Arabidopsis and *Nicotiana* Anthocyanin Production Activated by Maize Regulators *R* and *C1*

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Anthocyanin pathway–specific transcriptional activators *R* and *C1* from the monocot maize were expressed in two dicots, *Arabidopsis thaliana* and *Nicotiana tabacum*. Expression of *R* caused augmented anthocyanin pigmentation in both plant species and augmented trichome (hair) production in *Arabidopsis*. Alone, *C1* had no effect. Hybrid transgenic *Arabidopsis* expressing both *C1* and *R* produced anthocyanins in root, petal, and stamen tissues that normally never express anthocyanins. When *R* was expressed in the transparent testa glabrous (without anthocyanins and trichomes) mutant of *Arabidopsis*, the deficiency was complemented and both anthocyanins and trichomes were restored.

Anthocyanins are the prominent red, purple, and blue pigments of flowering plants. The chemically diverse anthocyanins are flavonoid derivatives; more than 1000 chemical moieties have been identified in angiosperms. Among other functions, anthocyanins in flowers attract pollinators and in fruit they attract dispersal agents. They also are elicited during antipathogen response and contribute to protective responses to environmental stresses such as cold, nutrient deprivation, mechanical damage, and excess ultraviolet or intense white light (*1–3*). Anthocyanins have been used as visible markers in the study of recombination, transposition, and genetic linkage in maize (*4*). Anthocyanins were among the markers in the first genetic experiments by Mendel (*5*).

Anthocyanin production in *Arabidopsis thaliana* is specifically controlled: Anthocyanins are normally produced in leaves, stems, sepals, pistils, and trichomes but not in roots, petals, or stamens. Anthocyanin production is also temporally regulated in germinating seedlings (*2*). Mutants for most of the genes in the anthocyanin biosynthetic pathway have been isolated in *Arabidopsis* and *Zea mays*. Together, these mutants form a group named transparent testa (*tt*) in *Arabidopsis*, and they each produce yellow or buff-colored seeds (wild-type seeds are brown) as a result of the absence or reduction of pigment in the outer seed coat (*6*). The *tt* mutants have reduced concentrations of or no anthocyanins in the rest of the plant. One mutant, *ttg* (transparent testa glabrous), completely lacks anthocyanins and is glabrous (devoid of trichomes or hairs) (*7*).

In maize, production of anthocyanin in the aleurone (epidermal layer of the kernel endosperm) requires functional copies of the regulatory genes *C1* and *R*, or an *R* homologue, *B* (*4*). The *C1* and *R* gene products probably interact (*8*). Consistent with its proposed role as transcriptional activator, the deduced *C1* protein product contains both acidic and basic domains and has homology to the mammalian *myb* proto-oncogenes (*9*). The *R* gene encodes a protein with an acidic and a basic domain and shares homology to the helix-loop-helix DNA binding and dimerization motif characteristic of *L-myc* and other mammalian transcriptional regulators (*10*).

The cDNAs for *C1* (2.1 kb) (*9*) and *R* (*Lc* allele) (2.4 and 2.2 kb) (*10*) were placed under transcriptional control of the cauliflower mosaic virus 35S promoter in the Ti binary vector pKYLX71 to produce plasmids pAL71, pAL69, and pAL144, respectively (*11*). The 35S promoter is active in most cell types, albeit to varying degrees (*12*). Plasmid pAL69 had the entire cloned *R* untranslated leader (20 bp short of the native transcription start site) that contained three upstream, out-of-frame AUG start codons. However, the plasmid pAL144 had a truncated untranslated leader (196 bp short of the native transcription start site) that lacked any out-of-frame AUG codons. Each construct was individually integrated into *Nicotiana tabacum* cultivar *xanthi* (*13*) or *Arabidopsis* (*14*) by *Agrobacterium tumefaciens* Ti-mediated transfer. Segregation of the transferred DNA (T-DNA) in progeny of primary transformants was monitored by the resistance to kanamycin conferred by the linked neomycin phosphotransferase II gene. The correct structure of the T-DNA in plants was confirmed by DNA blot hybridization. Transcription of the transferred genes was monitored by ribonuclease protection assay.

The tobacco cultivar used here produces anthocyanins exclusively in floral organs. There is light pink pigmentation in the corolla tube and anther filaments and darker pink in the corolla collar (Fig. 1A). Four independent transgenic tobacco lines that express *C1* (pAL71) were produced, and each of them exhibited a phenotype indistinguishable from wild type. A single transgenic tobacco line expressing *R* (pAL69) was produced. This transformant showed intense red pigmentation in the corolla tube and collar as well as in the anther filaments (Fig. 1A). This altered pigmentation intensity cosegregated with the kanamycin resistance marker on the T-DNA. This phenotype suggests that *R* expression augmented the activity of an *R*-like product in wild-type tobacco, which is limiting for pigment synthesis.

Three independent *C1*-expressing (pAL71) lines of transgenic *Arabidopsis* were produced, all of which were indistinguishable from the wt parent. Multiple *R*-expressing (pAL69) lines of transgenic *Arabidopsis* were produced, of which seven lines were followed in detail. Anthocyanins in *Arabidopsis* are normally found in leaf, stem, sepal (outer whorl of the flower), and pistil (stigma, style, and ovary) tissues but never in root, petal, or stamen tissues. This pattern coincides with that of green tissue that contains chlorophyll. All of the *R* transformants that expressed the plasmid with the full-length leader (pAL69) produced more than normal amounts of anthocyanins (Fig. 1B), but pigment production was restricted to tissues normally pigmented in the wt.

The pAL69 transformants showed two- to eightfold increases in absorption over that of wt at the 510-nm anthocyanin absorption peak (Table 1). However, the production of anthocyanins in these plants is not constitutive but remains regulated. When germinated in the dark, progeny of these transformants lack anthocyanins, which is a normal response.

One *R*-expressing (pAL69) *Arabidopsis* transformant was used to cross-pollinate the three transformants that express *C1*. It was determined that all four parents in these crosses were heterozygous for the T-DNA insertion by the segregation of the kanamycin resistance marker in the selfed transformants. The hybrid F1 seed that was produced was plated in petri dishes on germination medium (*14*) without selection. Of 36 progeny from one of these crosses, six had unusual pigmentation in the roots of seedlings (Fig. 1C). These F1 progeny, when grown to maturity, produced a small amount of anthocyanin in petal and stamen tissues (Fig. 1D). In addition, 3 of 38 progeny from one other *C1* × *R* cross also produced anthocyanins in petal tissue. The third cross produced no progeny with pigmentation in root or petal tissue. Considered together, these results suggest that the *Arabidopsis* homologue of *R* is limiting in most tissues but that sufficient C1-like product is present to allow pigmentation when *R* is added. When large amounts of *R* and *C1* are introduced, in-

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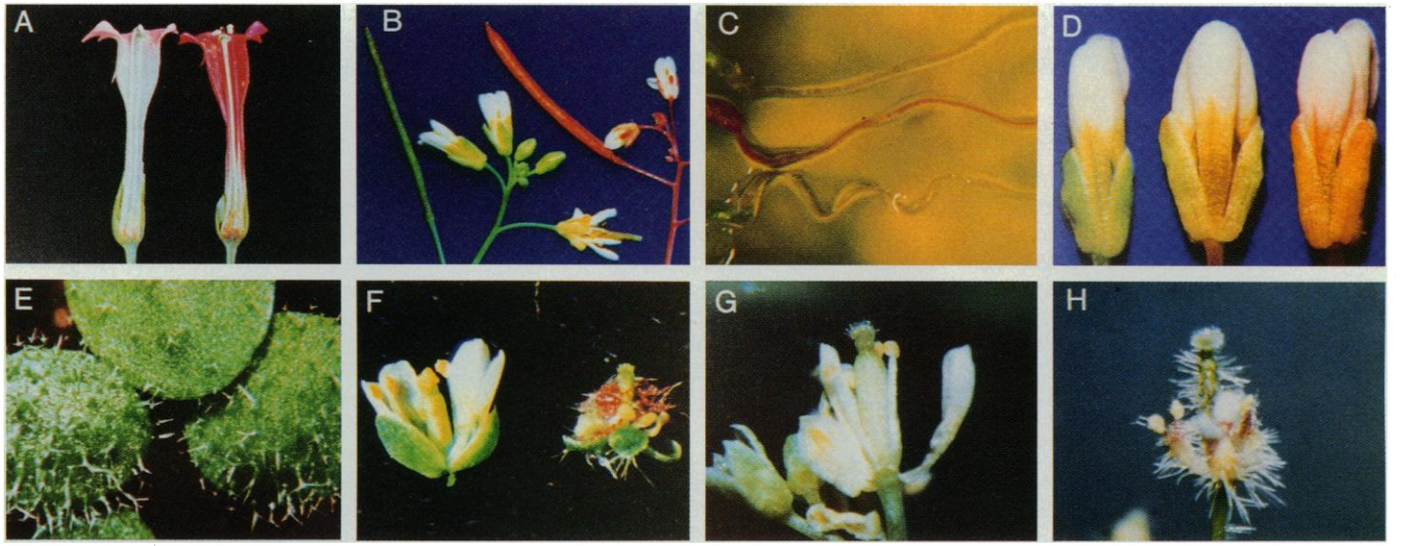

Fig. 1. Tobacco and *Arabidopsis* transformants. (**A**) Tobacco flowers: left, wild type; right, *R* (pAL69) transformant. (**B**) Eight-week-old *Arabidopsis* inflorescences: left, wild-type Rschew; right, *R* (pAL69) transformant. (**C**) Roots of *Arabidopsis*: top, *C1* (pAL71) transformant; middle, *C1* (pAL71) × *R* (pAL69) hybrid; bottom, *R* (pAL69) transformant. (**D**) *Arabidopsis* flowers: left, *C1* (pAL71) transformant; middle, *R* (pAL69) transformant; right, *C1* (pAL71) × *R* (pAL69) hybrid. (**E**) First true leaf of *Arabidopsis* seedlings: top, wild type; lower left and right, *R* (pAL69) transformants. (**F**) Flowers of the mutant *ttg*: left, untransformed; right, *R* (pAL144) transformant. (**G**) Wild-type Rschew flower. (**H**) Flower of *R* (pAL144) transformant of Rschew.

tense pigmentation is possible and occurs in a pattern that probably reflects where the 35S promoter is active.

Transgenic *Arabidopsis* plants that express R produce extra trichomes. In progeny of plants transformed with pAL69, the first true leaf to appear after the expansion of cotyledons (which are seed leaves that lack trichomes) has from two- to fivefold more trichomes than does the wt (Fig. 1E and Table 2). The additional trichomes are found only on tissues that normally produce these structures (leaves, stems, and sepals).

The *Arabidopsis* mutant *ttg* lacks both anthocyanins and trichomes. This mutant has been isolated at least ten times, and the two phenotypes are closely linked, which indicates that a lesion at the same locus may be responsible for both phenotypes (*7*).

Table 1. Optical density of extracts of *Arabidopsis R* transformants. Leaf tissue from *ttg*, Rschew wild type (wt), and Rschew transformed with pAL69 was assayed as described (*21*). Anthocyanins have an absorption peak in the range of 510 nm. Accordingly, absorption data at 510 nm (A_{510}) is reported. Line numbers refer to progeny of individual transformants.

Arabidopsis line	A_{510}
ttg	<0.005
Rschew wt	0.052
906	0.175
918	0.415
978	0.126
1411	0.233
1412	0.342
1413	0.212
1415	0.151

The simultaneous increase in both anthocyanins and trichomes in the wild-type *R* (pAL69) transformants suggests that the *ttg* gene might be an *R* homologue.

The *ttg* mutant was transformed with pAL144, the construct of *R* that lacked most of the untranslated leader. These transformants express both anthocyanins and trichomes (Fig. 1F). When wild-type, Rschew ecotype *Arabidopsis* plants were transformed with pAL144, more trichomes were expressed than in pAL69 transformants. The distribution of trichomes in both the *ttg* and Rschew pAL144 transformants was different than in wild-type plants transformed with pAL69. Trichomes grew on stem, leaf, and sepal tissue where trichomes are normally present but also on petal, stamen, and pistil structures (Fig. 1, F to H). Many of these excess trichomes were red, even when growing from nonpigmented tissues such as petals and stamens. These differential effects indicate post-transcriptional control of expression associated with the longer leader in pAL69 (*15*).

Some functions similar to those of *R* and possibly *C1*, which regulate the transcription of anthocyanin biosynthesis enzymes in maize (*8*), are also found in *Arabidopsis*. Between these maize activators and *Arabidopsis* is an evolutionary separation of an estimated 200 million years (*16*). Wild-type *Arabidopsis* may have homologues of *R* and *C1* that normally regulate pigment synthesis and trichome formation. The gene responsible for the *gl-1* (*glabrous-1*) mutation in *Arabidopsis* is a *myb* homologue (*17*), as is *C1*, although no anthocyanin phenotype is reported for gl-1. The gene responsible for the *delila* mutation of *Antirrhinum majus* (snapdragon) is homologous to *R* (*18*) and regulates anthocyanin production.

Members of the *R* family interact with the promoter sequence 5′-CAGGTG-3′ in structural genes of the anthocyanin pathway of maize (*8*). Similar sites are found in promoters of three structural genes for anthocyanin biosynthesis from *Arabidopsis*: chalcone synthase, 5′-CACGTA-3′ (*3*); chalcone isomerase, 5′-CACGTG-3′ (*19*); and dihydroflavanol 4-reductase, 5′-CACGTG-3′ (*19*). Thus it seems plausible that the transferred *R* in *Arabidopsis* functions in the same way that it does in maize: as a trans-acting factor that activates promoters of biosynthetic genes in the antho-

Table 2. Number of trichomes on the first expanded leaf on seedlings of *Arabidopsis R* transformants. Strains are as in Table 1 and seedlings were grown as described (*21*). Trichomes on the first leaf to emerge after cotyledon expansion were counted from five seedlings for each line.

Line	Trichome count on seedling					Average
	1	2	3	4	5	
ttg	0	0	0	0	0	0
Rschew wt	38	35	32	36	33	35 ± 2
906	70	77	67	84	98	79 ± 12
918	64	49	87	69	77	69 ± 13
978	130	125	73	103	94	105 ± 21
1411	146	220	196	129	172	173 ± 33
1412	131	150	109	148	130	134 ± 15
1413	93	89	93	96	62	87 ± 13
1415	99	139	116	116	103	115 ± 14

cyanin pathway. The transferred *C1* may only be functional in *Arabidopsis* when it interacts with the transferred *R*.

The observed effect of *R* on both trichomes and anthocyanin suggests that the *ttg* locus in *Arabidopsis* encodes an *R* homologue. Alternatively, the *ttg* protein may activate an *R* homologue. The activator *R* is a useful visual marker of transformation in maize (*20*). Both *R* and *C1* may provide visual genetic markers in *Arabidopsis* and other flowering plants by controlling production of both pigment and trichomes.

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22. We thank F. Ausubel, M. Campbell, E. Dement, M.-I. Benito, W. Kubasek, K. Luehrsen, B. Osborne, D. Preuss, M. Schena, and M. Thomas for helpful discussion; A. Hunt, C. Koncz, M. Koornneef, C. Schardl, S. Wessler, and U. Wienand for donations of plasmids and strains; and M. Campbell, G. Hwang, A. B. Lloyd, and G. Yen for reading the manuscript. Supported by NIH grants GM 32422 (V.W.) and R37-H600198 (R.W.D.) and NSF grant DMB-910601 (R.W.D.).

21 July 1992; accepted 8 October 1992

Interaction of U6 snRNA with a Sequence Required for Function of the Nematode SL RNA in Trans-Splicing

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Nematode trans-spliced leader (SL) RNAs are composed of two domains, an exon [the 22-nucleotide spliced leader] and a small nuclear RNA (snRNA)–like sequence. Participation in vitro of the spliced leader RNA in trans-splicing reactions is independent of the exon sequence or size and instead depends on features contained in the snRNA-like domain of the molecule. Chemical modification interference analysis has revealed that two short sequence elements in the snRNA-like domain are necessary for SL RNA activity. These elements are sufficient for such activity because when added to a 72-nucleotide fragment of a nematode U1 snRNA, this hybrid RNA could participate in trans-splicing reactions in vitro. One of the critical sequence elements may function by base-pairing with U6 snRNA, an essential U snRNA for both cis- and trans-splicing.

In nematodes, a subset of mRNAs contains a common 5′ terminal 22-nucleotide SL sequence. The SL sequence is acquired from a small (~100 nucleotides) SL RNA by trans-splicing. The SL RNA represents an unusual type of U snRNA that contains an exon, the 22-nucleotide SL, and an snRNA-like domain (*1*). Availability of ahomologous trans-splicing system in which synthetic SL RNA can serve as the trans-splice donor in vitro has facilitated a mutational analysis to identify sequences in the SL RNA that are important for its function (*2*). Such an analysis has shown that SL RNA participation in trans-splicing in vitro depends on assembly into an Sm small nuclear ribonucleoprotein (snRNP) and does not require specific exon sequences (*2*, *3*). These observations indicate that determinants of SL RNA function reside in the snRNA-like domain of the molecule.

We used chemical modification interference analysis as a probe to identify functionally important sequence elements in the snRNA-like domain of SL RNA. SL RNA that was 3′ end-labeled was treated with diethylpyrocarbonate (which modifies purines) under conditions in which less than one modification per molecule is introduced. The population of the SL RNAs carrying single modifications at random sites served as the substrate for trans-splicing (Fig. 1B). Because the SL RNA was 3′ end-labeled, only the Y-intron + exon intermediate (produced by the first step of trans-splicing) and the Y-intron product (produced by the second step of trans-splicing) were visualized by autoradiography of gel-fractionated trans-splicing reactions (Fig. 1, A and B). After recovery from preparative gels, the two Y-branched species, as well as unreacted SL RNA, were

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