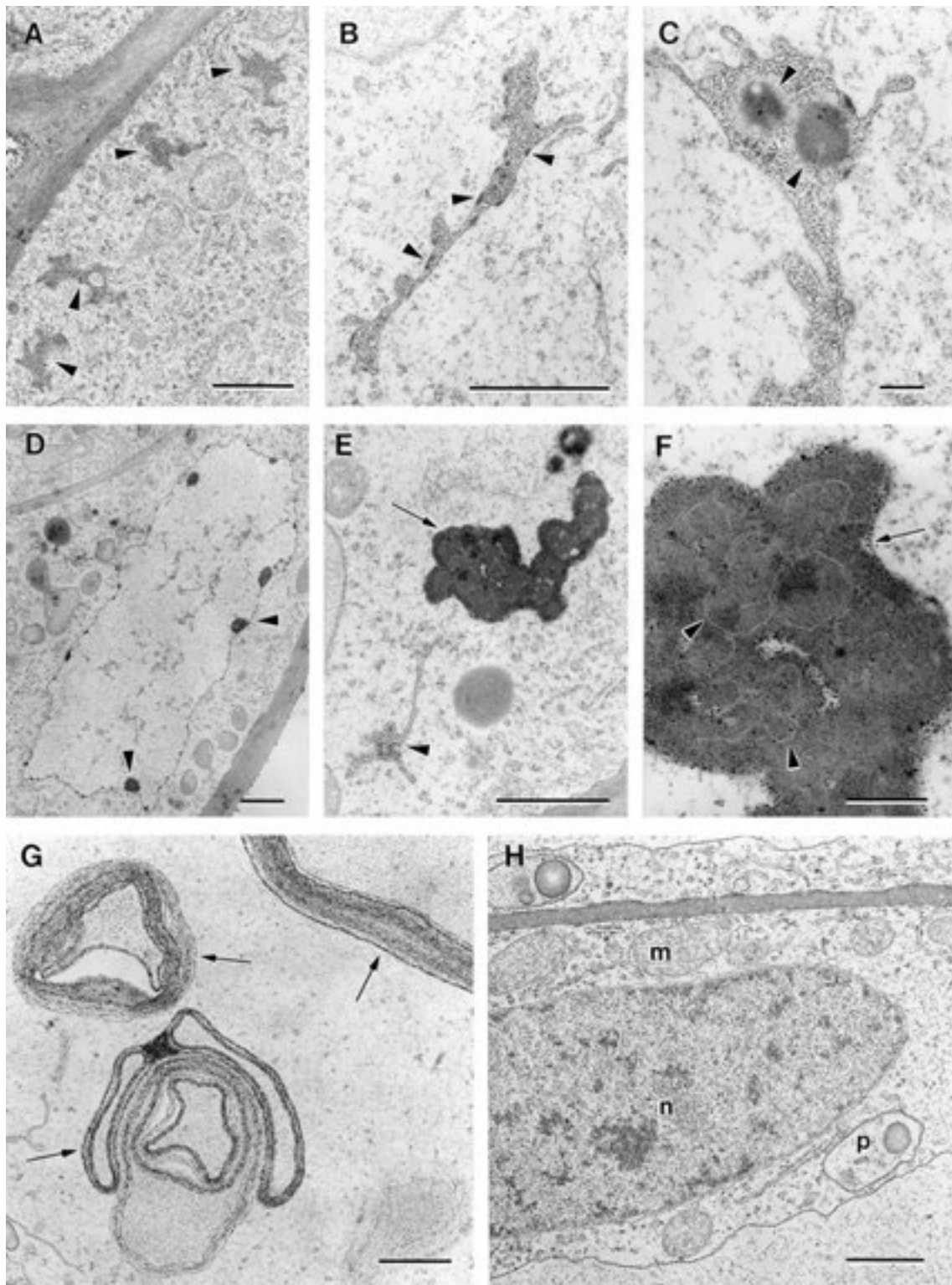
In maize floral organs, the *p1* gene, encoding the P protein, is necessary for the accumulation of C-glycosyl flavones derived from flavanones, and the 3-deoxyanthocyanidin and phlobaphene pigments derived from flavan-4-ols (e.g., apiferol and luteoforol, labeled A and L, respectively, in Figure 8) (Styles and Ceska, 1977). Previously, we demonstrated that *p1* encodes a Myb domain transcription factor that can bind directly to and transactivate the *a1* gene, which is involved in both 3-deoxy and 3-hydroxy flavonoid biosynthesis (Grotewold et al., 1991a, 1994). Here, we demonstrate that ectopic expression of *p1* in callus cells is sufficient to induce two branches of 3-deoxy flavonoid biosynthesis known to occur in maize floral organs (Figure 8). Specifically, we confirmed the accumulation of the flavan-4-ol luteoforol and several C-glycosyl flavones (Figures 4 and 8). In contrast to *p1*-expressing plant tissues, however, pigmented flavan-4-ol derivatives such as phlobaphenes and 3-deoxyanthocyanins did not accumulate to visible levels in callus. In BMS cells, P may be unable to activate one or more of the genes that are necessary for 3-deoxyanthocyanin and phlobaphene biosynthesis in maize floral organs (e.g., *bp1*; Figure 8; Byrne et al., 1996b). However, we also were unable to detect either phlobaphenes or 3-deoxyanthocyanins after in-

roduction of 35S::P by microprojectile bombardment into other maize cell types, including cells of *P-ww* pericarps at several stages of development (Grotewold et al., 1994; data not shown). Thus, we suspect that phlobaphene formation might be associated with the formation of secondary or tertiary cell walls or other physiological factors associated with terminal differentiation of maize inflorescence tissues. Formation of 3-deoxyanthocyanins may require the presence or absence of additional factors preferentially associated with silks, such as the *salmon silk (sm)* gene (Figure 8; Coe et al., 1988; Styles and Ceska, 1989).

The main difference between the predominant flavones induced by P in BMS and those found in *p1*-expressing silks resides in the glycosyl substituents (Figure 4). Many of the C-glycosyl flavones found in silks are toxic to the insect pests corn earworm and fall armyworm (Waiss et al., 1979; Elliger et al., 1980). The most active compound, maysin, is analogous to isoorientin, except that the glucose moiety is replaced with 2''-O-rhamnosyl deoxy-xylo-hexos-4-ulose (Figure 4). Two other compounds analogous to isovitexin and isoscoparin, but with the same disaccharide and similar insecticidal activity to maysin, have also been reported in silks (Snook et al., 1995).

C-Glycosyl flavones are widely distributed among the plant kingdom, but unlike the much better characterized anthocyanin pathway, relatively little is known about the enzymology or regulation of their biosynthesis (Heller and Forkmann, 1988; Kerscher and Franz, 1988). In some plants, they have been implicated as UV protectants (Les and Sheridan, 1990; Tevini et al., 1991), which is consistent with an increase in UV tolerance exhibited by P-expressing callus in culture (X. Li, personal communication). In maize, isoorientin and isovitexin have been previously isolated from silks, where they are known to be partially responsible for silk browning upon oxidation (Levings and Stuber, 1971). This trait has been associated previously with the *p1* locus (Coe et al., 1988; Byrne et al., 1996a) and presumably is related to the susceptibility of P-expressing cells to browning in vitro (Figures 1D and 1E). Phenolic oxidation could contribute to the slow growth rate of P-expressing callus and to the comparatively low levels of P expression, as could accumulation of isovitexin, which is known to be phytotoxic at high concentrations (Wagner and Van Brederode, 1996). Isovitexin has also been identified in other cereals as a pharmacologically active antioxidant (Ramarathnam et al., 1989; Shibamoto et al., 1994).

The glycosyl substituents of cyanidin produced by expression of C1/R in plant tissues and the transgenic cell lines were also different (Figure 4). These observations suggest either that the sugars added in callus and plants are determined by different glycosyl transferases with very strict substrate specificities or that similar enzymes that can accept a variety of substrates are responsible, and the sugars added reflect the availability of specific UDP-sugar conjugates within the different cell types. The substrate specificities of flavonoid C-glycosyl transferases are reportedly quite



**Figure 5.** Ultrastructure of BMS Cells Expressing C1 and R.

**(A)** Dilated and irregular-shaped SER (arrowheads) with fine granular material within their lumens.

broad (Kerscher and Franz, 1988), but those of *O*-glycosyl transferases generally are not (Heller and Forkmann, 1988). Thus, it is surprising to find that C1 and R regulate the accumulation of arabinosyl cyanidins in BMS cells, in contrast to the glucosyl cyanidins found in plant tissues (Figure 4). Whether the C1/R-regulated flavonoid *O*-glycosyl transferase encoded by *bz1* (Larson and Coe, 1977), which is expressed in C1/R-expressing BMS cells (Figure 7A), can utilize UDP-arabinose as a substrate or whether another enzyme might be responsible for arabinosylation of cyanidin in BMS is not clear.

### Novel Regulatory Roles for P

Previous genetic and biochemical studies have indicated that the *p1* gene is required for the accumulation of *C*-glycosyl flavones that are toxic to corn earworm and fall armyworm in maize silks (Styles and Ceska, 1977; Byrne et al., 1996a, 1996b). Our results show unequivocally that P is sufficient for regulating the biosynthesis of *C*-glycosyl flavones in maize cells. These findings suggest that manipulation of P function in vivo could decrease damage caused by these insect pests.

The elevation in ferulic acid level in cells expressing P was unexpected but demonstrates that *p1* expression can affect the levels of specific phenylpropanoids that are not direct flavonoid precursors (Figure 8). It is not clear whether P regulates directly at the transcriptional level any of the genes required for phenylpropanoid or ferulic acid biosynthesis or if the accumulation of ferulic acid is merely a consequence of mass action, feedback, or homeostasis at the biochemical level. Recently, it was demonstrated that snapdragon Myb proteins can regulate genes involved in both flavonoid and phenylpropanoid biosynthesis (Moyano et al., 1996). Among the metabolites that accumulate in BMS cells expressing P, however, there is a striking similarity between the structure of ferulic acid and the *C*-glycosyl flavone isoscaparin (Figure 4). This suggests that one way P could elevate the level of ferulic acid is by upregulating the activity of a catechol *O*-methyltransferase that can utilize either a flavonoid or a phenylpropanoid as a substrate (Figure 8).

Ferulic acid is a precursor of lignin (reviewed in Whetten and Sederoff, 1995), and in grasses such as maize, ferulate moieties play an important role in other cell wall polymerization processes, such as cross-linking between polysaccharides (Ralph et al., 1994). The pericarp is a tissue in which secondary and tertiary cell wall changes involving lignification occur at late developmental stages (Randolph, 1936). Although immature pericarps accumulate significant amounts of ferulic acid independently of *p1* expression (data not shown), P could conceivably target ferulic acid or its derivatives toward different fates or even different cellular compartments. One possibility that remains to be investigated, for example, is whether ferulic acid is a component of or is required in the formation of phlobaphene polymers.

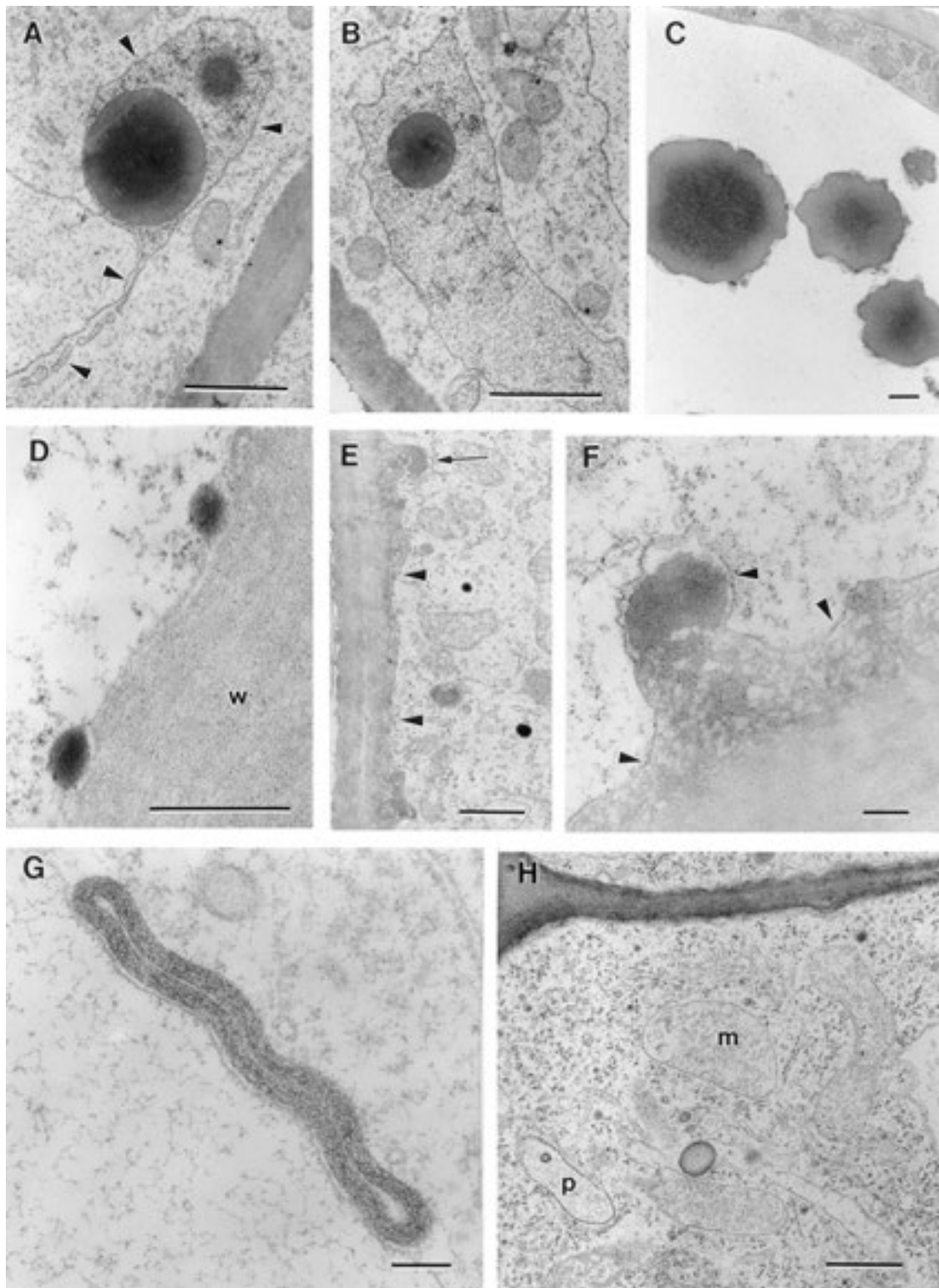
Recently, the *p1* locus also has been implicated in controlling the levels of a ferulic acid-related phenylpropanoid ester, chlorogenic acid, in maize silk tissue (Byrne et al., 1996b). This observation was corroborated by the appearance of a peak having a UV spectrum indistinguishable from chlorogenic acid in the P-expressing cells examined in the current study. Recently, it was shown that chlorogenic acid also accumulated in transgenic tobacco plants overexpressing phenylalanine ammonia-lyase (Howles et al., 1996), suggesting that upregulation of flux through the phenylpropanoid pathway could perhaps result in accumulation of this compound by mass action.

In addition to the accumulation of *C*-glycosyl flavones luteoforol and ferulic acid, BMS lines expressing P accumulated at least two autofluorescent compounds, found in distinct subcellular compartments (see below). This unanticipated phenotypic property of ectopic P expression suggests that P could be used as an alternative visual marker for tracking transformed cell lineages in maize, much as anthocyanin pigmentation induced by C1 and/or R has been used previously (Ludwig et al., 1990; Bowen, 1992).

A small number of radiolabeled proteins that were absent from untransformed BMS cells were detected in either the P- or C1/R-expressing BMS cell lines by two-dimensional gel electrophoresis (data not shown). This number was of the order of magnitude expected, if the sole functions of P or C1/R are *de novo* induction of some or all of the enzymes and trafficking components required for 3-deoxy and 3-hydroxy

**Figure 5.** (continued).

- (B) Osmiophilic material (arrowheads) condensed inside the SER membrane.  
 (C) Osmiophilic spherical deposits (arrowheads) within the lumen of the SER. Bar = 0.2  $\mu$ m.  
 (D) Highly dilated SER with electron-dense deposits (arrowheads) inside the membrane. SER nearly fills the lumen of the cell.  
 (E) SER filled with electron-dense material (arrow) and an irregular-shaped SER (arrowhead) similar to those seen in (A).  
 (F) Fine detail of SER shown in (E) highlighting the delimiting membrane (arrow) and spherical outlines of membrane-like substructures (arrowheads) within the SER lumen. Bar = 0.2  $\mu$ m.  
 (G) Multilamellar bodies (arrows) within the cytosol of C1/R-expressing cells.  
 (H) Nontransformed BMS cell for comparison with (A) through (G). m, mitochondria; n, nucleus; p, amyloplast.  
 Bars = 1  $\mu$ m, unless noted otherwise.



**Figure 6.** Ultrastructure of P-Expressing BMS Cells.

**(A)** Partially dilated SER (arrowheads) containing spherical deposits of osmiophilic material.

flavonoid accumulation, respectively. No proteins were obviously downregulated by either P or C1/R.

### Chalcone Isomerase

In this study, we found that no chalcone isomerase transcript or enzymatic activity could be detected in C1/R- or P-expressing BMS cells (Figure 7A). This is in sharp contrast to what has been found in the plant, where *chi* transcripts are found in several tissues expressing P or C1/R homologs (Grotewold and Peterson, 1994; Deboo et al., 1995; Chopra et al., 1996). We suggest that *chi* gene activation in maize may require factors in addition to either P or C1/R that may not be present in the BMS cells used here. Thus, *chi* genes may be regulated differently from *c2* and *a1* in maize. In dicots, genes encoding chalcone synthase and chalcone isomerase are also regulated differently from other flavonoid biosynthetic genes (Koes et al., 1994; Shirley, 1996).

Whatever the mechanisms underlying *chi* regulation in maize, our data show that flavonoids can form in vivo without chalcone isomerase. Presumably, isomerization of chalcones to flavanones can occur spontaneously, as it can in vitro (Mol et al., 1985). Previous evidence for in vivo isomerization of chalcones in the absence of chalcone isomerase has been obtained in *Dianthus* (Forkmann and Dangelmayr, 1980) and *petunia*, in which the *po* mutation inhibits *chiA* expression in anthers (Van Tunen et al., 1991), but flavonoids essential for male fertility still accumulate (Ylstra et al., 1994). In maize, isomerization of chalcones in vivo in the absence of *chi* enzymatic function could provide an explanation for the lack of available *chi* mutants (Grotewold and Peterson, 1994).

### Evolutionary Implications and Role of *p1* as a Quantitative Trait Locus

Silk maysin content was analyzed recently as a quantitative trait by using probes linked to flavonoid biosynthetic and regulatory loci to detect restriction fragment length polymor-

phisms (Byrne et al., 1996b). A major QTL accounting for >50% of the observed genetic variation in an F<sub>2</sub> population derived from a hybrid cross was found to cosegregate with restriction fragment length polymorphisms associated with the *p1* locus. Gene action at this locus was additive. Very little is known about the effect of different levels of *p1* expression on the activation of downstream genes, despite the large number of available *p1* alleles (Athma et al., 1992; Moreno et al., 1992). In transgenic cell lines expressing P at different levels, we found that P dictated the level of downstream biosynthetic gene expression and that the relationship between the levels of P mRNA accumulation and the expression of the downstream genes was close to linear over a broad range. These observations demonstrate how two alleles that differ in the expression level of *p1* could be sufficient to account for not only the variation observed in the QTL study but also the additive gene action. Alternatively, the alleles scored by Byrne et al. (1996b) could express similar levels of *p1* mRNA but encode proteins with different abilities to activate downstream genes such as *a1* and *c2*. Precedence for this possibility also exists in the literature (Chopra et al., 1996). With either hypothesis, it seems reasonable to conclude from these studies that *p1* itself, and not a closely linked gene, can act as a QTL for 3-deoxy flavonoid biosynthesis in maize silks. Candidate loci for other QTL in maize had been suggested previously, but none has been shown to correspond as definitively by the criteria outlined here.

Although P is necessary and sufficient for C-glycosyl flavone accumulation, C1 clearly is not. This may explain why maize has retained two very similar Myb-related transactivators that regulate redundant target genes (i.e., *c2* and *a1*). Expression of *p1* in maize floral organs could conceivably increase reproductive fecundity by reducing insect predation, thereby conferring a selective advantage. In contrast, expression of C1/R or PI/B would activate genes, in addition to those regulated by P, that would lead to accumulation of anthocyanins, which are not known to have insecticidal properties analogous to those conferred by C-glycosyl flavones. Thus, despite their similarity in structure and function and the apparent redundancy in the target genes they regulate, P

Figure 6. (continued).

(B) Fully dilated SER with an electron-dense body within its lumen.

(C) Portion of a vacuole containing large spherical osmiophilic deposits.

(D) Pockets of osmiophilic material between the plasmalemma and cell wall (w). Bar = 0.2 μm.

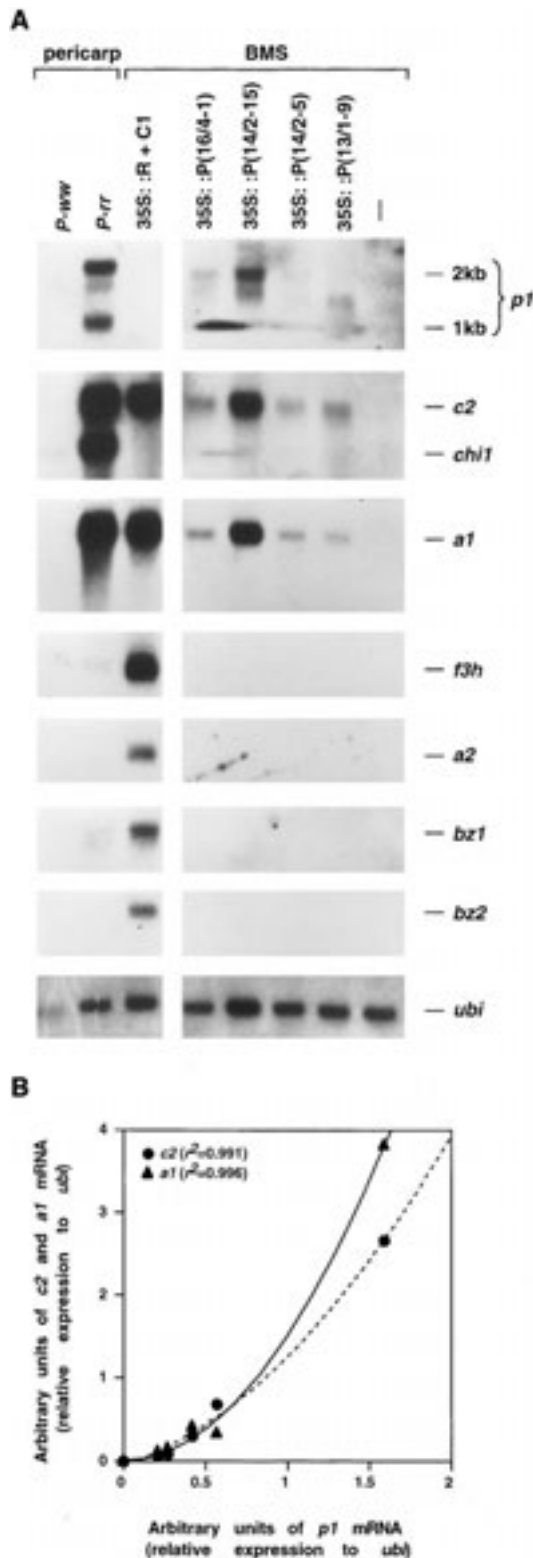
(E) Large pocket of osmiophilic material (arrow) and extensive accumulation of osmiophilic material between the cell wall and plasmalemma (arrowheads).

(F) A portion of (E) at higher magnification showing the formation of a matrix of electron-dense material between the cell wall and plasmalemma (arrowheads). Bar = 0.2 μm.

(G) Multilamellar body within the cytosol of P-expressing cells. Bar = 0.2 μm.

(H) Nontransformed BMS cells for comparison with (A) through (G). m, mitochondria; p, amyloplast.

Bars = 1 μm, unless noted otherwise.



**Figure 7.** Flavonoid Biosynthetic Gene Transcripts in P- and C1/R-Expressing BMS.

and C1 can confer very different phenotypes that could potentially have been retained by unique selective constraints.

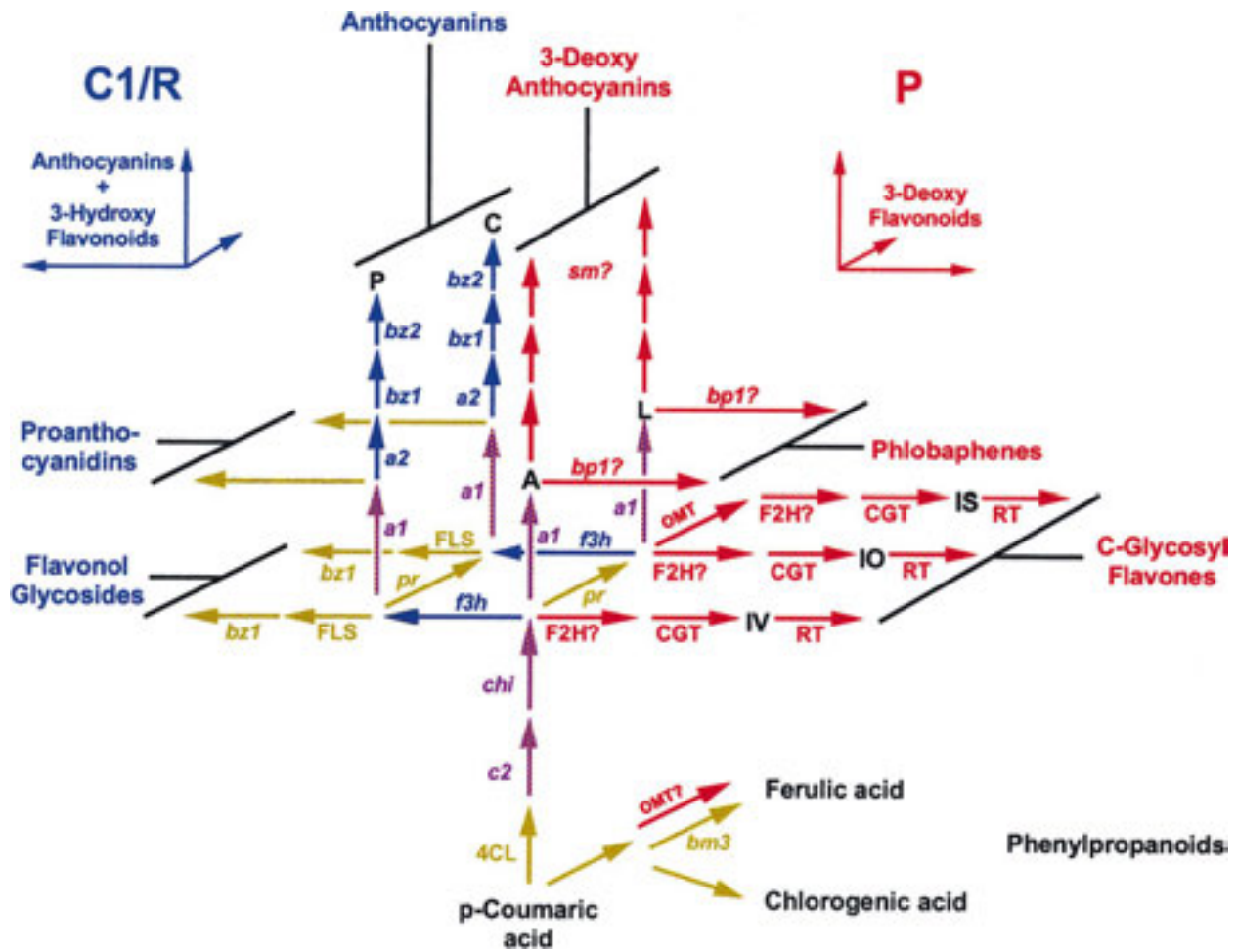
### Differential Trafficking of Secondary Metabolites

The fluorescent compounds found in P-expressing cell lines accumulated in vesicles derived from the SER and deposited their contents in either the central vacuole or the cell wall (Figure 5). Thus, secretion of P-induced secondary metabolites via ER-derived vesicles closely resembles exocytosis of low molecular weight compounds (e.g., neurotransmitters) in animal cells (Bennett and Scheller, 1994; Bennett, 1995). In contrast to P-expressing cells, no evidence for secretion was seen in C1/R-expressing cells; anthocyanoplasts derived from the SER were destined exclusively for the central vacuole. In general, glycosylated flavonoids are sequestered in the vacuole, whereas the more reactive flavonoid aglycones are thought to be secreted (Stafford, 1990). Because flavan-4-ols are thought to be precursors of the phlobaphenes found in the cell walls of mature floral tissues of maize (Styles and Ceska, 1989), the aglycone of luteoforol might be one of the compounds secreted in P-expressing cells. It is possible that esterified forms of ferulic acid, similar to those thought to be exported by cells undergoing lignification (Campbell and Sederoff, 1996), may also be secreted in P-expressing cells. Further work is needed to determine which compounds induced by P accumulate intracellularly and which are secreted.

Although much is known about the sorting of proteins and membrane components inside eukaryotic cells, secondary metabolite trafficking has received very little study. Plants frequently accumulate much higher levels of low molecular weight compounds than animals, and an enormous number (>100,000) of plant secondary metabolites has been described (Luckner, 1990). Thus, mechanisms for ensuring differential

**(A)** Poly (A)<sup>+</sup> RNA from a C1/R-expressing BMS line (35S::R+C1), P-expressing BMS lines (35S::P), untransformed BMS cells (-), or pericarp (*P-rr* or *P-ww*) was hybridized with probes corresponding to *p1* (P; the 2- and 1-kb *P-rr* transcripts are indicated), *c2* (chalcone synthase), *chi1* (chalcone isomerase), *a1* (flavanone/dihydroflavonol NADPH-dependent reductase), *f3h* (flavanone 3-hydroxylase), *a2* (proanthocyanidin synthase), *bz1* (UDP-glucose:flavonoid 3-O-glucosyltransferase), and *bz2* (a glutathione S-transferase required for vacuolar uptake). A probe for ubiquitin (*ubi*) was used as a loading control.

**(B)** Levels of *c2*, *a1*, and *p1* mRNA were quantified by using a PhosphorImager to analyze RNA gel blots (images not shown) that were normalized to the level of *ubi* mRNA. The graph shows normalized values for *c2* (circles) and *a1* (triangles) transcript levels plotted against the level of P transcript seen in five BMS cell lines independently transformed with 35S::P (the cluster of data points at the lower left) and *P-rr* pericarps (the two highest data points). Correlation coefficients ( $r^2$ ) are shown for the quadratic curves that best fit the two data sets.



**Figure 8.** Summary of 3-Deoxy and 3-Hydroxy Flavonoid Biosynthesis in Maize.

Enzymatic steps required for the formation of either C1/R-regulated anthocyanin pigments or P-regulated phlobaphene pigments, 3-deoxyanthocyanins, and C-glycosyl flavones are color-coded blue or red, respectively. Enzymatic steps common to both branches of the pathway are colored purple, and steps not known to be regulated by P or C1/R, or not directly involved in the formation of the P and C1/R-regulated flavonoids, are colored olive. Genes known to be responsible for specific steps in maize are shown in lowercase letters (*c2*, chalcone synthase; *chi*, chalcone isomerase; *f3h*, flavanone 3-hydroxylase; *pr*, flavonoid 3'-hydroxylase; *a1*, flavanone/dihydroflavonol NADPH-dependent reductase; *a2*, proanthocyanidin synthase; *bz1*, UDP-glucose:flavonoid 3-O-glucosyltransferase; *bz2*, a glutathione S-transferase required for vacuolar uptake; *bm3*, caffeic acid 3-O-methyltransferase; *bp1*, *brown pericarp*; *sm*, *salmon silk*), whereas enzymatic activities for which corresponding genes have not been characterized in maize are shown in capital letters (FLS, flavonol synthase; OMT, flavonoid 3' O-methyltransferase; F2H, flavanone 2-hydroxylase; RT, rhamnosyl transferase; CGT, C-glycosyl transferase; 4CL, 4-coumarate:coenzymeA ligase). Key products or intermediates of relevance to this study are indicated by capital letters in black (IS, isoscaparin; IV, isovitexin; IO, isoorientin; A, apiferol; L, luteoforol; C, cyanidin; and P, pelargonidin). The derivation of ferulic and chlorogenic acid from *p*-coumaric acid, a phenylpropanoid precursor of flavonoids, is also indicated.

compartmentalization of secondary metabolites may have evolved that might not be found in other organisms. Further study of the cell lines described here, particularly those in which P is under inducible control, could shed light on this neglected and potentially fruitful area of plant cell biology. Clearly, more than one type of endosomal compartment is found in plant cells (Paris et al., 1996), but factors that determine either

sorting of metabolites to specific vesicle types or fusion of vesicles to target membranes have not been well characterized. One way in which sorting of metabolites may occur is via vesicle-specific transporters. In maize, the *bz2* gene conjugates anthocyanins with glutathione, and the glutathione conjugates are then specifically imported into a vacuolar compartment by a glutathione transporter (Marrs et al., 1995). In barley,

however, the C-glycosyl flavone isovitexin is transported into vacuoles by a glutathione-independent mechanism requiring a vacuolar H<sup>+</sup>-ATPase (Klein et al., 1996). It is not clear from these reports whether both transporters reside on the same endosomal compartment in vivo or whether they are segregated into separate vesicles. If specific vesicle types are differentiated at this level, they could also harbor or recruit different v-SNAREs that might target them for fusion either with the tonoplast or the plasmalemma (Bennett, 1995). Thus, it will be interesting to see if genes encoding transporters or SNARE components are regulated by either P or C1/R in a fashion similar to those encoding flavonoid biosynthetic enzymes.

### Engineering Secondary Metabolite Production in Plant Cells

Classic approaches used to manipulate the production of metabolites in plant cells that require the genetic engineering of specific enzymes in a given pathway are often limited either by flux considerations (Kacser and Burns, 1973, 1981; Niederberger et al., 1992) or by the plasticity of plant metabolism (reviewed in ap Rees, 1995; Herbers and Sonnewald, 1996). In this study, we have shown clearly that ectopic expression of appropriate regulatory genes constitutes a viable strategy for engineering secondary metabolite production in plant cell cultures. It is likely that transcriptional control and metabolic flux considerations each play a role in dictating the final levels of flavonoids and phenylpropanoids observed in these transgenic cell lines. As predicted by theory (Kacser and Burns, 1973, 1981), however, and validated previously by experimentation in lower organisms (Niederberger et al., 1992), coordinate upregulation of many or all of the necessary enzymes in each flavonoid biosynthetic pathway led to dramatic increases in product accumulation. Overexpression of one or more rate-limiting branch point enzymes in either pathway (e.g., chalcone synthase or flavanone 3-hydroxylase) would not have been a viable strategy in this instance, because few, if any at all, of the remaining enzymes are expressed in untransformed BMS cells. Extension of our study to commercially valuable secondary metabolite production will first require identification of transcription factors or other signal transducers that regulate the biosynthesis of targeted compounds. As genes encoding key biosynthetic enzymes from a variety of interesting biosynthetic pathways unique to plants are cloned and characterized, this will become increasingly feasible.

## METHODS

### Plant Materials

Maize stocks containing either *P-rr-4B2* (red pericarp and cob glumes; Grotewold et al., 1991b) or *P-ww-1112* (white pericarp and

white cob; Athma and Peterson, 1991) were grown under standard field or glasshouse conditions. The Black Mexican Sweet (BMS) cells (P2 No. 10) used in this study were maintained in Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2,4-D at 1 to 2 mg/L, either as callus on medium solidified with Phytigel (Sigma) or as suspension cultures in liquid medium shaken at 150 rpm. Both types of cultures were grown in the dark at 27°C. The genotype of BMS is *P-ww A1 A2 C1 C2 R-g b pl* (D. Styles, personal communication), but the cultures were derived originally from cambial tissue in the hypocotyl that would not express P, C1, Pl, B, or R in this genetic background (Sheridan, 1982; Coe et al., 1988).

### Transformation

BMS cells were pretreated for 12 to 18 hr in liquid media containing 3% polyethylene glycol (8000 molecular weight) and transformed by microprojectile bombardment with pPHP610 (35S::BAR) and either pPHP687 (35S::R + 35S::C1) or pPHP1962 (formerly pPHI687 and pPHI1962; Grotewold et al., 1994). Plasmids have been described previously (Grotewold et al., 1994), except for pPHP610, which is similar to pPHP611 (formerly pPHI611; Grotewold et al., 1994), but has the nopaline synthase polyadenylation fragment from pPHP459 (formerly pPHI459; Ludwig et al., 1990) instead of the *pinII* sequence, and the plasmids used for expressing P under estradiol-inducible control, which will be described in more detail elsewhere (B.A. Roth, C. Brouwer, C. Brooke, B. Bowen, and P. Solan, manuscript in preparation). Approximately 40 ng of each plasmid was used per bombardment. After bombardment, cells were transferred to fresh solid media, resuspended in a small volume of liquid medium after 48 hr, and replated on solid media containing 5 mg/L BASTA (AgrEvo, Wilmington, DE). BASTA-resistant colonies were identified between 4 and 6 weeks later and maintained on selective media.

### Identification and Characterization of Compounds Accumulating in Transgenic BMS Cell Lines

#### Identification of Luteoforol

To detect the presence of flavan-4-ols, ~100 mg of callus was macerated with a plastic grinder in an Eppendorf tube containing 30% HCl/70% butanol (v/v) at room temperature (Watterson and Butler, 1983). After 1 hr, the cell debris was pelleted by centrifugation and the relative flavylum ion concentration was measured by absorbance at 550 nm. For comparison between samples, absorbance values were adjusted according to the mass of tissue used. For spectral analysis, ~500 mg of fresh callus cells or frozen pericarp tissue was ground in a tube with 1 mL of 30% HCl/70% butanol (v/v) and incubated for 60 min at 37°C. Samples were then spun for 10 min at ~14,000g, and the absorption spectra of the supernatants were determined. Acidic butanol extracts from P-expressing cells had a  $\lambda_{\max}$  of 559 nm, similar to the  $\lambda_{\max}$  of 564 nm found in extracts made from immature *P-rr* pericarps (Figures 2A and 2B). Extracts made from C1/R-expressing cells had a  $\lambda_{\max}$  of 533 nm (Figures 2A and 2C). The 559-nm peak in extracts from either the P-expressing cells or *P-rr* pericarps was eliminated by boiling for 10 min (Figures 2D and 2E), whereas the 533-nm peak in the C1/R-expressing cell extract was unaffected (Figure 2F). This indicated that the major flavylum ions in extracts from cells expressing P were different from those found in the C1/R-expressing cell extract and likely to be de-



rived from one or more heat-labile flavan-4-ols. These compounds are notoriously unstable and readily give rise to oligomeric 3-deoxyproanthocyanidin oligomers (Stich and Forkmann, 1988; Stafford, 1990) that form flavylium ions in acidic butanol only at higher temperature.

Apiferol and luteofol are flavan-4-ols previously described from maize and sorghum that give flavylium ions in acidic butanol with a  $\lambda_{\text{max}}$  of 535 and 552 nm, respectively (Watterson and Butler, 1983). To confirm the identity of the major flavan-4-ol in P-expressing BMS cells, a methanol extract was hydrolyzed with aqueous HCl. This should convert flavan-4-ols such as apiferol and luteofol to their corresponding 3-deoxyanthocyanidins (i.e., apigeninidin and luteolinidin). The treated extract had a  $\lambda_{\text{max}}$  of 498 nm that shifted in alcoholic  $\text{AlCl}_3$  to a shoulder at 546 nm. The addition of HCl restored the absorption at 498 nm. An authentic sample of apigeninidin had a  $\lambda_{\text{max}}$  of 475 nm and did not respond to  $\text{AlCl}_3$ , whereas luteolinidin had a  $\lambda_{\text{max}}$  of 495 nm that shifted in  $\text{AlCl}_3$  to 546 nm and reverted to 498 nm upon readdition of HCl. Additional work is required to distinguish whether monomers or oligomers of luteofol accumulate preferentially in P-expressing cells (Stafford, 1990).

#### Identification and Isolation of Ferulic Acid and C-Glycosyl Flavones

Maize callus phenolic acids and flavones were analyzed by reverse-phase HPLC, as described previously (Snook et al., 1989), on an Altex Ultrasphere C18 column (5 micron,  $4.6 \times 250$  mm; Beckman, Fullerton, CA) by using a water/methanol linear gradient from 10 to 90% methanol in 35 min at a flow rate of 1 mL/min and detection at 340 nm. All solvents contained 0.1%  $\text{H}_3\text{PO}_4$ .

For each callus sample, ~7 g (dry weight) of lyophilized material was extracted in methanol three times with 20 min of ultrasonication. After filtration to remove residual debris, extracts were evaporated until only water remained. The water residue was submitted to preparative reverse-phase chromatography. Approximately 30 g of the packing material from a Waters PrepPAK 500 C18 cartridge (Millipore, Bedford, MA) was repacked into a smaller chromatography column ( $54 \times 2.54$  cm, 15 psi nitrogen pressure used to aid flow), washed with methanol, and recycled to water. Salts and sugars were washed out with water, and elution was done in steps of 10% methanol, 20% methanol, and 50% methanol. Phenolic acids and their ester derivatives eluted with 20% and flavones with 50% methanol. Ferulic acid was identified by its UV spectrum and by coelution with standard ferulic acid. Fraction e (Figure 3) had a UV spectrum indistinguishable from chlorogenic acid. After evaporation, the 50% eluant containing the flavones was dissolved in water and chromatographed on the C18 column described above, which was sequentially eluted with 30, 35, and 40% methanol in water. The eluant was monitored at 340 nm, and fractions were tested for flavones by HPLC. Three eluant fractions were identified and designated flavone fractions b, c, and d (Figure 3). Flavone fraction b (70% purity of flavone b) and c (50% purity of flavone c) contained luteolin derivatives, whereas flavone fraction d (90% purity of flavone d) was shown to contain mainly an apigenin derivative as determined by UV spectra.

Isoorientin (6-C-glucosylluteolin) was isolated from flavone fraction b by reverse-phase chromatography as above by loading in 35% methanol and eluting with 38% methanol, yielding 3 mg of isoorientin, fast atom bombardment mass spectrometry (FAB-MS) 448 (M+H, for ion mass plus hydrogen ion). Flavone fraction b comigrated on HPLC with isoorientin isolated from maize silks. Flavone fraction b also contained ~12% of 2''-O-rhamnosylisoorientin (FAB-MS 594).

Iso scoparin (3'-methoxyisoorientin) was obtained from flavone fraction d by reverse-phase chromatography as above, yielding 6 mg of 95% purity, FAB-MS 463 (M+H), in addition to small amounts of the 2''-O-rhamnosyl derivative.

Flavone fraction c contained isovitexin (6-C-glucosylapigenin) as judged from its comigration with authentic isovitexin obtained from maize silks. The low amount obtained did not permit mass spectroscopy analysis.

#### Identification of O-Arabinosyl Cyanidin

To determine the accumulation of anthocyanidin pigments, we extracted ~250 mg of tissue by using ethyl acetate, and the ethyl acetate volume was reduced to 1 mL. The residue remaining after the extraction with ethyl acetate was then extracted with methanol containing 1% HCl, and the extract volume was reduced to 1 mL. The acidic methanol extracts were analyzed by HPLC and thin-layer chromatography. HPLC was done on C18  $\mu$ Bondpack analytical columns (Waters, Milford, MA). Sample extracts of 0.05 mL were eluted at a flow rate of 1 mL/min, in a linear gradient from 0 to 40% solvent B (10% acetic acid in methanol) in 20 min, followed by a second linear gradient to 100% solvent B in 10 min. Elutions were monitored at 280, 320, 475, and 495 nm. Thin-layer chromatography studies were done on cellulose plates (SigmaCell Type 100; Sigma) in one direction, using any of three solvent systems: acetic acid-water-HCl (30:10:3 [v/v]); formic acid-water-HCl (25:15:10 [v/v]); or butanol-acetic acid-water (4:1:5 [v/v]). Spots were observed under UV light. By these criteria and by comparison with standards, two forms of cyanidin, possible glycosides, were identified in the C1/R transgenic callus. Hydrolysis in mineral acid (10 min at 100°C or 30 min at 60°C) converted both derivatives to free cyanidin, consistent with O-glycosylation. After hydrolysis, the cyanidin was removed and the residue remaining was analyzed for sugars by using a mass spectrometer (Bioion KB, Stockholm, Sweden). By comparison with authentic standards, only arabinose was detected in the extracts.

#### Chalcone Isomerase Enzyme Assay

Chalcone was prepared from 4',5',7-trihydroxy flavanone as described previously (Moustafa and Wong, 1967). Isomerase activity was measured in extracts from BMS transgenic cell lines prepared by grinding ~200 mg of callus with liquid nitrogen and extracting proteins by using a plastic grinder in an Eppendorf tube in 100 mM potassium phosphate buffer, pH 8.0, containing 1.4 mM  $\beta$ -mercaptoethanol. Assays were performed in 100 mM potassium phosphate buffer, pH 8.0, 1.4 mM  $\beta$ -mercaptoethanol, 10 mM KCN, and 17 mM chalcone. Activity was measured by disappearance of absorption at 385 nm and corrected by subtracting the amount of flavanone formed via spontaneous isomerization.

#### Light Microscopy

Fresh tissue was mounted on glass slides in phosphate-buffered saline, pH 7.4, and viewed with a Leica (Wetzlar, Germany) DM RB microscope equipped with bright-field, phase contrast, and epifluorescence optics. Filter cubes used were from Leica (I3; excitation, 450 to 490 nm; dichroic beam splitter, 510 nm; and emission, 515 nm) or Chroma

Technology Corp. (Brattleboro, VT) (fluorescein isothiocyanate: excitation, 465 to 495 nm; dichroic beam splitter, 505 nm; and emission, 515 to 555 nm). Photomicrographs were recorded on Fuji (Tokyo, Japan) Sensia film or captured digitally with a Photometrics (Tucson, AZ) CH250 CCD camera.

### Electron Microscopy

Tissue was fixed in 2% glutaraldehyde–2% paraformaldehyde in 50 mM phosphate buffer, pH 7.2, for 4 hr at room temperature and then rinsed three times in buffer and postfixed in 1% osmium tetroxide for 2 hr at room temperature. After several further buffer rinses, tissue was dehydrated in a graded ethanol series, and acetone was used as a transitional solvent into Spurr's resin. Infiltration into pure resin was accomplished on a rotator by adding fresh resin daily over a 4-day period before casting. Thin 60- to 90-nm sections were made using a diamond knife on a Reichert (Vienna, Austria) Ultracut S microtome and placed on copper grids. Sections were stained with 5% uranyl acetate in 70% ethanol for 1 hr, then in aqueous lead citrate for 1 hr, and observed on a JEOL (Tokyo, Japan) 1200EX-II scanning transmission electron microscope at 80 kV. Images were recorded on Kodak (Rochester, NY) SO-163 film.

### RNA Purification and RNA Gel Blot Analysis

Frozen tissue was pulverized in liquid nitrogen, and total RNA was isolated from pericarp tissue or BMS cells using the Trizol reagent (Life Technologies Inc., Gaithersburg, MD), according to the manufacturer's instructions. mRNA was isolated using streptavidin-coated paramagnetic beads (PolyAtract mRNA isolation system III; Promega). Three to five micrograms of poly(A)<sup>+</sup> RNA was separated on formaldehyde-containing 1% agarose gels and probed with a *p1* cDNA fragment containing the Myb-homologous region (Grotewold et al., 1991a), a *c2* cDNA (Wienand et al., 1986), a *chi1* cDNA (Grotewold and Peterson, 1994), an *a1* cDNA (Schwarz-Sommer et al., 1987), an *f3h* cDNA (Deboo et al., 1995), an *a2* cDNA (Menssen et al., 1990), a *bz1* genomic fragment (Dooner et al., 1985), a *bz2* cDNA (Nash et al., 1990), and a ubiquitin (*ubi*) probe (Christensen et al., 1992). Quantification of hybridization bands on RNA gel blots was done using a Fuji (Tokyo, Japan) PhosphorImager.

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### REFERENCES

- Alfermann, A.W., and Petersen, M. (1995). Natural product formation by plant cell biotechnology. *Plant Cell Tiss. Organ Cult.* **43**, 199–205.
- ap Rees, T. (1995). Prospects of manipulating plant metabolism. *Trends Biotechnol.* **13**, 375–378.
- Athma, P., and Peterson, T. (1991). *Ac* induces homologous recombination at the maize *P* locus. *Genetics* **128**, 163–173.
- Athma, P., Grotewold, E., and Peterson, T. (1992). Insertional mutagenesis of the maize *P* gene by intragenic transposition of *Ac*. *Genetics* **131**, 199–209.
- Bennett, M.K. (1995). SNAREs and the specificity of transport vesicle targeting. *Curr. Opin. Cell Biol.* **7**, 581–586.
- Bennett, M.K., and Scheller, R.H. (1994). A molecular description of synaptic vesicle membrane trafficking. *Annu. Rev. Biochem.* **63**, 63–100.
- Bodeau, J.P., and Walbot, V. (1992). Regulated transcription of the maize *Bronze-2* promoter in electroporated protoplasts requires the *C1* and *R* gene products. *Mol. Gen. Genet.* **233**, 379–387.
- Bowen, B. (1992). Anthocyanin genes as visual markers in transformed maize tissues. In *GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression*, S.R. Gallagher, ed (San Diego, CA: Academic Press), pp. 163–177.
- Britsch, L. (1990). Purification and characterization of flavone synthase I, a 2-oxoglutarate-dependent desaturase. *Arch. Biochem. Biophys.* **282**, 152–160.
- Byrne, P.F., Darrah, L.L., Snook, M.E., Wiseman, B.R., Widstrom, N.W., Moellenbeck, D.J., and Barry, B.D. (1996a). Maize silk-browning, maysin content, and antibiosis to the corn earworm, *Helicoverpa zea* (Boddie). *Maydica* **41**, 13–18.
- Byrne, P.F., McMullen, M.D., Snook, M.E., Musket, T.A., Theuri, J.M., Widstrom, N.W., Wiseman, B.R., and Coe, E.H., Jr. (1996b). Quantitative trait loci and metabolic pathways: Genetic control of the concentration of maysin, a corn earworm resistance factor, in maize silks. *Proc. Natl. Acad. Sci. USA* **93**, 8820–8825.
- Campbell, M.M., and Sederoff, R.R. (1996). Variation in lignin content and composition. *Plant Physiol.* **110**, 3–13.
- Chopra, S., Athma, P., and Peterson, T. (1996). Alleles of the maize *P* gene with distinct tissue specificities encode Myb-homologous proteins with C-terminal replacements. *Plant Cell* **8**, 1149–1158.
- Christensen, A.H., Sharrock, R.A., and Quail, P.H. (1992). Maize polyubiquitin genes: Structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. *Plant Mol. Biol.* **18**, 675–689.
- Coe, E.H., Jr., Neuffer, M.G., and Hoisington, D.A. (1988). The genetics of corn. In *Corn and Corn Improvement*, 3rd ed, G.F. Sprague and J. Dudley, eds (Madison, WI: American Society of Agronomy), pp. 81–258.
- Deboo, G.B., Albertsen, M.C., and Taylor, L.P. (1995). Flavanone 3-hydroxylase transcripts and flavonol accumulation are temporally coordinate in maize anthers. *Plant J.* **7**, 703–713.
- Dooner, H.K., Weck, E., Adams, S., Ralston, E., Favreau, M., and English, J. (1985). A molecular genetic analysis of insertions in the *bronze* locus in maize. *Mol. Gen. Genet.* **200**, 240–246.
- Dooner, H.K., Robbins, T.P., and Jorgensen, R.A. (1991). Genetic and developmental control of anthocyanin biosynthesis. *Annu. Rev. Genet.* **25**, 173–199.

- Dörnenburg, H., and Knorr, D. (1996). Generation of colors and flavors in plant cell and tissue cultures. *Crit. Rev. Plant Sci.* **15**, 141–168.
- Eberhardt, T.L., Bernards, M.A., He, L., Davin, L.B., Wooten, J.B., and Lewis, N.G. (1993). Lignification in cell suspension cultures of *Pinus taeda*. *J. Biol. Chem.* **268**, 21088–21096.
- Elliger, C.A., Chan, B.G., Waiss, A.C., Lundin, R.E., and Haddon, W.F. (1980). C-Glycosyl flavones from *Zea mays* that inhibit insect development. *Phytochemistry* **19**, 293–297.
- Forkmann, G. (1991). Flavonoids as flower pigments: The formation of the natural spectrum and its extension by genetic engineering. *Plant Breed.* **106**, 1–26.
- Forkmann, G., and Dangelmayr, B. (1980). Genetic control of chalcone isomerase activity in flowers of *Dianthus caryophyllus*. *Biochem. Genet.* **18**, 519–527.
- Goff, S.A., Klein, T.M., Roth, B.A., Fromm, M.E., Cone, K.C., Radicella, J.P., and Chandler, V.L. (1990). Transactivation of anthocyanin biosynthetic genes following transfer of *B* regulatory genes into maize tissues. *EMBO J.* **9**, 2517–2522.
- Grotewold, E., and Peterson, T. (1994). Isolation and characterization of a maize gene encoding chalcone flavanone isomerase. *Mol. Gen. Genet.* **242**, 1–8.
- Grotewold, E., Athma, P., and Peterson, T. (1991a). Alternatively spliced products of the maize *P* gene encode proteins with homology to the DNA binding domain of Myb-like transcription factors. *Proc. Natl. Acad. Sci. USA* **88**, 4587–4591.
- Grotewold, E., Athma, P., and Peterson, T. (1991b). A possible hotspot for *Ac* insertion in the maize *P* gene. *Mol. Gen. Genet.* **230**, 329–331.
- Grotewold, E., Drummond, B., Bowen, B., and Peterson, T. (1994). The Myb-homologous *P* gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. *Cell* **76**, 543–553.
- Harborne, J.B., ed (1988). *The Flavonoids: Advances in Research since 1980*. (New York: Chapman and Hall).
- Heller, W., and Forkmann, G. (1988). Biosynthesis. In *The Flavonoids: Advances in Research since 1980*, J.B. Harborne, ed (New York: Chapman and Hall), pp. 399–425.
- Herbers, K., and Sonnewald, U. (1996). Manipulating metabolic partitioning in transgenic plants. *Trends Biotechnol.* **14**, 198–205.
- Herman, E.M., and Lamb, C.J. (1992). Arabinogalactan-rich glycoproteins are localized on the cell surface and in intravacuolar multivesicular bodies. *Plant Physiol.* **98**, 264–272.
- Howles, P.A., Sewalt, V.J.H., Paiva, N.L., Elkind, Y., Bate, N.J., Lamb, C., and Dixon, R.A. (1996). Overexpression of L-phenylalanine ammonia-lyase in transgenic tobacco plants reveals control points for flux into phenylpropanoid biosynthesis. *Plant Physiol.* **112**, 1617–1624.
- Hrazdina, G., and Jensen, R.A. (1992). Spatial organization of enzymes in plant metabolic pathways. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 241–267.
- Kacser, H., and Burns, J.A. (1973). The control of flux. *Symp. Soc. Exp. Biol.* **27**, 65–104.
- Kacser, H., and Burns, J.A. (1981). The molecular basis of dominance. *Genetics* **97**, 639–666.
- Kerscher, F., and Franz, G. (1987). Biosynthesis of vitexin and isovitexin: Enzymatic synthesis of the C-glucosyl flavones vitexin and isovitexin with an enzymatic preparation from *Fagopyrum esculentum* M. seedlings. *Z. Naturforsch.* **42c**, 519–524.
- Kerscher, F., and Franz, G. (1988). Isolation and some properties of an UDP-glucose:2-hydroxyflavanone-6(or 8)-C-glucosyltransferase from *Fagopyrum esculentum* M. cotyledons. *J. Plant Physiol.* **132**, 110–115.
- Klein, M., Weissenboeck, G., Dufaud, A., Gaillard, C., Kreuz, K., and Martinoia, E. (1996). Different energization mechanisms drive the vacuolar uptake of a flavonoid glucoside and a herbicide glucoside. *J. Biol. Chem.* **271**, 29666–29671.
- Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: Function and evolution. *Bioessays* **16**, 123–132.
- Larson, R.L. (1989). Flavonoid 3'-O-methylation by a *Zea mays* L. preparation. *Biochem. Physiol. Pflanz.* **184**, 453–460.
- Larson, R.L., and Coe, E.H. (1977). Gene-dependent flavonoid glucosyltransferase in maize. *Biochem. Genet.* **15**, 153–156.
- Larson, R.L., Bussard, J.B., and Coe, E.H., Jr. (1986). Gene-dependent flavonoid 3'-hydroxylation in maize. *Biochem. Genet.* **24**, 615–624.
- Les, D.H., and Sheridan, D.J. (1990). Biochemical heterophyly and flavonoid evolution in North American *Potamogeton* (Potamogetonaceae). *Am. J. Bot.* **77**, 453–465.
- Levings, C.S., and Stuber, C.W. (1971). A maize gene controlling silk browning in response to wounding. *Genetics* **69**, 491–498.
- Lloyd, A.M., Walbot, V., and Davis, R.W. (1992). *Arabidopsis* and *Nicotiana* anthocyanin production activated by maize regulators R and C1. *Science* **258**, 1773–1775.
- Luckner, M., ed (1990). *Secondary Metabolism in Microorganisms, Plants, and Animals*, 3rd ed. (Berlin: Springer-Verlag).
- Ludwig, S.R., Bowen, B., Beach, L., and Wessler, S.R. (1990). A regulatory gene as a novel visible marker for maize transformation. *Science* **247**, 449–450.
- Marrs, K.A., Alfenito, M.R., Lloyd, A.M., and Walbot, V. (1995). A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene *Bronze-2*. *Nature* **375**, 397–400.
- Menssen, A., Hohmann, S., Martin, W., Schnable, P.S., Peterson, P.A., Saedler, H., and Gierl, A. (1990). The *En/Spm* transposable element of *Zea mays* contains splice sites at the termini generating a novel intron from a *dSpm* element in the *A2* gene. *EMBO J.* **9**, 3051–3057.
- Middleton, E. (1996). The flavonoids as potential therapeutic agents. In *Immunopharmaceuticals*, E.S. Kimball, ed (Boca Raton, FL: CRC Press), pp. 227–257.
- Mol, J.N.M., Robbins, M.P., Dixon, R.A., and Veltkamp, E. (1985). Spontaneous and enzymatic rearrangement of naringenin chalcone to flavanone. *Phytochemistry* **24**, 2267–2269.
- Moreno, M.A., Chen, J., Greenblatt, I., and Dellaporta, S.L. (1992). Reconstitutive mutagenesis of the maize *P* gene by short range *Ac* transpositions. *Genetics* **131**, 939–956.
- Moustafa, E., and Wong, E. (1967). Purification and properties of chalcone-flavanone isomerase from soya bean seed. *Phytochemistry* **6**, 625–632.
- Moyano, E., Martinez-Garcia, J.F., and Martin, C. (1996). Apparent redundancy in *myb* gene function provides gearing for the control of flavonoid biosynthesis in *Antirrhinum* flowers. *Plant Cell* **8**, 1519–1532.

- Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.
- Nash, J., Luehrsen, K.R., and Walbot, V.** (1990). *Bronze-2* gene of maize: Reconstruction of a wild-type allele and analysis of transcription and splicing. *Plant Cell* **2**, 1039–1049.
- Niederberger, P., Prasad, R., Miozzari, G., and Kacser, H.** (1992). A strategy for increasing an *in vivo* flux by genetic manipulations. *Biochem. J.* **287**, 473–479.
- Nozzolillo, C., and Ishikura, N.** (1988). An investigation of the intracellular site of anthocyanoplasts using isolated protoplasts and vacuoles. *Plant Cell Rep.* **7**, 389–392.
- Paris, N., Stanley, C.M., Jones, R.L., and Rogers, J.C.** (1996). Plant cells contain two functionally distinct vacuolar compartments. *Cell* **85**, 563–572.
- Peckett, C.R., and Small, C.J.** (1980). Occurrence, location and development of anthocyanoplasts. *Phytochemistry* **19**, 2571–2576.
- Quattrocchio, F., Wing, J.F., Leppen, H.T.C., Mol, J.N.M., and Koes, R.E.** (1993). Regulatory genes controlling anthocyanin pigmentation are functionally conserved among plant species and have distinct sets of target genes. *Plant Cell* **5**, 1497–1512.
- Ralph, J., Quideau, S., Grabber, J.H., and Hatfield, R.D.** (1994). Identification and synthesis of new ferulic acid dehydromers present in grass cell walls. *J. Chem. Soc. Perkin Trans.* **1**, 3485–3498.
- Ramarathnam, N., Osawa, T., Namiki, M., and Kawakishi, S.** (1989). Chemical studies on novel rice hull antioxidants. 2. Identification of isovitexin, a C-glycosyl flavonoid. *J. Agric. Food Chem.* **37**, 316–319.
- Randolph, L.F.** (1936). Developmental morphology of the caryopsis in maize. *J. Agric. Res.* **53**, 881–917.
- Reid, L.M., Mather, D.E., Arnason, J.T., Hamilton, R.I., and Bolton, A.T.** (1992). Changes in phenolic constituents of maize silk infected with *Fusarium graminearum*. *Can. J. Bot.* **70**, 1697–1702.
- Schwarz-Sommer, Z., Shepherd, N., Tacke, E., Gierl, A., Rohde, W., Leclercq, L., Mattes, M., Berndtgen, R., Peterson, P.A., and Saedler, H.** (1987). Influence of transposable elements on the structure and function of the *A1* gene of *Zea mays*. *EMBO J.* **6**, 287–294.
- Sheridan, W.F.** (1982). Black Mexican Sweet corn: Its use for tissue culture. In *Maize for Biological Research*, W.F. Sheridan, ed (Charlottesville, VA: Plant Molecular Biology Association), pp. 385–388.
- Shibamoto, T., Hagiwara, Y., Hagiwara, H., and Osawa, T.** (1994). Flavonoid with strong antioxidative activity isolated from young green barley leaves. *Am. Chem. Soc. Symp. Ser.* **547**, 153–163.
- Shirley, B.W.** (1996). Flavonoid biosynthesis: 'New' functions for an 'old' pathway. *Trends Plant Sci.* **1**, 377–382.
- Snook, M.E., Widstrom, N.W., and Gueldner, R.C.** (1989). Reversed-phase high-performance liquid chromatographic procedure for the determination of maysin in corn silks. *J. Chromatogr.* **477**, 439–447.
- Snook, M.E., Widstrom, N.W., Wiseman, B.R., Byrne, P.F., Harwood, J.S., and Costello, C.E.** (1995). New C-4'-hydroxy derivatives of maysin and 3'-methoxymaysin isolated from corn silks (*Zea mays*). *J. Agric. Food Chem.* **43**, 2740–2745.
- Snyder, B.A., and Nicholson, R.L.** (1990). Synthesis of phytoalexins in sorghum as a site-specific response to fungal ingress. *Science* **248**, 1637–1639.
- Stafford, H.A.** (1990). *Flavonoid Metabolism*. (Boca Raton, FL: CRC Press).
- Stich, K., and Forkmann, G.** (1988). Biosynthesis of 3-deoxyanthocyanins with flower extracts from *Sinningia cardinalis*. *Biochemistry* **27**, 785–789.
- Styles, E.D., and Ceska, O.** (1975). Genetic control of 3-hydroxy- and 3-deoxy-flavonoids in *Zea mays*. *Phytochemistry* **14**, 413–415.
- Styles, E.D., and Ceska, O.** (1977). The genetic control of flavonoid synthesis in maize. *Can. J. Genet. Cytol.* **19**, 289–302.
- Styles, E.D., and Ceska, O.** (1989). Pericarp flavonoids in genetic strains of *Zea mays*. *Maydica* **34**, 227–237.
- Tevini, M., Braun, J., and Fieser, G.** (1991). The protective function of the epidermal layer of rye seedlings against ultraviolet-B radiation. *Photochem. Photobiol.* **53**, 329–333.
- Tobias, R.B., and Larson, R.L.** (1991). Characterization of maize pollen flavonoid 3'-O-methyltransferase activity and its *in vivo* products. *Biochem. Physiol. Pflanz.* **187**, 243–250.
- Tuerck, J.A., and Fromm, M.E.** (1994). Elements of the maize *A1* promoter required for transactivation by the anthocyanin *B/C1* or phlobaphene *P* regulatory genes. *Plant Cell* **6**, 1655–1663.
- Van der Meer, I.M., Stuitje, A.R., and Mol, J.N.M.** (1993). Regulation of general phenylpropanoid and flavonoid gene expression. In *Control of Plant Gene Expression*, D.P.S. Verma, ed (Boca Raton, FL: CRC Press), pp. 125–155.
- Van Tunen, A.J., Mur, L.A., Recourt, K., Gerats, A.G.M., and Mol, J.N.M.** (1991). Regulation and manipulation of flavonoid gene expression in anthers of petunia: The molecular basis of the *po* mutation. *Plant Cell* **3**, 39–48.
- Wagner, A.M., and Van Brederode, J.** (1996). Inhibition of mitochondrial respiration by the flavone aglycone isovitexin causes aberrant petal and leaf morphology in *Silene latifolia*. *Plant Cell Rep.* **15**, 718–722.
- Waiss, A.C., Jr., Chan, B.G., Elliger, C.A., Wiseman, B.R., McMillian, W.W., Widstrom, N.W., Zuber, M.S., and Keaster, A.J.** (1979). Maysin, a flavone glycoside from corn silks with antibiotic activity toward corn earworm. *J. Econ. Entomol.* **72**, 256–258.
- Watterson, J.J., and Butler, L.G.** (1983). Occurrence of an unusual leucoanthocyanidin and absence of proanthocyanidins in sorghum leaves. *J. Agric. Food Chem.* **31**, 41–45.
- Whetten, R., and Sederoff, R.** (1995). Lignin biosynthesis. *Plant Cell* **7**, 1001–1013.
- Wienand, U., Weydemann, U., Niesbach-Klöggen, U., Peterson, P., and Saedler, H.** (1986). Molecular cloning of the *c2* locus of *Zea mays*, the gene encoding chalcone synthase. *Mol. Gen. Genet.* **203**, 202–207.
- Yeoman, M.M., and Yeoman, C.L.** (1996). Manipulating secondary metabolism in cultured plant cells. *New Phytol.* **134**, 553–569.
- Ylstra, B., Busscher, J., Franken, J., Hollman, P.C.H., Mol, J.N.M., and Van Tunen, A.J.** (1994). Flavonols and fertilization in *Petunia hybrida*: Localization and mode of action during pollen tube growth. *Plant J.* **6**, 201–212.