Expression of Anthocyanins and Proanthocyanidins after Transformation of Alfalfa with Maize *Lc*[1,2]

Heather Ray[3], Min Yu[3], Patricia Auser[4], Laureen Blahut-Beatty, Brian McKersie[5], Steve Bowley, Neil Westcott, Bruce Coulman, Alan Lloyd, and Margaret Y. Gruber*

Saskatoon Research Centre, Agriculture and Agri-Food Canada, 107 Science Place, Saskatoon, Saskatchewan, Canada S7N 0X2 (H.R., M.Y., P.A., L.B.-B., N.W. B.C., M.Y.G.); Plant Biotechnology Institute, 110 Gymnasium Place, Saskatoon, Saskatchewan, Canada S7N 0W9 (H.R.); Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada N1G 241 (S.B., B.M.); and Department of Botany, Institute for Cellular and Molecular Biology, University of Texas, Austin, Texas 78713 (A.L.)

Three anthocyanin regulatory genes of maize (*Zea mays*; *Lc*, *B-Peru*, and *C1*) were introduced into alfalfa (*Medicago sativa*) in a strategy designed to stimulate the flavonoid pathway and alter the composition of flavonoids produced in forage. *Lc* constructs included a full-length gene and a gene with a shortened 5′-untranslated region. *Lc* RNA was strongly expressed in *Lc* transgenic alfalfa foliage, but accumulation of red-purple anthocyanin was observed only under conditions of high light intensity or low temperature. These stress conditions induced chalcone synthase and flavanone 3-hydroxylase expression in *Lc* transgenic alfalfa foliage compared with non-transformed plants. Genotypes containing the *Lc* transgene construct with a full-length 5′-untranslated region responded more quickly to stress conditions and with a more extreme phenotype. High-performance liquid chromatography analysis of field-grown tissue indicated that flavone content was reduced in forage of the *Lc* transgenic plants. Leucocyanidin reductase, the enzyme that controls entry of metabolites into the proanthocyanidin pathway, was activated both in foliage and in developing seeds of the *Lc* transgenic alfalfa genotypes. Proanthocyanidin polymer was accumulated in the forage, but (+)-catechin monomers were not detected. *B-Peru* transgenic and *C1* transgenic populations displayed no visible phenotypic changes, although these transgenes were expressed at detectable levels. These results support the emerging picture of *Lc* transgene-specific patterns of expression in different recipient species. These results demonstrate that proanthocyanidin biosynthesis can be stimulated in alfalfa forage using an *myc*-like transgene, and they pave the way for the development of high quality, bloat-safe cultivars with ruminal protein bypass.

The ability to manipulate flavonoid biosynthesis in crop plants is gaining rapidly in importance as new economically important uses are found in the areas of nutraceuticals, food quality, and feed quality. The introduction of proanthocyanidin (PA, a flavonoid polymer) into alfalfa (*Medicago sativa*) forage is particularly important to ruminant livestock producers. Proanthocyanidins eliminate pasture bloat, improve the efficiency of conversion of plant protein into animal protein (ruminal protein bypass), reduce greenhouse gases, reduce gastrointestinal parasites, and inhibit insect feeding (Waghorn, 1990; Neizen et al., 1995, 1998; Aerts et al., 1999; Muir et al., 1999; McMahon et al., 2000). Alfalfa forage (leaf and stem tissues) accumulate anthocyanins only at senescence or locally under some stress conditions such as insect feeding (Goplen et al., 1980). No known conditions induce proanthocyanidins in alfalfa forage, although they are structurally related to anthocyanins. However, these compounds do accumulate in seed coats (Koupai-Abyazani et al., 1993).

Anthocyanins and proanthocyanidins share early and middle steps of the flavonoid biosynthetic pathway, including chalcone synthase (CHS), chalcone isomerase, flavanone 3β-hydroxylase (F3H), and dihydroflavonol reductase (DFR; Winkel-Shirley, 2001; Marles et al., 2003). After this point, the paths diverge. Some of the remaining structural genes for anthocyanins are well known, whereas those specific to proanthocyanidin synthesis are only now being characterized. Until recently, regulatory genes affecting proanthocyanidin accumulation were largely unknown. However, the first basic helix-loop-helix *myc*-like genes that stimulate legume proanthocyanidin biosynthesis have now been isolated from *Lotus ul-*

[1] This work was supported in part by grants from the Alberta Agriculture Research Institute, the Alberta Cattle Commission, and the Saskatchewan Agricultural Development Fund. This is Institute paper no. 1547.

[2] This paper is dedicated to the memory of Dr. Bernie Goplen and Dr. Ron Howarth. Together, they had a vision for improving alfalfa forage quality by introducing proanthocyanidins and faith that it could be accomplished.

[3] These authors contributed equally to the paper and to the assessment of the transgenic plants.

[4] P.A. has retired from Agriculture and Agri-Food Canada.

[5] Present address: BASF Plant Science, 26 Davis Drive, Research Triangle Park, Raleigh-Durham, North Carolina 27709-3528.

* Corresponding author; e-mail gruberm@agr.gc.ca; fax 306–956–7247.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.025361.

iginosus and related species (Ray and Gruber, 2001). Other types of proanthocyanidin regulatory genes such as *TT*8 are also being recovered from Arabidopsis seed coat mutants (Nesi et al., 2000, 2001; for review, see Marles et al., 2003).

Myc-like genes that regulate anthocyanin biosynthesis are well known and include the *B* series, the *R* series, and *Lc* (leaf color) of maize (*Zea mays*), all of which have very similar sequences (Ludwig et al., 1989; Ludwig and Wessler, 1990; Radicella et al., 1991). *Lc* induces the expression of enzymes which form red anthocyanin pigments in several plant tissues (midrib, ligule, auricle, glume, pericarp, palea, and lemma) of maize (Ludwig et al., 1989; Ludwig and Wessler, 1990). In contrast, the numerous alleles of *B* vary greatly in expression, from seed only to the whole aboveground plant (Ludwig and Wessler, 1990). The *myb* class of anthocyanin regulatory genes, including the *C*1 gene of maize (Paz-Arez et al., 1987) is also well known. Plants appear to have well over 100 *myc* and *myb* genes, some of which are functionally interchangeable (Quattrocchio et al., 1993, 1998; Mol et al., 1996; Winkel-Shirley, 2001).

Several of the anthocyanin *myc* and *myb* regulatory genes have been tested for their ability to influence anthocyanin or proanthocyanidin accumulation when expressed in heterologous plants, but the success of this strategy has varied. For example, *Sn* was shown to stimulate or inhibit proanthocyanidin accumulation in *Lotus corniculatus*, depending on the tissue type, transformed line, and gene copy number (Damiani et al., 1998, 1999; Robbins et al., 2003). *B-Peru* and *C*1 stimulated anthocyanin synthesis when cobombarded into white clover (*Trifolium repens*) or pea (*Pisum sativum*) in transient assays, but the expression in white clover was very low (de Majnik et al., 1998). *Lc* has stimulated anthocyanin biosynthesis on its own or together with *C*1 in several different plant species, but in some species *Lc* has no visible effect (Boase et al., 1998; Bradley et al., 1999). Reviews of this field can be found in Mol et al. (1998) and Winkel-Shirley (2001).

We tested the ability of *Lc* to stimulate the flavonoid pathway and to alter the composition of flavonoid end products produced in alfalfa forage and seed coat. Two *Lc* constructs (each under the control of the same single cauliflower mosaic virus [CaMV] 35S promoter) were tested, including one with a nearly full-length leader region and a second one with the leader region truncated by about 200 bp. Observations in Arabidopsis and tobacco (*Nicotiana tabacum*) suggested that the longer leader region may limit expression (Lloyd et al., 1992). We also transformed alfalfa with *B-Peru* (Radicella et al., 1991) and with the *C1 myb* gene of maize (Cone et al., 1986; Paz-Arez et al., 1987) under the control of an enhanced CaMV 35S (35Sx2-AMV) promoter. Also, we transformed alfalfa with *B-Peru* under the control of the promoter from the small subunit of Rubisco (ssRBC). This range of genes enabled us to test the specificity of regulatory sequences required to stimulate the flavonoid pathway in alfalfa. Flavonoid composition, visible phenotype, and gene expression were examined in the transgenic plants grown under several different environmental conditions.

RESULTS

Development of Transgenic Alfalfa Genotypes

After transformation and regeneration from tissue culture, five small populations of new alfalfa genotypes exhibiting resistance to kanamycin and a positive response in an *npt*II PCR assay were recovered. These populations contained either a 2.2- kb *Lc* gene, a 2.4-kb *Lc* gene, a *B-Peru* gene under one of two

Table I. *Development of transgenic alfalfa genotypes containing maize anthocyanin regulatory genes*

Population Name[a]	Regulatory Transgene/ Promoter Names	No. of *npt*II$^+$ Genotypes[b]	No. of Transgene-Containing Genotypes[c]	No. of Transgene- Expressing Genotypes and (Expression Pattern)[d]		No. of Anthocyanin-Stimulating Genotypes[e]
				Immature leaves	Mature leaves	
A01-88	2.4-kb *Lc*/CaMV 35S	18	6	n.d.[f]	4 (S), 2 (M)	5++
A01-90	2.2-kb *Lc*/CaMV 35S	24	13	n.d.	2 (S), 2 (M), 2 (WN)	4+
A01-045	2.0-kb *B-Peru*/35Sx2-AMV	13	5[g]	3 (MS)	1 (MS), 3 (MW), 1 (WN)	0
A01-047	1.1-kb *C1*/35Sx2-AMV	13	4[h]	1 (SW), 3 (WN)	2 (MS), 2 (MW)	0
A04-100	2.0-kb *B-Peru*/ssRBC	11	6	n.d.	3 (M)	0

[a] Transgenic population names indicate the non-transformed parent genotype (i.e. A01 or A04). [b] Each *npt*II$^+$ genotype arose from an independent transgenic event that was selected for kanamycin resistance and tested for the presence of the *npt*II gene using a PCR assay. [c] Subset of *npt*II$^+$ genotypes that contained a maize anthocyanin regulatory transgene confirmed using Southern-blot analysis. [d] Subset of maize transgene-containing genotypes that expressed detectable levels of transgene using northern blots. Parentheses indicate approximate transgene RNA accumulation based on three independent experiments using growth cabinet-grown or field-grown material. S, Strong; M, medium; MS, medium-strong; MW, medium-weak; W, weak; N, none. [e] Subset of maize transgene-expressing genotypes that accumulated anthocyanin in field-grown material. ++, Strong anthocyanin accumulation; +; moderate-weak anthocyanin accumulation. [f] n.d., Not determined. [g] Five genotypes contained both promoter and maize transgene. An additional *npt*II$^+$ genotype contained only the promoter. [h] Four *npt*II$^+$ genotypes contained both promoter and maize transgene. One additional *npt*II$^+$ genotype (not indicated) contained only the maize transgene, and four additional *npt*II$^+$ genotypes(not indicated) contained only the promoter.

Table II. *Growth/test conditions for transgenic and non-transformed alfalfa genotypes*

Condition	Location	Growth Medium	Temperature Day/Night	Light/Photoperiod	Moisture
			°C	μE/h	
A	Unshaded greenhouse A	Brick chips and hydroponic solution	20/17	600/16	Daily watering
B	Shaded greenhouse B	Soil-less mix	20/17	300–500/16	Daily watering
C	Unshaded greenhouse C	Soil-less mix	20/17	600/16	Daily watering
D	Growth cabinet	Soil-less mix	4	40/16	Daily watering
E	Growth cabinet	Soil-less mix	4	200/16	Daily watering
F	Growth cabinet	Soil-less mix	20/16	650/Continuous	Daily watering
G	Growth cabinet	Soil-less mix	20/16	650/16	Daily watering
H	Outdoors in pots	Clay soil	14–22/16	1,100–1,400/16	Daily watering
I	Outdoors in Saskatoon field plots	Dark-brown clay soil	Summer, 18–37; fall, 5–20	1,100–1,400/summer 18/fall 12	Dry land (2–7 cm season^{-1})
J	Outdoors in Elora field plots	Rich loam soil	Summer, 20–30; fall 10–20	1,100–1,400/summer 14/fall 12	Moist soil conditions
K	Culture room	Murashige and Skoog culture medium, 0.7% (w/v) agar	22/Continuous	105–120/16	NA

[a] NA, Not applicable.

promoters, or a *C1* gene from maize (Table I). Several of the *Lc* genotypes developed deep red-green leaves when the newly transformed plantlets were grown in a greenhouse under bright lights (Condition A, Table II), in contrast to the non-transformed parent (control) plantlets that remained bright green under these conditions. New leaflets of *Lc* plants were bright green and did not display the red phenotype when the young plantlets were transferred to lower light intensity (Condition B, Table II). Also, red color in older leaves of the *Lc* genotypes disappeared when the plants were removed from the brighter light conditions. Novel color phenotypes were not observed under either of these conditions for the *B-Peru* and *C1* genotypes.

The presence of the introduced genes, and in some cases their promoters, was confirmed by Southern blotting (Fig. 1, top, illustrating the *Lc* genotypes; promoter and transgene data for *B-Peru* and *C1* genotypes not shown). The blots indicated that a large number of these primary transgenic events (kanamycin resistance and *npt*II$^+$ plants) had no flavonoid transgene or were missing a promoter (Table I). For example, in the *C1* population, 50% of the *npt*II$^+$ plants had a promoter, whereas only 30% had a flavonoid transgene and a promoter (Table I). In addition, only one-half of the *npt*II$^+$ genotypes recovered in population 88 actually contained the *Lc* transgene, whereas a 2-fold larger number of genotypes were recovered with the smaller construct (population 90). Although intact independent transgenic events were easily recovered from *Brassica napus* using the same maize regulatory gene constructs used in this investigation (S. Wang and M.Y. Gruber, unpublished data), our experience suggests that alfalfa may be particularly sensitive to construct design, transgene type, or transgene size and may sometimes require the development of very large sets of primary transgenic events to select a range of suitable transgenic genotypes.

Northern blots of RNA extracted from immature (folded) and mature (open and expanded) leaf tissue were used to select those primary transgenic events confirmed to have both a flavonoid transgene and a promoter and to place them into transgene expression categories (Fig. 1, bottom; Table I). A similar number of genotypes expressing a maize flavonoid transgene were recovered for the *B-Peru* and *Lc* populations, whereas a smaller number were recov-

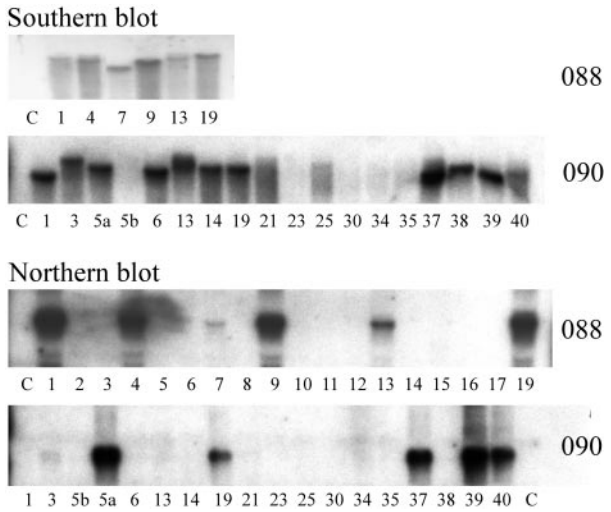

Figure 1. Molecular characterization of *Lc* transgenic alfalfa in populations 88 and 90. Top, Representative Southern blot confirming the presence of the *Lc* transgene in *npt*II$^+$ alfalfa. Lanes represent 15 μg of DNA extracted from individual genotypes (each from an independent transgenic event). DNA was digested with *Hin*dIII, blotted, and probed with *Lc*. Samples from population 88 only illustrate *Lc*$^+$ genotypes, whereas samples from population 90 illustrate a range of *npt*II$^+$ genotypes with or without the *Lc* transgene. Bottom, Representative northern blot of *Lc* transgenic alfalfa under non-stress greenhouse conditions (Condition B). Lanes represent 15 μg RNA extracted from individual transgenic genotypes, blotted, and probed with *Lc*. Lane C represents nucleic acid extracted from A01 non-transgenic parental genotype.

ered for C1. Selected genotypes were multiplied by rooting shoot cuttings, and these established clones were used to assess response to a variety of conditions.

Transgenic Alfalfa Phenotypes under Stress Conditions

Several clones (i.e. rooted cuttings) of each of the selected alfalfa genotypes that expressed a maize transgene were tested for phenotypic color changes in response to several different environmental conditions. A minimum of two mature clones of each selected genotype were placed under Conditions C to H (Table II), and 24 young clones (4 months old) for each genotype were placed in the Saskatoon field trial (Condition I). Twenty-four clones of representative genotypes of *B-Peru* and *C*1, but not *Lc*, were also placed in a field trial in Elora (ON, Canada; Condition J). *Lc* genotypes were not developed early enough to be tested in the latter field trial. Throughout growth under all of the test conditions, *B-Peru* transgenic and *C*1 transgenic genotypes (populations 45, 47, and 100) remained green and did not show phenotypic color change (data not shown), although transcripts for these two transgenes were clearly detected on the northern blots (data not shown). Even after 1 month under cold conditions, $C1^+$ genotypes had green leaves and stems, whereas $B\text{-}Peru^+$ genotypes had green leaves and only a light-red stem coloration. The untransformed parent genotype A01 (Fig. 2A), clones of untransformed varieties Peace and Beaver, and several transgenic genotypes that poorly expressed the *Lc* transgene also remained green under all conditions tested (data not shown).

In contrast, genotypes that strongly expressed the 2.4-kb *Lc* transgene (from population 88, Table I) developed a light-red coloration on newly developed leaf tips under the increased light intensity of Condition C (data not shown), but plants expressing the 2.2-kb *Lc* transgene (from population 90, Table I) could not be detected under these latter conditions. Immature and mature leaves of the 2.4-kb *Lc* plants consistently developed a deep red-purple color when the plants were placed under cold temperature with moderate light intensity (Condition E) or under moderate temperature with either high-intensity artificial light (Conditions F and G) or natural sunlight conditions (Condition H, Fig. 2, A and C; Tables II and II). In one clone, 88-19, the red phenotype became visible within 6 h, whereas other selected plants of population 88 required about 24 h. The forage color was less intense when the 2.2-kb *Lc* plants (from population 90) were exposed to cold or high-intensity light. For example, genotypes 90 developed a faint red leaf phenotype and a red or deep red stem phenotype by 72 h under cold conditions, whereas leaves and stems from genotypes from population 88 turned deep red within 24 h under these conditions. Return to a green phenotype occurred quickly when plants were returned to Condition B. Usually, the "greening" occurred within 2 to 3 d of growth if the plants had been subjected to a photoperiod but took longer (approximately 7 d) if plants had been subjected to continuous light (condition F). In addition to phenotypic color changes under cold or high light conditions, all plants from both Lc^+ populations developed a red phenotype while developing roots from cuttings under Condition B.

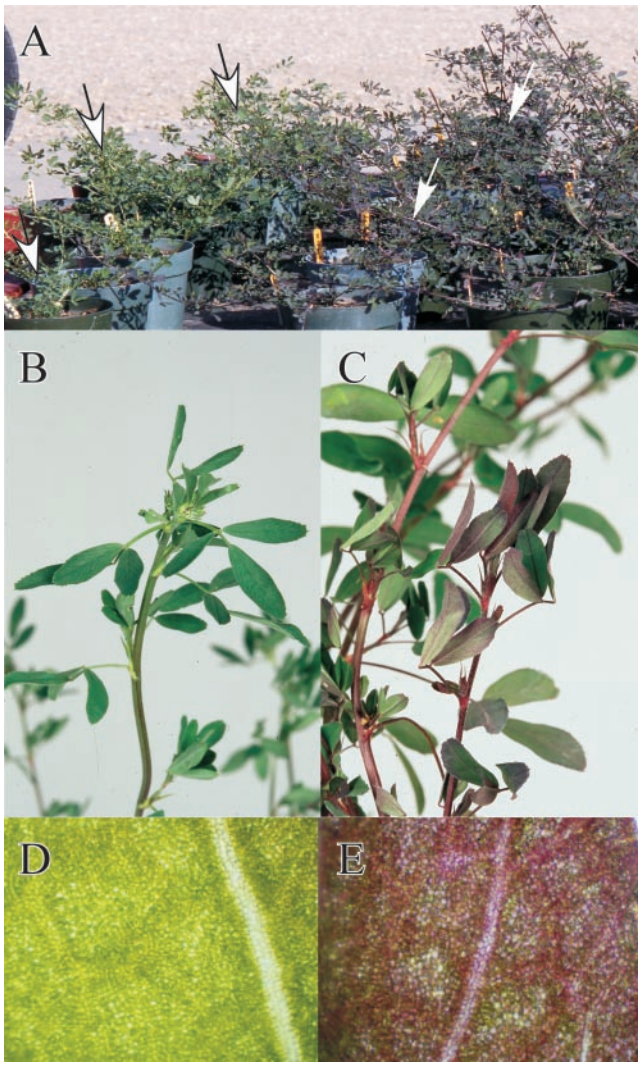

Figure 2. Phenotype of *Lc* transgenic alfalfa growing in natural sunlight under Condition H (Table I). A, Whole plants. Green nontransformed parental control plant A01 (white/black arrows), Clones of deep red *Lc* transgenic plant A01-88-19 (white arrows). B, Close-up of harvested shoots from A01. C, Close-up of harvested shoots from 88-19. D, Green mesophyll cells from A01 abaxial leaf surface. E, Red mesophyll cells from 88-19 abaxial leaf surface. Data are representative of two or more experiments.

The red coloration was only visible on surfaces of the *Lc* transgenic plants exposed to light and was particularly noticeable on the abaxial leaf surface of folded leaves, over veins, and on stems (Fig. 2C). In emerging leaflet clusters, the outer abaxial surface

was deep red, whereas areas of inner leaflets protected from the light remained green with a sharp boundary between the red and green regions. When plants were placed in the cold room under low-fluence fluorescent light (Condition E), the red color was most intense close to the light source. Under strong natural sunlight either in pots (Condition H in May or September) or planted in a field trial (Condition I) or under high-fluence artificial light (Conditions F and G), the red hue of population 88 genotypes strengthened to a deep red-purple and was present on all exposed shoots (Fig. 2C). In contrast, genotypes of population 90 always displayed a lighter red color even under field conditions. Color development in leaves resulted primarily from pigment accumulated in mesophyll cells (Fig. 2, D and E). Under growth cabinet conditions where flowering was permitted (Conditions F and G), *Lc* transgenic flower buds had a distinct red color compared with the green buds of the non-transformed parent plant (data not shown). However, the natural deep purple coloration of the mature flower was not visibly altered under these latter conditions.

Growth habit and morphology of the *Lc* transgenic clones at the Saskatoon field location (Condition I) were very similar to the A01 non-transformed parent plant, except for rooted clones of genotype 88-4. Clones of this genotype consistently grew more slowly than all the other plants in the field trial and appeared to have shortened internodes. One of the *C*1 genotypes (47-6) also had a short phenotype. *Lc*-expressing clones from genotypes that displayed a moderate or deep red-green phenotype indoors (Conditions C and E–H) consistently maintained a deeper coloration when grown outdoors throughout two field seasons in Saskatoon (Condition I) and through some light frost in the fall. *Lc* transgenic plants that did not express *Lc* remained green under field conditions and appeared phenotypically similar to the non-transformed parent clones and to the *B-Peru*-transgenic and *C*1-transgenic clones.

Expression of *Lc* and Flavonoid Structural Genes under Extreme Conditions

Because genotypes expressing *B-Peru* or *C*1 did not display any red color under laboratory or field conditions (a phenotype that signaled stimulation of the lower portion of the flavonoid pathway), expression of endogenous flavonoid structural genes was not examined in either of these populations. Instead, selected genotypes containing the *Lc* transgene were tested by northern blotting for accumulation of *Lc*, *CHS*, *F3H*, or *DFR* transcripts under conditions (outlined above) that generated the red phenotype. *Lc* transgenic genotypes that constitutively expressed either the 2.2- or 2.4-kb *Lc* transgene under Condition B did not display significant changes in expression of *CHS*, *F3H*, or *DFR* under these conditions (results not shown).

After acclimation of plants expressing the 2.2-kb *Lc* gene for 7 d to cold temperature (Condition E), an altered pattern of flavonoid gene expression was observed in several of these genotypes Under the latter conditions, the red stems of these genotypes clearly accumulated more *CHS* and *F3H* transcripts compared with stems of plants under noninducing conditions (Table III). Genotypes in which stem *F3H* was not induced did not display a red stem phenotype, i.e. 90-3. Coordinate expression of *F3H* and *CHS* was difficult to assess in leaves after cold acclimation due to weak gene expression (data not shown). RNA accumulation and phenotypic changes were also very similar in plants of population 88 (data not shown), although *CHS* gene expression was stronger in population 88 genotypes than in plants from population 90, and the phenotype developed faster. *DFR* transcript levels were not detectably affected in any of these plants after cold acclimation (data not shown). Gene expression and color were also unchanged under cold conditions in the non-transformed parent (control) plants (data not shown).

Expression of flavonoid genes was next examined over 48 h in two of the *Lc* transgenic genotypes (88-19 and 90-5a) after a shift from growth under moderate

Table III. *Expression of Lc and flavonoid structural genes in stems of genotypes A01-90 after acclimation to cold temperature*

Genotype[a]	Relative Expression of Flavonoid Biosynthetic Genes[b]			
	Stem phenotype	*Lc*	CHS	F3H
90-3[c]	Green	3.50	4.28	1.07
90-19[d]	Deep red	1.06	5.12	2.23
90-39[d]	Deep red	0.99	11.19	9.45
90-40[d]	Red	2.27	8.78	1.88

[a] Leaves from these genotypes had very weak flavonoid gene expression under Condition E. [b] Relative expression in plants grown at 4°C under Condition E compared with expression in noninduced plants grown under Condition B (Table II). Northern blots of 15 μg of total RNA hybridized to *Lc, CHS,* or *F3H* probes. Band signal strength was standardized by comparison with the intensity of rRNA bands. Table is representative of data from two or more experiments. [c] Displayed a green phenotype. [d] Displayed a faint-red leaf phenotype.

light intensity (Condition B) into high light intensity (either Condition F or G). Northern blots indicated that *CHS* transcripts were moderately abundant by the earliest time period (5 h) in clones of the non-transformed A01 parent plant and maintained at that level until 24 h, after which transcript levels declined (Fig. 3, top). Initially, *CHS* was accumulated to a similar extent in clones of *Lc* transgenic genotypes 88-19 and 90-5a and the non-transgenic A01, but by 24 h *CHS* accumulation was stronger in the transgenic genotypes. A low abundance of *F3H* transcripts was detected by 24 h both in 90-5a and 88-19 clones, after which transcript levels declined. *F3H* did not accumulate at all in A01 plants. *DFR* transcripts were barely detectable in transgenic and non-transgenic clones.

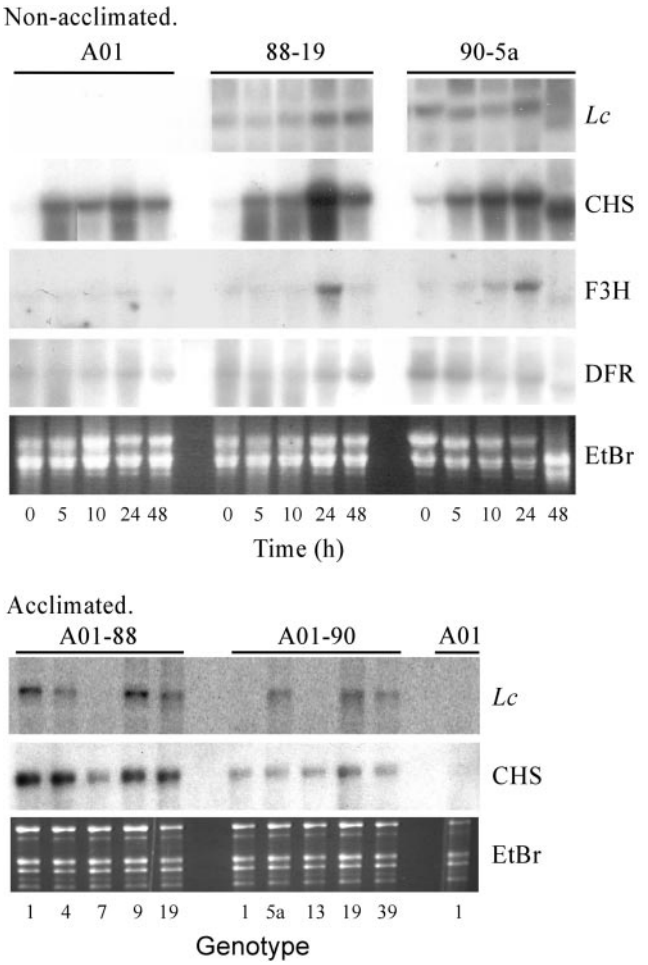

Figure 3. Flavonoid structural gene expression in *Lc* transgenic alfalfa under high light intensity. Top, Time course of RNA transcript accumulation in transgenic genotype 88-19 and 90-5a undergoing acclimation to continuous high light intensity (Condition F, up to 48 h). Lanes represent 15 μg of RNA extracted, blotted, and probed with Lc, CHS, F3H, and DFR probes. Bottom, RNA transcript accumulation in transgenic genotypes acclimated to growth under Saskatoon field conditions (Condition I). Lanes represent 15 μg of RNA extracted from individual transgenic genotypes, blotted, and probed with *Lc* or *CHS*. EtBr, Ethidium bromide-stained RNA loaded in each lane. *Lc* and *CHS* blots were exposed overnight. *F3H* and *DFR* blots were exposed for 6 d. Blots are representative of four or more experiments.

Expression of flavonoid biosynthetic genes was also evaluated in acclimated tissues of *Lc* transgenic alfalfa forage collected from the field plot at the end of September 2001, 3 months after planting (Condition I). Little change was observed in RNA from the non-transformed parent plant after acclimation. However, plants that had expressed *Lc* under moderate or cold temperatures and moderate light intensities and had displayed a red or deep red-purple phenotype in the field plot accumulated *CHS* RNA when acclimated to these more extreme conditions (Fig. 3, bottom). CHS was more intensely expressed in population 88 containing the strongly expressed 2.4-kb *Lc* gene, compared with population 90 containing the more weakly expressed 2.2-kb construct without the 5′-untranslated region (Fig. 3, bottom). *CHS* was also weakly expressed under field conditions in transgenic genotypes that poorly expressed *Lc*, i.e. 88-7, 90-1, and 90-13 (Fig. 3, bottom). Neither *F3H* nor *DFR* accumulated significantly after acclimation under Condition I in any of the transgenic or non-transgenic populations (data not shown).

Anthocyanin Analysis

Quantification of total anthocyanin content of leaf was carried out on forage harvested from potted plants grown under Condition H and from field material harvested at the end of September (Condition I). Spectrophotometric scans had a very broad peak typical of anthocyanins and centered at about 523 nm. Total anthocyanins correlated strongly with field observations of the color phenotype (Table IV). Green plants, i.e. A01 and *Lc* transgenics with limited transgene expression such as 88-7, had little or no detectable anthocyanins. Deep red field plants expressing the *Lc* transgene had 3- to 5-fold higher anthocyanin compared with plants that poorly expressed *Lc* (e.g. 88-7 and 90-13). The weight of forage harvested at the end of September had no correlation to the amount of anthocyanin present in leaf (data not shown).

Anthocyanin composition was examined in aqueous extracts of one transgenic genotype, 88-19, using mild hydrolysis conditions. The extraction resulted in two small red spots that cochromatographed on thin-layer chromatography (TLC) plates with cyanidin chloride and pelargonidin chloride in 15% (w/v) acetic acid and Forestal's reagent (spot A, reagent flows [Rfs], 0.06 and 0.39; spot B, Rfs, 0.29 and 0.65, respectively). These spots also displayed identical colors to these standards when TLC plates were viewed under visible and UV light (data not shown). Under mild to moderate hydrolysis conditions, a large red spot that chromatographed with pelargonin chloride in 15% (w/v) acetic acid and Forestal's re-

Table IV. *Anthocyanin accumulation in leaves of plants exposed to natural light*

Plant Population	Plant Genotype	Leaf Phenotype of Field Plants	Leaf Anthocyanin[a]	
			Mixture of leaves from field plants[b]	Mature leaves from outdoor potted plants[c]
			μg g⁻¹ fresh wt	
A01-88	1	Deep red	145	26
	4	Deep red	85	26
	7	Green	30	ND[d]
	9	Deep red	n.d.[e]	39
	19	Deep red	158	20
A01-90	1	Green	ND	n.d.
	5a	Light red	41	n.d.
	13	Green	15	n.d.
	19	Light red	47	n.d.
	39	Light red	67	n.d.
A01	Parent	Green	ND	ND
var. Pickseed	8920MF	Green	n.d.	3
var. Beaver	Breeder clone 3	Green	n.d.	2

[a]Anthocyanin concentration was calculated using a standard curve of absorbance of cyanidin chloride measured at 523 nm. Absorbance not due to red anthocyanins in the extracts was normalized before calculation by subtracting the yellow-colored background absorbance obtained for the green non-transformed Eastern Canadian breeding genotype A01 used to develop the *Lc* transgenic population. All plants with a green phenotype had this yellow background absorbance regardless of whether they were transgenic or not. [b] Anthocyanin was extracted from leaf tissue bulked from 24 clones of each *Lc* genotype harvested from a Saskatoon dry land field trial at the end of September 2001. [c] Anthocyanin was extracted from leaf tissue bulked from two established clones for each *Lc* genotype. Clones were grown in pots outdoors under Condition H for 4 d in May 2001 in a protected Saskatoon farm yard. [d] ND, Not detected. [e] n.d., Not determined. Non-transgenic parent genotype A01 and clones from two western Canadian varieties from Pickseed (Ontario, Canada) and Agriculture and Agri-Food Canada (Ottawa, Ontario, Canada) were also included in the test.

agent (Rfs, 0.65 and 0.75, respectively), was visible in the 88-19 water extract. This spot decreased in size under harsh hydrolysis conditions and even under mild conditions did not exhibit the same color characteristics as pelargonin on the TLC plate.

Flavonoid Analysis

Forage of three *Lc* transgenic genotypes (88-4, 88-19, and 90-5a) and the non-transformed parent genotype A01 was harvested from the Saskatoon field trial (Condition I) and then analyzed by C_{18}-HPLC after acid hydrolysis and separation into four subfractions of increasing polarity on an Oasis C_{18} cartridge. Total flavonoid content was reduced in the three transformed plants compared with the non-transformed A01, as visualized in the 100% (w/v) MeOH subfraction by the overall reduction in UV absorbance on the HPLC traces (Fig. 4). In addition, luteolin was disproportionately reduced in leaves and stems of all three transgenic genotypes tested, whereas apigenin was only affected in leaves of two of the transgenic plants and not noticeably affected in stems (Table V). Other changes to the UV-absorbing HPLC peaks were also observed, including a reduction of a peak that had a luteolin-like spectrum but a retention time different from our available standards, suggesting that other flavonoids were also reduced. These other HPLC peaks will be the subject of a more comprehensive analysis at a later date using selective extraction and ion cyclotron mass spectroscopy to document metabolic changes in the *Lc* transgenic alfalfa in greater detail.

Influence of *Lc* on Leucocyanidin Reductase (LCR) Activity and Proanthocyanidin Accumulation

LCR activity was examined in leaves from two selected *Lc* transgenic genotypes, 88-19 and 90-5a, that were grown under Condition B, then shifted for up to 48 h into Condition F or G. Leaf LCR specific activity was detectable after 12 h of continuous light exposure but rose to a maximum in 88-19 leaves after 24 h (Fig. 5A; Table VI) and then declined but was still detectable after 48 h. LCR enzyme activity had the same profile but was lower in genotype 90-5a compared with 88-19 and could not be detected in A01 non-transgenic leaves under stringent assay conditions (see "Materials and Methods" and footnotes from Table VI).

LCR activity was also examined in developing seed of *Lc* transgenic alfalfa. Two batches of seeds (11 DAP) were dissected under sterile conditions from pods of 88-19 and 90-5a plants that had been acclimated to Condition F for 1 week before cross-pollination and then maintained under the same conditions during seed development. A third batch of 14-d-old seeds was dissected from plants that had

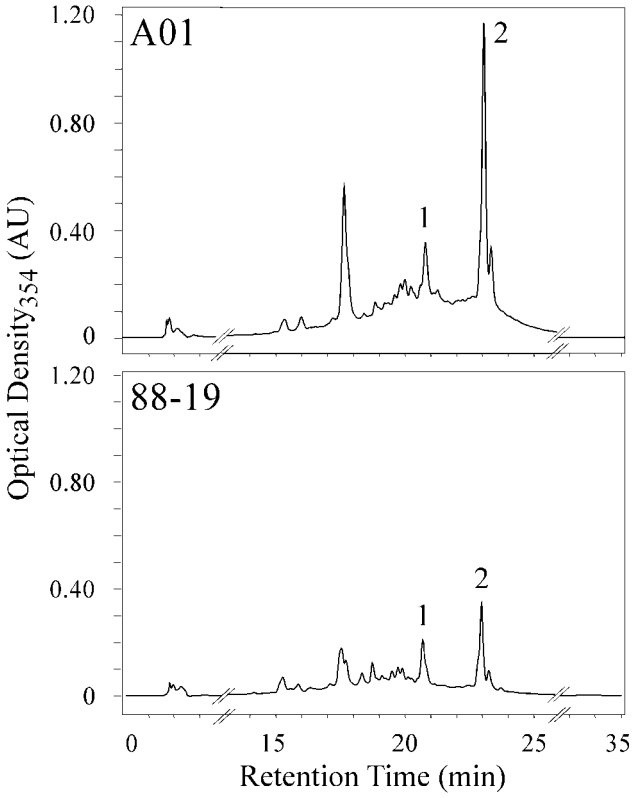

Figure 4. Flavonoid composition in leaves of *Lc* transgenic alfalfa genotype 88-19. Representative photodiode array UV detector (PDA; UV) trace eluting from a Waters Symmetry C_{18} HPLC column from a 10-μL injection of 100% (w/v) MeOH-solubilized compounds (300-μL total volume). Before HPLC, the compounds were eluted from a C_{18} Oasis cartridge loaded with acid-hydrolyzed tissue extract loaded at an equivalent weight for each genotype. Each trace is representative of two injections. Samples were prepared once from leaves harvested and bulked at the end of the field season from 24 clones of each genotype. Peak 1, Luteolin (retention time 20.6 min). Peak 2, Apigenin (retention time 22.9 min).

been maintained under Condition B and then shifted into Condition G shortly before seed harvesting. After dissection, all harvested seeds were immediately incubated under sterile conditions on Murashige and Skoog medium for 48 h under supplemented light (Condition K), then frozen in liquid N_2 and assayed for LCR activity. LCR activity was preferentially stimulated in 11-d-old developing seeds of the transgenic plants exposed to continuous high-intensity light. Seeds from genotype 88-19 displayed 4-fold higher activity than the A01 control plant, whereas seed activity from genotype 90-5a was 10-fold higher than the control (Fig. 5B; Table VI). LCR activity remained higher in the 14-d-old transgenic seed than in the control but displayed no difference between the two genotypes.

Forage tissue was also analyzed to determine proanthocyanidin polymers, dimers, and flavan-3-ol monomer content. Initially, individual leaves from field-grown material or from plants grown under continuous light (condition F) were stained with dimethylaminocinnamylaldehyde (DMACA; McMurrough and McDowell, 1978; Xie et al., 2003) as a means of avoiding color interference in the assay from the anthocyanin present in the transgenic tissues. However, this method was not sensitive enough to detect proanthocyanidin in the transgenic alfalfa tissue (data not shown). In addition, a background of compounds that are normally present in untransformed alfalfa forage reacted to produce a red interfering color over time with the DMACA staining. It is possible that these compounds could be similar to those found by Sarkar and Howarth (1976) that interfere with the vanillin:HCl assay.

Subsequently, plants grown for 32 or 48 h under continuous light were tested for proanthocyanidin using more sensitive heat-dependent PVPP:butanol:HCl assays after tissue fractionation into 80% (w/v) MeOH-extractable and unextractable fractions (Watterson and Butler, 1983). Extractable fractions from the 48-h samples were further partitioned into aqueous and ethyl acetate subfractions. Each OD in the heat-dependent portion of the transgenic tissue assays was accurately determined for all three types of fractions against a large color background arising from anthocyanin or other contaminating compounds. The aqueous-extractable and unextractable fractions were then related to a standard curve using purified alfalfa seed polymer (Koupai-Abyazani et al., 1993).

A low concentration of 80% (w/v) MeOH-extractable proanthocyanidin (approximately 0.06%–0.1% fresh weight) was selectively bound to PVPP and was detected in both transgenic genotypes after heating the PVPP-bound material in butanol:HCl (Table VI; Watterson and Butler, 1983). Trace amounts of 80% (w/v) MeOH-extractable proanthocyanidin (<0.0005% fresh weight) could also be detected in non-transformed A01 leaves. Heat-dependent butanol:HCl-hydrolyzable material was also found in ethyl acetate-partitioned, 80% (w/v) MeOH-extractable leaf material of both transgenic genotypes exposed for 48 h to continuous light (approximately 2-fold higher for 88-19 and similar in amount for 90-5a compared with aqueous-partitioned extractable PA; data not shown). In addition, leaves from genotype 88-19 exposed to 48 h of continuous light accumulated a 4-fold higher amount of unextractable proanthocyanidin compared with aqueous-extractable PA (Table VI). Under the same inducing conditions, genotype 90-5a accumulated 2-fold higher unextractable PA polymer compared with extractable material (Table VI). This unextractable PA was determined after hydrolyzing residual cell debris in hot butanol:HCl after exhaustively washing the residue in MeOH to remove contaminating soluble (extractable) PA (Terrill et al., 1992). Unextractable polymer and ethyl acetate-partitioned material could not be detected in transgenic leaves of either 88-19 or 90-5a exposed for 32 h to

Table V. *Relative content of luteolin and apigenin flavones in Lc transgenic alfalfa*

Genotype	Leaves			Stems		
	Extract wt[a]	Luteolin[b]	Apigenin[b]	Extract wt[a]	Luteolin[b]	Apigenin[b]
	mg			*mg*		
A01	36.2	100[c]	100[d]	41.9	100	100
88-4	36.9	41	120	31.3	8	100
88-19	36.4	44	24	32.5	33	80
90-19	35.0	17	47	32.1	9	91

[a] Wt of extracted plant material loaded onto the Oasis™ C_{18} cartridge. [b] The HPLC PDA (UV) response for luteolin and apigenin peaks in the non-transformed parent plant A01 was normalized to 100, then the response for the corresponding transgenic plant peaks was expressed proportionately. Data are representative of two injections, each from one bulked extraction of 24 field clones for each genotype. [c] Total apigenin (263 $\mu g\ g^{-1}$ dry wt). [d] Luteolin (824 $\mu g\ g^{-1}$ dry wt) is included for A01 leaves because anthocyanin and proanthocyanidin assays (per gram fresh wt) were also conducted on this tissue.

continuous light nor in the non-transformed genotype A01 under the inducing conditions.

DISCUSSION

Stimulation of Anthocyanin Biosynthesis by *Lc* Is a Function of Environmental Conditions and Transgene Specificity

Alfalfa was transformed with three maize anthocyanin regulatory genes, *Lc*, *B-Peru*, and *C1*, as part of a strategy to stimulate flux through the forage flavonoid pathway into late products such as anthocyanins and proanthocyanidins. Plants grown under moderate greenhouse conditions expressed abundant transcripts of these transgenes in leaves when tested by northern blotting (results not shown). However, no anthocyanin or proanthocyanidin was accumulated under these conditions, and the flavonoid structural genes *CHS*, *F3H*, and *DFR* were not induced. These data suggested that the maize regulatory transgenes were insufficient on their own to stimulate the lower branches of the flavonoid pathway.

Induction of the flavonoid pathway was highly dependent on interactions between the environment and a specific transgene sequence. When the four transgenic populations were stressed by cold or strong light, only plants from the two *Lc* populations developed a red or deep red-purple color in leaves and stems, and the intensity of the color depended on the *Lc* construct within the plants. This phenotypic change was accompanied by a rapid accumulation of *CHS* and *F3H* transcripts, although the *F3H* was transiently induced. Our results imply that the flavonoid pathway in alfalfa is induced only in the presence of an unknown stress-responsive alfalfa factor and that the induction is strongly enhanced by *Lc*. The strong induction of *CHS* transcripts, coupled with transient induction of *F3H*, suggests that this stress-responsive factor in combination with *Lc* acts predominantly but not exclusively at the entrance into the flavonoid pathway (although we have not tested expression of late anthocyanin genes). The weak expression of *CHS* and *F3H* and the green phenotype in non-transgenic alfalfa under strong light conditions suggests that a critical level of gene expression is required to stimulate anthocyanin, and this may be particularly true for *F3H*. For example, genotypes from population 90 that induce both *CHS* and *F3H* under cold conditions display a red stem phenotype. In contrast, a green stem phenotype occurs in genotype 90-3 under cold conditions because stem *CHS* but not *F3H* is induced.

Lc may interact with a stress-induced myc-like factor to override repression and to stimulate anthocyanin production in alfalfa forage, although we have not excluded other factors such as inhibitory myb-like factors. Inhibitory *myb* and *myc* genes, or those that may not be able to form a functional complex, have been identified in the past (Hope et al., 1988; Schnittger et al., 1999; Szymanski et al., 2000). They include the *C1-1* allele of maize, an myb-like repressor factor with a 3′ activation region that is less acidic than the equivalent region in the *C1* activator allele (Paz-Arez et al., 1990). Stress has rarely been considered as a factor interacting with a transgene. It is possible that some of the apparent "failures" to accumulate anthocyanin in *Lc* transgenic plants merely require identification of specific stress-inducing growth conditions.

Red color was visibly induced within hours by either cold temperatures or bright light in most *Lc* transgenic alfalfa genotypes, and the plants changed to the new phenotype completely within about 3 d. The red color faded quite quickly when these stresses were removed. However, plants placed outdoors in pots or in the field remained red to deep red on every occasion they were observed over a 3- to 4-month period, including light frost. This suggests that the stress of natural outdoor light is more than sufficient to maintain a stress-responsive factor. The rapid appearance and subsequent disappearance of red color is suggestive of a high turnover rate for anthocyanin in transgenic alfalfa, possibly incorporating an active mechanism of destruction. The anthocyanins (cyanidin and pelargonidin) that we detected in the trans-

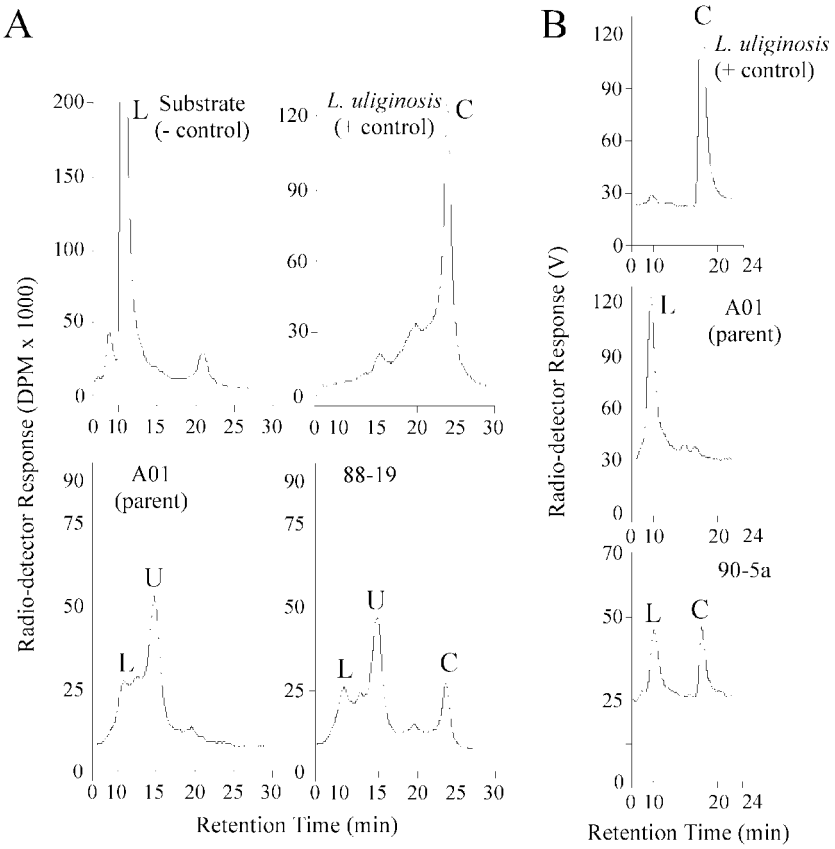

Figure 5. Representative radiochemical-HPLC traces illustrating the stimulation of LCR activity in *Lc* transgenic alfalfa tissues. Peak L, [^{3}H]cis-leucocyanidin substrate. Peak C, (+) [^{3}H]catechin (product of LCR activity). (+) [^{3}H]Catechin was designated "not detected" when the peak height was <1,000 dpm above the baseline in a smoothed HPLC chromatogram trace. LCR activity was not detected in A01 leaves, even when the A01 leaf extract was assayed using a 2-fold higher protein concentration than extracts from the transgenic genotypes. Peak U, Unidentified peak occurring with concentrated plant extracts that have weak LCR activity. A, Shoot tip containing mature (expanded) and immature (folded) leaves of genotype 88-19. HPLC elution program for A (t in min): 1, t_0 to t_{26}, 3% (w/v) HOAc, 0.7 mL min^{-1}; 2, t_{26} to t_{27}, 3% (w/v) HOAc, 1.0 mL min^{-1}; 3, t_{27} to t_{28}, linear gradient to 100% (w/v) MeOH, 1 mL min^{-1}; and 4, t_{28} to t_{33}, 100% (w/v) MeOH, 1 mL min^{-1}. Peak L (A), 11.2 min. Peak C (A), 23.6 min. Peak U (A), 14.5 min. HPLC traces include a negative control (substrate only) assay, a positive control assay (leaves of *Lotus uliginosis*), leaves of A01 non-transformed control plant, and leaves of *Lc* transgenic genotype 88-19. B, Developing seed (11 d after pollination [DAP]) of genotype 90-5a. Includes positive control assays (*L. uliginosus* leaves) and 11-d developing seeds of A01 non-transformed plants. HPLC elution program for B (t_n in min): 1, t_0 to t_{21}, 3% (w/v) HOAc, 0.7 mL min^{-1}; 2, t_{21} to t_{22}, 3% (w/v) HOAc, 1.0 mL min^{-1}; 3, t_{22} to t_{23}, linear gradient to 100% (w/v) MeOH, 1 mL min^{-1}; and 4, t_{23} to t_{28}, 100% (w/v) MeOH, 1 mL min^{-1}. Peak L (B), 9.7 min. Peak C (B), 17.3 min. Each trace is representative of two or more experiments. Elevated initial radioactivity on the trace reflects nonspecific background isotope adhering to the column matrix and radiodetector cell.

genic forage differ in composition from the 3,5-diglucoside derivatives of delphinidin, petunidin, and malvidin previously detected in petals of different alfalfa varieties and *Medicago* spp. (Lesins, 1955; Gupta, 1968). Additional analysis is being carried out to clarify the other major pelargonin-like anthocyanin that is accumulated in *Lc* transgenic leaves.

Lc has been somewhat unpredictable as a transgene among different recipient species, with effects ranging from no phenotype to strong induction of anthocyanin and ectopic effects. The 2.4-kb *Lc* construct in transgenic alfalfa population 88 contained an additional 200 bp of upstream transcribed, non-translated sequence that was absent in the 2.2-kb construct in population 90. This additional sequence includes a short open reading frame containing three in-frame start codons (Damiani and Wessler, 1993; Wang and Wessler, 1998, 2001). When transformed into rice (*Oryza sativa*), maize, Arabidopsis, or tobacco, the 2.2-kb *Lc* construct was found to be more highly expressed and more effective at inducing anthocyanin than the 2.4-kb construct (Lloyd et al., 1992; Bloor et al., 1998). The 2.2-kb construct also resulted in a broader range of pigmented tissue in *Petunia hybrida* (Bradley et al., 1998). These findings led to the conclusion (in some labs) that the upstream open reading frame repressed translation, chiefly by reducing the efficiency of ribosome re-initiation at the *Lc* start

Table VI. *LCR activity and PA concentration in Lc transgenic alfalfa genotypes*

Plant Genotype	Leaf PA[a] (32/48 h Continuous Light)[a]		LCR-Specific Activity (+)[^{3}H]Catechin			
			Leaves		Developing seed	
	Extractable[b]	Nonextractable[c]	Continuous light[d]	Photoperiod[e]	11 DAP[f] (650 μE)	14 DAP[g] (400 μE)
	μg g^{-1} fresh wt		*dpm h^{-1} mg^{-1} fresh wt $\times$ 10^3*			
A01	ND[h]/0.4	ND/ND	ND	ND	8.2	4.1
88-19	104/57.0	ND/250.0	86.2	40.4	35.7	27.3
90-5a	29.3/61.7	ND/140.7	32.8	n.d.[i]	82.0	22.0

[a] Plants exposed to 650 μE of continuous artificial light for 32 or 48 h (Condition F, Table I). [b] Extractable PA was measured by butanol:HCl hydrolysis of 80% (w/v) MeOH-extracted material bound directly to polyvinylpolypyrrolidone (PVPP) from 32-h light-exposed plants or after removal of an ethyl acetate-partitioned subfraction (from 48-h light-exposed plants). [c] Nonextractable PA was detected by butanol:HCl hydrolysis of cell particulate residue remaining after extensive MeOH washing (at least five times) to remove contaminating extractable PA. [d] Plants exposed to 650 μE of continuous artificial light for 24 h before leaf harvest (Condition F, Table I). Average of two independent experiments. [e] Plants exposed to 650 μE of artificial light for 16-h photoperiod before leaf harvest (Condition G, Table I). Average of two experiments. [f] Plants maintained under 650 of μE artificial light with 16-h photoperiod (Condition G, Table I) during forage growth, flowering, and seed development. Developing seeds (11 DAP) were harvested from two different batches of cross-pollinated seed. Average of two experiments. [g] Plants maintained under 400 μE in a greenhouse (Condition B, Table I) during forage growth, flowering, and most of seed development. Data are from one experiment from a third batch of cross-pollinated seed harvested 14 DAP. Seeds dissected from all cross-pollinations were incubated under sterile conditions for 48 h under Condition K (Table I). [h] ND, PA not detected in 2 g leaves; LCR activity, i.e. (+)[^{3}H]catechin, not detected in A01 leaves at 2-fold higher total protein compared with 88-19 and 90-5a leaves. [i] n.d., Not determined.

codon but also by RNA secondary structure (Wang and Wessler, 1998, 2001). In contrast, the introduction of a 2.2-kb *Lc* construct under the CaMV 35S promoter had no observed effect on anthocyanin accumulation in chrysanthemum (*Dendranthema* $\times$ *grandiflorum*), lisianthus (*Eustoma grandiflorum*), and pelargonidin (*Pelargonium* $\times$ *domesticum*; Boase et al., 1998; Bradley et al., 1999). However, in alfalfa, the additional 200-bp leader sequence led to enhanced *CHS* expression and enhanced anthocyanin accumulation. Although these findings need to be interpreted with caution until a larger number of transgenic genotypes have been evaluated, nevertheless they support the hypothesis that the *Lc* gene interacts with alfalfa in a manner different from its behavior in other plants, at least in vegetative tissue.

Populations expressing *B-Peru* or C1 did not display the red phenotype under any indoor or outdoor conditions tested, even though *B-Peru* is highly homologous to *Lc* (Colliver et al., 1997). *B-Peru*, but not *C*1 on its own, stimulated anthocyanin production when introduced into white clover, pea, and *B. napus* (de Majnik et al., 1998; S. Wang and M.Y. Gruber, unpublished data). Another laboratory also found that anthocyanins did not develop in other transgenic alfalfa populations containing *B-Peru* or C1 genes, even when these two genes were combined (P. Larkin, personal communication). This pattern is consistent with the hypothesis that interactions between native factors and transgenes are necessary and specific and that the successful stimulation of flavonoid accumulation is dependent on such interactions. The hypothesis is also supported by the fact that the *myc* transgene *delila* of snapdragon (*Antirrhynum majus*) has different expression patterns in each of three recipient plant species (Mooney et al., 1995).

Stimulation of Proanthocyanidin Biosynthesis in Alfalfa by the *Lc* Transgene

Because the maize *Lc* transgene was successful at stimulating anthocyanins in alfalfa forage, one genotype from each of the two *Lc* alfalfa populations was selected to test for LCR activity and proanthocyanidin content in forage after plant growth under high light intensity. LCR activity was selectively detected in leaves of genotypes 88-19 and 90-5a, but not in leaves of the non-transgenic parent A01. Activity profiles between the two transgenic genotypes tested had a similar pattern, but the activity of 88-19 was always higher than 90-5a. In addition, proanthocyanidin was accumulated in the transgenic leaves, and could be fractionated into extractable and unextractable fractions. When measured after 32 h of high-intensity light, all of the detectable proanthocyanidin of the transgenic plants was found in the extractable fraction, and a 3-fold higher concentration was recovered from genotype 88-19 leaves compared with 90-5a. After 48 h of exposure, a larger portion of detectable proanthocyanidin was unextractable except under extremely harsh conditions (butanol:HCL hydrolysis of the extracted and washed cell residue) or was partitioned into ethyl acetate.

Accurate determination of the concentration of different fractions of PA in *Lc* alfalfa forage may not be possible if their behavior differs from the alfalfa seed polymer standard (for review, see Marles et al., 2003). Although the ethyl acetate-extracted portion from the transgenic tissue was significant, its solubility characteristics differed with those of the alfalfa polymer standard and may be due to a shorter chain length. Not all positions in PA dimers, trimers, and polymers will be equally available for reaction, and color de-

velopment is reduced unless the polymer is completely acid hydrolyzed (Price et al., 1978; Mole and Waterman, 1987). Depending on how it is bound to the cell debris, the unextractable polymer may be less available for hydrolysis and, as a consequence, be underestimated in the transgenic tissue. Underestimation of PA has been observed with seed in other plant species (Marles, 2001). Although we have not measured the polymer size within these different fractions, the fact that a significant amount of extractable material partitioned both into ethyl acetate and water suggests that a range of polymer sizes is likely to be recovered when the polymers are analyzed in more detail. The proportion of extractable and unextractable *PA* is also likely to change when the forage is grown under different conditions.

LCR activity was also stimulated in developing alfalfa seed from *Lc* transgenic genotypes 88-19 and 90-5a relative to A01 non-transgenic control seed when these three genotypes were cross-pollinated with cv Peace. Seed coat is the only alfalfa tissue that normally accumulates proanthocyanidin. The LCR activity we observed with our A01 non-transgenic seed falls within the range detected for seed from cv Beaver (Skadhauge et al., 1997). Although LCR analysis was conducted only on one representative genotype from each of the two *Lc* populations, it was curious to note that the stimulation of LCR activity by the 2.2-kb construct was higher in 11-d-old transgenic seed relative to the 2.4-kb construct. This contrasts with the higher LCR activity and proanthocyanidin concentration observed for the 2.4-kb construct in forage, an observation that is consistent with forage CHS expression profiles for the two constructs.

The stimulation of LCR activity and proanthocyanidin accumulation in alfalfa forage is likely to raise strong interest in the worldwide forage seed sector. LCR activity and proanthocyanidin has not been detected previously in alfalfa leaf tissue (Skadhauge et al., 1997), even after an exhaustive search for these compounds in alfalfa and alfalfa-related germplasm (Goplen et al., 1980). Although *Lc* has been used for many years to manipulate anthocyanin biosynthesis in plants, to our knowledge, our results are also the first demonstration of proanthocyanidin stimulation by *Lc* and highlight the importance of *myc* genes in the regulation of proanthocyanidin in alfalfa. The maize *myc*-like gene *Sn* has also been shown to regulate proanthocyanidin biosynthesis in *L. corniculatus* (Damiani et al., 1998, 1999) but in leaf and root tissues that already contain easily detected amounts of proanthocyanidin (G. Lees and M.Y. Gruber, unpublished data).

Effect of the *Lc* Gene on the Products of the Upper Flavonoid Pathway in Alfalfa

The effect of the *Lc* gene on alfalfa flavonoid biosynthesis extended beyond anthocyanins and proanthocyanidins. Alfalfa forage normally accumulates a range of small flavonoids, predominantly flavones and isoflavones (Ingham, 1979; Saleh et al., 1982). Luteolin and apigenin are the main flavones induced in non-transformed alfalfa when exposed to artificial UV light (H. Ray and M.Y. Gruber, unpublished data), whereas isoflavones are induced by fungal elicitors (Ingham, 1979).

The total content of flavones was reduced in leaves and stems in field-grown clones of our *Lc* genotypes relative to the parent non-transformed genotype. Luteolin, a flavone with a di-hydroxylated B ring, was strongly reduced in all *Lc* genotypes tested. In some cases, apigenin (a monohydroxylated relative of luteolin), was also decreased, but distribution between apigenin and luteolin depended on individual transgenic genotypes and tissue type. The reduction in flavone content may be due to a draw by anthocyanin and proanthocyanidin biosynthesis on the metabolic capacity of the pathway. Alternatively, the *Lc* gene may displace or interfere with a gene that normally stimulates the flavone pathway in alfalfa. Because the flavonoid pathway is involved in plant defense, we are now conducting tests to determine whether the reduction in flavones has resulted in any changes to the stress-coping mechanisms of the *Lc* transgenic alfalfa plants.

CONCLUSIONS

The transgenic alfalfa populations expressing maize anthocyanin regulatory genes offer a unique opportunity to study the interactions between closely related introduced genes and the environment and to test strategies aimed at developing anthocyanins and proanthocyanins in forage crop species. We have shown that the *Lc* gene, but not *B-Peru* or *C1*, can stimulate both anthocyanin and proanthocyanidin biosynthesis in alfalfa forage but that stimulation only takes place in the presence of an unknown stress-responsive alfalfa factor. In addition, we have shown that the 200-bp *Lc* 5′-untranslated region sequence enhances expression of *CHS* and LCR and anthocyanin and proanthocyanin accumulation in alfalfa forage. These findings demonstrate that it will be possible to introduce proanthocyanidins into alfalfa and develop forage with improved ruminant digestibility, a technology that is highly sought by the forage industry worldwide. In the future, we will examine the interaction of *Lc* transgenic genotypes in combination with other transgenes to see if anthocyanins can be diverted into additional proanthocyanidin production. It may be possible to stimulate proanthocyanidin biosynthesis further by directly introducing a proanthocyanidin-specific regulatory transgene, such as *TAN1* (Ray and Gruber, 2001) or the *LCR* and *ANR* biochemical transgenes that have been cloned recently (Tanner et al., 2002; Xie et al., 2003).

MATERIALS AND METHODS

Alfalfa (*Medicago sativa*) Transformation

Two *Lc* sense constructs, controlled by a single CaMV 35S promoter in *Agrobacterium tumefaciens* vectors pAL69 and pAL144 (Lloyd et al., 1992), were introduced an advanced Eastern Canadian breeding genotype N4-4-2 of alfalfa (called A01 in this investigation) according to established protocols (McKersie et al., 1993). One construct contained a 2.4-kb *Lc* gene that contained all but 20 bp at the 5′ end of the full-length cDNA (Lloyd et al., 1992). This gave rise to plant population 88. The second construct was 2.2 kb in length, lacking 200 bp of the 5′ transcribed non-translated leader region (Lloyd et al., 1992). This latter construct gave rise to plant population 90. Both constructs stimulated anthocyanin accumulation in tobacco and Arabidopsis, but differed in their expression patterns (Lloyd et al., 1992). *B-Peru* (Radicella et al., 1991) and *C1* cDNAs (Cone et al., 1986) were introduced into the same breeding genotype A01 under the control of an enhanced CaMV 35S promoter (35Sx2-AMV, a doubled CaMV 35 promoter with an alfalfa mosaic virus enhancer; Gruber and Crosby, 1993). *B-Peru* was also introduced into a second Eastern Canadian breeding genotype (called A04 in this investigation) under the control of a promoter from the pea (*Pisum sativum*) ssRBC. Shoots regenerating on kanamycin were rooted and tested for the presence of *NPT*II by PCR.

Plant Maintenance and Test Conditions

Initially, selected *ntp*II$^+$ shoots were transferred to a medium of fine brick chips and grown hydroponically in Guelph (ON, Canada) in a greenhouse supplemented with high pressure halogen lights (Condition A, Table I). Later, plants were transferred to a soil-less potting mixture and maintained as stock plants in a Saskatoon greenhouse supplemented with high-pressure halogen lights (Condition B, Table I). Plants were propagated through rooted shoot cuttings to test growth and phenotype and gene expression under a range of environmental conditions. One or two internodes of stem were trimmed free of mature leaves, dipped in rooting powder (Root Stim No. 1, Plant Products Co. Ltd., Brampton, ON, Canada), and placed in florist's foam wetted with water. Rooted cuttings were grown in 3.5- × 3.5-inch pots under Condition B until they were well-established plants and then transferred to a range of additional growth conditions (C–J) outlined in Table I to test their response.

Field Plots

Established rooted cuttings (4–6-month-old clones) from selected primary transformed plants from all four populations were inoculated with a commercial preparation of *Rhizobium meliloti* and transplanted into dry land, dark-brown soil in a 16- × 24-m field plot in early July 2001 at the Agriculture and Agri-Food Canada farm (Saskatoon, SK, Canada; Condition I; Table I). Six clones per primary transgenic plant were evenly spaced within a 2-m distance in rows 1 m apart. Each planting was replicated in four locations within the plot (total of 24 clones per genotype). Seven centimeters of rainfall over the first field season was supplemented by hand watering three times while plantlets were establishing. Young flower buds and 4 cm of vegetative tissue were removed from each floral shoot before floral opening. Forage was clipped and discarded in late August. At the end of the growing season (late September 2001 and early October 2002), material longer than about 7 cm was harvested, weighed, and quick frozen in liquid nitrogen at the field site. The plants were then allowed to recover without additional harvesting before the onset of winter in November. A fence was erected after the growing season to trap a light layer of blowing snow on the plots over the winter, but plots were often bare. Plant phenotype was observed over two growth seasons. Winter temperatures averaged −20°C at the field site. Rainfall (less than 3 cm) in the second field season was not supplemented by hand watering.

The *C1* population and one of the *B-Peru* populations (45) were also grown for 3 consecutive years in a replicated trial under milder south-central Canadian conditions at the Elora farm (University of Guelph) beginning in the 1998 field season (Condition J, Table I). Six plants grown from rooted cuttings of each primary transgenic plant were transplanted evenly into short (2-foot) rows spaced 6 feet apart. Each of these plantings was replicated four times. Rainfall was plentiful, and plots were mowed when necessary to prevent flowering. Winter temperatures averaged −10°C at the Elora trial, and a heavy blanket of wet snow covered the trial in the winter.

Southern and Northern Blots

Nucleic acids were extracted from quick-frozen young or mature leaf material harvested from field- or greenhouse-grown plants using Qiagen plant DNA or RNA kits (Qiagen USA, Valencia, CA). Except where indicated, all molecular biology methods were conducted using standard techniques (Sambrook et al., 1989). After nucleic acid quantification, Southern and northern blots were prepared with 15 μg of nucleic acid following standard protocols and hybridized with an excised *Lc* gene labeled with alpha[^{32}P]dCTP using a random primer labeling kit from Invitrogen (Carlsbad, CA). Northern blots were also blotted with fragments of alfalfa *CHS*, *F3H*, or *DFR* genes that had been cloned in our laboratory. Band density was quantified by densitometry using a Bio-Rad Molecular Imager F-X with Quantity I software (Bio-Rad Laboratories, Hercules, CA) and compared with either actin or major ribosomal RNA bands. Blotting experiments were repeated at least twice.

Anthocyanin Assays

Deep frozen leaf tissue (approximately 1 g comprised mainly of leaf, petiole, and a small amount of fine stem material) was ground in liquid N_2, heated to 55°C for 10 min in 10 mL of 2 N HCl, then cooled and incubated overnight in the dark at 24°C to extract anthocyanidins. Extraction under extremely harsh hydrolysis conditions (90°C, 15 min) was avoided because it caused the red extract color to turn brown. The extract was clarified by centrifugation and then an aliquot was scanned from 240 to 600 nm in a Varian Cary 3 spectrophotometer using 2 N HCl as a blank. Anthocyanidin hydrolysates were also partitioned with water-saturated ethyl acetate, after which the red aqueous phase was extracted into isoamyl alcohol, dried, and reconstituted in methanol-1% (w/v) HCl (MeOH-HCl; Harborne, 1998). Optical densities recorded at the wavelength maximum (523 nm) were recorded if they fell within the linear portion of a standard curve of commercial cyanidin chloride. Anthocyanins were extracted from forage harvested three times from plants growing under bright sunlight.

For analysis of anthocyanin composition using TLC, moderately harsh hydrolysis conditions were used to extract anthocyanidins from genotypes 88-19 and A01 according to Harborne (1998). After a 45-min incubation in 2 N HCl (81°C–84°C), the extracts were filtered with Miracloth (Calbiochem, San Diego, CA), clarified by centrifugation, and extracted with a 3-fold volume of water-saturated ethyl acetate to remove flavonoids. Ethanol was added to the resulting red water phase to assist drying in vacuo. The resulting syrup was solubilized into 80% (w/v) MeOH and separated by cellulose TLC together with several commercial anthocyanin standards. TLC plates were developed with either 15% (w/v) acetic acid (HOAc) or Forestal solution (concentrated 3:30:10 [v/v]HCl:HOAc:water) in the first dimension and by butanol:HOAc:water (4:1:5 [v/v]) or 15% (w/v) HOAc in the second dimension. R_F values and spot colors were compared with authentic standards and with published values of a range of anthocyanins (Harborne, 1967; Mabry et al., 1970).

Flavonoid Analysis

Deep-frozen forage collected from the field trial in October 2002 was freeze dried and separated manually into stem and leaf samples for analysis of flavonoids other than anthocyanins following an established protocol with variations (Stochmal et al., 2001). Similar tissues from 24 clones of each genotype were bulked and ground in liquid N_2. Stems were additionally ground frozen in a coffee grinder in small batches. Ground tissue was extracted with chloroform (stabilized with 1% [w/v] ethanol) for 24 h in a Soxhlet thimble, and the residue air was dried with periodic stirring. Dried residue was refluxed in 70% (w/v) MeOH (1 g:10 mL, respectively) while stirring for 2 h and then cooled at room temperature by stirring. The filtrate was clarified by centrifugation at 5,000*g* for 20 min and evaporated to dryness. The dried extract was reconstituted at 0.5 g L^{-1} in purified water, acidified to 2 N HCl (10-mL total volume), boiled at 100°C for 40 min, and neutralized to pH 7.0 with 5 N NaOH. The neutralized hydrolysate was loaded onto two Oasis C_{18} cartridges (Hydrophilic-Lipophilic Balance, 3 mL, Waters, Milford, MA), and then each sample (1 mL, 30–43 mg L^{-1}) was

sequentially extracted into four subfractions with water (1 mL repeated four times), 5% (w/v) MeOH (1 mL repeated four times), 70% (w/v) MeOH (1 mL repeated twice), and 100% (w/v) MeOH (1 mL repeated 15 times). Subfractions that were extracted similarly from each cartridge were either pooled or kept separate and injected onto a Symmetry RP C_{18} column (Waters, 3.0 × 150 mm, 5-μm particle size) using a Waters Integrity HPLC. Elution from the column was monitored by a programmable PDA detector and Millenium software (Waters, Milford, MA).

Proanthocyanidin and Flavan-3-ol Monomer Analysis

Proanthocyanidin and flavan-3-ol monomers were initially analyzed using the DMACA method outlined by McMurrough and McDowell (1978) but modified to use 0.1% (w/v) DMACA in mineral acid instead of acidified 1% (w/v) DMACA and then observed histochemically according to Xie et al. (2003). Five unfolded leaves from each genotype (A01, 88-19, and 90-5a) that had been grown for 48 h under condition F and frozen at −80°C were initially soaked in MeOH for 1 week. This extraction removed green pigments that could confound the detection of the DMACA-flavanol analogs. Although soaking in MeOH can extract flavonoids and reduce the titer of flavan-3-ols, soaking leaf tissue in hexane or chloroform could not remove these green pigments. Extracted leaves were then soaked in either 1% (w/v) HCL, 100% (w/v) ethanol, 1% (w/v) HCL in ethanol, 0.1% (w/v) DMACA in 100% (w/v) ethanol, or 0.1% (w/v) DMACA in 1% (w/v) HCL in ethanol, and color development was observed carefully under a dissecting microscope throughout a 2-h period. The acid treatments were compared with the DMACA treatments to detect differences between the red color that was enhanced by the acid (due to the presence of anthocyanin) and the bluish color that is characteristic of DMACA analogs of proanthocyanidin and flavan-3-ols monomers.

Bulked leaf samples (1 to 2 g from genotypes A01 and 88-19 and 1 g from genotype 90-5a) were subsequently extracted and analyzed by the more sensitive PVPP:butanol:HCl method (Watterson and Butler, 1983) to detect whether flavan-3-ols monomers, dimers/trimers, and larger polymeric proanthocyanidins were present in small quantities in the transgenic alfalfa plants. These genotypes had been grown for 32 or 48 h under condition F, and tissue samples were stored frozen at −80°C. *Lotus uliginosus* leaf extract (highly concentrated in PA but with little or no anthocyanin) was included as a positive control. Leaves were ground to a fine powder in liquid nitrogen using a mortar and pestle. Aqueous 80% (w/v) MeOH (5.3 mM sodium metabisulfite) was then added to extract flavonoids and proanthocyanidins (2:1 [w/v] tissue:solvent) and the mixture allowed to incubate with shaking for 5 h in the dark. Particulate matter (PM) was pelleted by centrifugation and reextracted overnight with 80% (w/v) MeOH in the dark. PM was retained for additional analysis of unextractable PA as outlined below. Pooled 80% (w/v) MeOH-soluble fractions (containing extractable compounds) were reduced in volume in a vacuum centrifuge to approximately 0.5 mL. MeOH-soluble fractions from tissue that was exposed to 32 h of continuous light was used directly in the PVPP:butanol:HCl assay described below. MeOH-soluble fractions from tissue exposed to 48 h of continuous light was partitioned into two subfractions by vortexing three times with an equal volume of water-saturated ethyl acetate and separating the phases by centrifugation (Harborne, 1998). The lower aqueous fraction (which should contain larger extractable PA polymers and smaller anthocyanin and flavonoid glycosides) and the pooled upper ethyl acetate fraction (which should include less polar flavan-3-ols, PA dimers/trimers, and other flavonoid aglycones) were each dried by vacuum centrifugation.

Each of the MeOH-extracted or dried, aqueous-partitioned MeOH phases from alfalfa leaves was reconstituted in 2 mL of MeOH and divided into two equal 1-mL samples. Each 1-mL sample was vortexed intermittently for 15 min with 50 mg of PVPP (Sigma, St. Louis) in a screw cap test tube in a protocol to selectively bind longer chain proanthocyanins and distinguish them from anthocyanins (Watterson and Butler, 1983; Skadhauge et al., 1997). Solids (i.e. PVPP and bound compounds) were pelleted by centrifugation and rewashed exhaustively (more than five times with MeOH) until the supernatant had no color. An equivalent portion of the washed solids from each sample was then covered fully with 1 mL of 100% (w/v) butanol:100% (w/v) HCL (70:30 [v/v]). One sample was retained at room temperature to determine the absorbance (OD_{530}) due to residual anthocyanins (which are easily hydrolyzed), whereas the other sample was heated at 70°C for 1 h to hydrolyze anthocyanidins from proanthocyanidin polymers. The absorbance baseline was reconstructed for each of the two samples to reduce interference from additional peaks in the extracts, according to Skadhauge et al. (1997). Proanthocyanidin was determined as the difference in micrograms between heated and unheated samples using a standard curve prepared from proanthocyanidin polymer that had been extracted from bulked alfalfa cv Rambler seed and treated with PVPP and butanol:HCl exactly like the alfalfa forage extracts.

Dried ethyl acetate-partitioned fractions from MeOH-extracted leaves were reconstituted in 80% (w/v) MeOH, and a small portion was analyzed in an HP1100 HPLC on a Waters Novapack C18 column (3.9 × 150 mm, 5-μm particle size) at 1 mL min^{-1} using a 3% (w/v) acetic acid isocratic solvent system for 30 min. Chromatography was monitored with a PDA set at 230, 250, and 280 nm to monitor UV-absorbing compounds, such as (+)-catechin and small, extractable PA polymers (e.g. dimers), and at 550 nm to detect any residual contaminating anthocyanin. The remainder of the ethyl acetate-partitioned fraction was divided into two equal portions and hydrolyzed in 100% (w/v) butanol:100% (w/v) HCL (70:30 [v/v]) either at room temperature or at 70°C for 1 h to detect anthocyanidins arising from any small, extractable PA polymers.

Cell residue, i.e. PM retained after the initial MeOH extraction to remove extractable PA, was washed at least five times with 100% (w/v) MeOH (2:1 [w/v] PM:MeOH) throughout a 24-h period until the supernatant was colorless. The PM was tested in a butanol:HCl assay as outlined above (but without the PVPP step) to detect unextractable PAs.

LCR Assays

LCR activity assays were conducted on young and mature fresh leaf material that was harvested from duplicate primary *Lc* transgenic plants that had been grown in a growth cabinet under Condition B and then transferred to Conditions F or G for 12, 24, or 48 h. Assays were also conducted on young developing alfalfa seed harvested from genotypes 88-19, 09-5a, and A01 that had been grown under Condition G and cross-pollinated on three separate occasions with a clone of the western Canada-adapted alfalfa cv Peace. At 11 DAP, pods were dissected open, and seeds were removed under sterile conditions. Young seeds were incubated for 2 d on sterile filter paper placed on solid Murashige and Skoog media under Condition K. LCR enzyme activity was assayed in the alfalfa tissues by measuring (+)[^{3}H]catechin extracted into ethyl acetate after incubating [^{3}H]cis-leucocyanidin, NADPH, and aqueous tissue extracts for 30 min at 30°C under reducing conditions (Tanner and Kristiansen, 1993; Skadhauge et al., 1997). [^{3}H]flavonoids were separated in 3% (w/v) acetic acid using a Novapak C_{18} column and a Hewlett-Packard 1090 HPLC outfitted with a programmable PDA detector and a Berthold LB 506 radio-HPLC detector. The column was flushed with MeOH after each assay. (+)[^{3}H]Catechin was completely recovered in the ethyl acetate phase, whereas recovery of other [^{3}H]flavonoids was <50% due to partitioning into the aqueous phase and nonspecific binding to the column matrix. LCR activity was recorded as the area (disintegrations per minute) of the (+)[^{3}H]catechin HPLC peak in a 30-min assay using an analog/digital signal converter and HPChemStation software and was expressed as radio-specific activity (disintegrations per minute per milligram fresh weight per hour). [^{3}H]cis-leucocyanidin substrate was synthesized in the laboratory (Tanner and Kristiansen, 1993).

Several control assays were included routinely in each experiment. These included negative controls (assay buffer, NADPH, and substrate without plant extract or with boiled plant extract), a strong positive control (assay buffer, substrate, NADPH, and *Lotus uliginosis* leaf extract), tissue-specific controls (including leaf and cross-pollinated seed extracts of A01), and extracts spiked with nonradioactive (+)catechin. Some of the control assays were necessary because the continuous use of acetic acid in the elution buffer slowly stripped away the C_{18} column coating and increased retention times. The control assays were also necessary to monitor products unrelated to LCR activity that accumulated in assays containing a high proportion of concentrated plant extract with weak LCR activity. Assays that contained (+)[^{3}H]catechin HPLC peak heights <1,000 dpm above a smooth constructed baseline were regarded as "not detected." Plant extracts with high protein concentrations were developed to recover consistent, easily measured (+)[^{3}H]catechin peaks in samples with low activity.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes,

ACKNOWLEDGMENTS

We are grateful to Sandra Northrup, Terry Huebert, M.A. Susan Marles, Cecilio Gregorio, Ning Chen, Donna Hancock, Tim Nelson, Naomi Carriere, and Jim Elder for proficient technical assistance and advice. The authors acknowledge the gift of a pea ssRBC promoter and plasmid pBI525 containing the enhanced CaMV 35S promoter from the Plant Biotechnology Institute (National Research Council, Saskatoon, SK, Canada). Alfalfa seed polymer was purified earlier in the laboratory of Dr. Alister Muir at the Saskatoon Research Centre (Agriculture and Agri-Food Canada).

Received December 27, 2002; returned for revision February 20, 2003; accepted April 15, 2003.

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