Members of the *c1/pl1* Regulatory Gene Family Mediate the Response of Maize Aleurone and Mesocotyl to Different Light Qualities and Cytokinins[1]

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We investigated the role of transcription factors (R, SN, C1, and PL) in the regulation of anthocyanin biosynthesis by different light qualities (white, red, blue, and ultraviolet) and by cytokinin in maize (*Zea mays*). We analyzed anthocyanin accumulation, structural gene expression, and regulatory gene expression in the seed aleurone and the seedling mesocotyl. In the mesocotyl, white, blue, and ultraviolet-B light strongly induced anthocyanin accumulation and expression of two key structural genes. In contrast, red light had little effect. Cytokinin enhanced the response to light but was not sufficient to induce anthocyanin accumulation in darkness. Plants with the *pl-bol3* allele showed high levels of anthocyanin accumulation in response to light, whereas those with the *pl-W22* allele did not, demonstrating the importance of *pl1* in the light response. The expression of the *pl-bol3* gene, encoding an MYB-related transcription factor, was induced by light and enhanced by cytokinin in a very similar manner to the structural genes and anthocyanin accumulation. Expression of the *bHLH* (basic helix-loop-helix) *Sn1-bol3* gene was stimulated by several light qualities, but not enhanced by cytokinin, and was less well correlated with the induction of anthocyanin biosynthesis. In the aleurone, white, red, and blue light were effective in stimulating anthocyanin accumulation and expression of the *MYB*-related gene *C1*. The *bHLH R* gene was constitutively expressed. We conclude that specific members of the *MYB*-related *c1/pl1* gene family play important roles in the regulation of anthocyanin synthesis in maize in response to different light qualities and cytokinin.

Anthocyanins represent one of the most widespread classes of pigment in higher plants. They are important secondary metabolites produced in a range of organs. Anthocyanins are involved in a variety of processes during plant development and during interactions between the plant and its environment (for review, see Mol et al., 1996; Chalker-Scott, 1999).

The biochemical pathway leading to the synthesis of anthocyanin is well understood and the structural and regulatory genes involved have been cloned from many plants (for review, see Martin and Paz-Ares, 1997; Mol et al., 1998; Winkel-Shirley, 2001). In maize (*Zea mays*), at least 20 genes are involved in the synthesis as well as in the determination of the amount, type, and distribution of anthocyanins (Dooner et al., 1991). The structural genes, encoding enzymes catalyzing the different steps of the biosynthetic pathway and including *c2*, *chi*, *f3h*, *a1*, *a2*, *bz1*, and *bz2*, are controlled in a coordinated manner by the action of at least two families of regulatory genes, *r1/b1* and *c1/pl1*, responsible for the developmental and tissue-specific pigmentation of plant and seed tissues. The *r1/b1* family encodes functionally exchangeable proteins with sequence homology to the basic helix-loop-helix (bHLH) DNA-binding/dimerization domain found in the MYC oncoproteins. This family comprises the *b1* and *r1* genes and in certain accessions additional members such as *sn1*, *Lc*, and *Hopi*, distal to *r1* (Chandler et al., 1989; Ludwig et al., 1989; Tonelli et al., 1991; Consonni et al., 1993; Petroni et al., 2000). The *c1/pl1* family encodes proteins with sequence homology to the DNA-binding domain of the MYB-related oncoproteins (Cone et al., 1986, 1993a; Paz-Ares et al., 1986, 1987). This family shows less allelic diversity than *r1/b1* and its members are characterized by functional and structural similarity (Cone et al., 1993a). *c1* is required for anthocyanin synthesis only in seeds tissues such as the aleurone, the scutellum, and the embryo, whereas *pl1* is necessary for the pigmentation of several tissues of the plant body and of the pericarp, the outer seed integument.

Anthocyanin biosynthesis is modulated by environmental stimuli such as light, temperature, and nutrient supply, as well as by internal stimuli such as growth regulators, metabolites, and the particular

[1] This work was supported by Ministero Delle Politiche Agricole E Forestali Progetto Biotecnologie Vegetali (Area 1, Progetto N. 2) and by Ministero Dell' Istruzione, Dell' Università E Della Ricerca (Italy) Progetto Strategico Biotecnologie (to C.T.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.010799.

developmental stage of the competent tissue (Mol et al., 1996). Light is one of the most important environmental stimuli regulating anthocyanin accumulation and acts both as an essential stimulus and as a factor that modulates the intensity of pigmentation. Scheffler et al. (1994) demonstrated that the *C1* active allele is necessary for light induction of the *C2* structural gene in the aleurone of germinating seeds. In addition, expression of the *Sn1-bol3* gene is modulated by light in the pericarp layer of the seed (Procissi et al., 1997) and in the mesocotyl, according to the *sn1* allele studied (Tonelli et al., 1991, 1994). The *Pl* gene is unaffected by light (i.e. *Pl-Rh* allele), whereas some *pl1* alleles are induced by light (i.e. "sun red" *pl*; Cone et al., 1993b). Analogously, the *C1* gene appears to be constitutively activated in the aleurone during seed development, although some *c1* alleles show light inducibility during seed germination (Scheffler et al., 1994). Moreover, in pericarp, both *Sn1-bol3* and *pl* expression are light modulated, whereas in aleurone *R-sc* is constitutively expressed and *C1* shows light inducibility (Procissi et al., 1997). The light-induced expression of the *MYB* genes *C1* and *pl* was found to be the limiting factor for conferring the developmental competence of the pericarp and the aleurone layers to respond to light (Procissi et al., 1997). The expression of the *r1* gene *Hopi* in scutellum is not enhanced by light and is limited to the germination phase, whereas the accumulation of *C1* transcript is under both developmental and light control (Petroni et al., 2000).

Little is known about the role of different light qualities in the modulation of anthocyanin synthesis and accumulation in maize. Mereghetti et al. (1991) determined the kinetics of light-induced pigment accumulation in pericarp and aleurone. The aleurone responds to white, red, and blue light by increasing its pigment content up to 72 h of irradiation. Pericarp tissue responds to light to a lesser extent reaching the highest value between 24 and 48 h of continuous illumination with blue and white light; red light, on the other hand, induces only a negligible response. A similar analysis has been performed in maize roots. Irradiation of seedling root tissues with different light qualities resulted in a significant increase in anthocyanin only in response to blue light (Galbiati et al., 1994).

Plant growth regulators are also important in controlling anthocyanin biosynthesis (Mol et al., 1996). For instance, gibberellins stimulate anthocyanin accumulation in petunia (*Petunia hybrida*) corolla tissue (Weiss et al., 1992) and abscisic acid modulates anthocyanin accumulation in maize seeds by its ability to regulate *C1* gene expression (Kao et al., 1996). Cytokinin treatment stimulates anthocyanin accumulation in tissue culture and plant organs. In Arabidopsis seedlings, this increase is due to the coordinate increased accumulation of mRNAs encoded by four genes in the anthocyanin biosynthetic pathway that also appear to be controlled by a circadian clock (Deikman and Hammer, 1995).

Here, we address the regulatory mechanisms underlying the accumulation of anthocyanin in maize aleurone and mesocotyl tissues in response to different light qualities and cytokinin. We define the effects of different light qualities, show that the two tissues differ markedly in their responsiveness to red light, and demonstrate that cytokinin enhances the effect of light in mesocotyls. We report the regulation by light qualities and cytokinin of *MYB*-related and *bHLH* maize genes involved in the control of anthocyanin biosynthesis. We conclude that the accumulation of anthocyanin, and induction of anthocyanin structural genes, is most closely correlated with expression of the relevant *MYB* regulatory genes.

RESULTS

Different Effects of Red Light on Anthocyanin Gene Expression in Seeds and Seedlings

Because light regulation of anthocyanin biosynthesis is mediated through transcriptional activation of the biosynthetic genes, the question we addressed is whether light induces structural gene expression through the same transcription factors that control tissue-specific pigment accumulation, or through a different set of regulatory genes. Moreover, we asked whether genes of the *r1/b1* and *c1/pl1* families were themselves light regulated because this would implicate them as effectors of light signal transduction.

Although responses to light occur throughout the life of the plant, they are especially evident in the young seedling. We determined anthocyanin content in homozygous *r-Δ Sn1-bol3 pl-bol3* and homozygous *r-Δ Sn1-bol3 pl-W22* mesocotyls after exposure to different light qualities: white, blue, and red light (Table I). In each case, we measured pigment accumulation over 72 h illumination. In *r-Δ Sn1-bol3 pl-bol3* mesocotyls, no anthocyanin accumulation was detected in darkness, whereas both white and blue light induced a strong increase in pigment content. There was a difference in the effect of these light qualities because after 72 h of exposure, the anthocyanin content in white light exceeded that in blue light. In contrast, red light induced only a very weak response. Homozygous *r-Δ Sn1-bol3 pl-W22* mesocotyls showed a much smaller response to all three types of light

Table I. *Anthocyanin accumulation in the mesocotyl of 5-d-old dark-grown r-Δ Sn1-bol3 pl-bol3 and r-Δ Sn1-bol3 pl-W22 seedlings after 72 h subsequent treatment with different light quality*

Mean values are expressed as A_{530} per mesocotyl. Mean SEs are below 5%. For light treatments, see "Materials and Methods."

Line	Light Treatment					
	Dark	White	Blue	Red	UV-A	UV-B
pl-bol3	0.04	9.01	4.88	0.86	2.42	6.98
pl-W22	0.08	1.17	1.22	0.11	0.41	1.31

treatment than *r-Δ Sn1-bol3 pl-bol3* mesocotyls, confirming a higher accumulation in white- and blue-light treatments than in the red one (Table I).

Homozygous lines used in the experiments differed only in their *pl1* locus constitution (*pl-bol3* versus *pl-W22*). Therefore, the anthocyanin accumulation data suggest that the regulatory gene *pl1* could be the main factor determining the response of the tissue to different light qualities. It is known that *sn1* and *pl1* genes mediate the transcriptional control of structural genes involved in anthocyanin deposition (Procissi et al., 1997). To analyze anthocyanin gene expression, we measured transcript levels of two key structural genes, *C2* (chalcone synthase) and *A1* (dihydroflavonol reductase), and of *sn1* and *pl1* regulatory genes by reverse transcriptase (RT)-PCR in homozygous *r-Δ Sn1-bol3 pl-bol3* mesocotyls after exposure to white, red, and blue light for 24, 48, and 72 h (Fig. 1A). The expression of the *C2* gene was well correlated with anthocyanin accumulation after white- and blue-light treatments (Fig. 1B). Both treatments induced an increase in the steady-state level of the *C2* transcript up to 72 h of illumination. In contrast, after 24 h of exposure to red light, we observed a strong induction of the *C2* transcript that was followed by a severe reduction after 48 and 72 h. No expression was observed in the absence of light. Analysis of *A1* gene expression gave very similar results.

Analysis of the expression pattern of the *MYB* gene *pl-bol3* highlighted that the *pl-bol3* transcript was absent in the dark but was strongly induced after 24 h of exposure to white, blue, and red light. We observed a further increase of *pl-bol3* gene expression

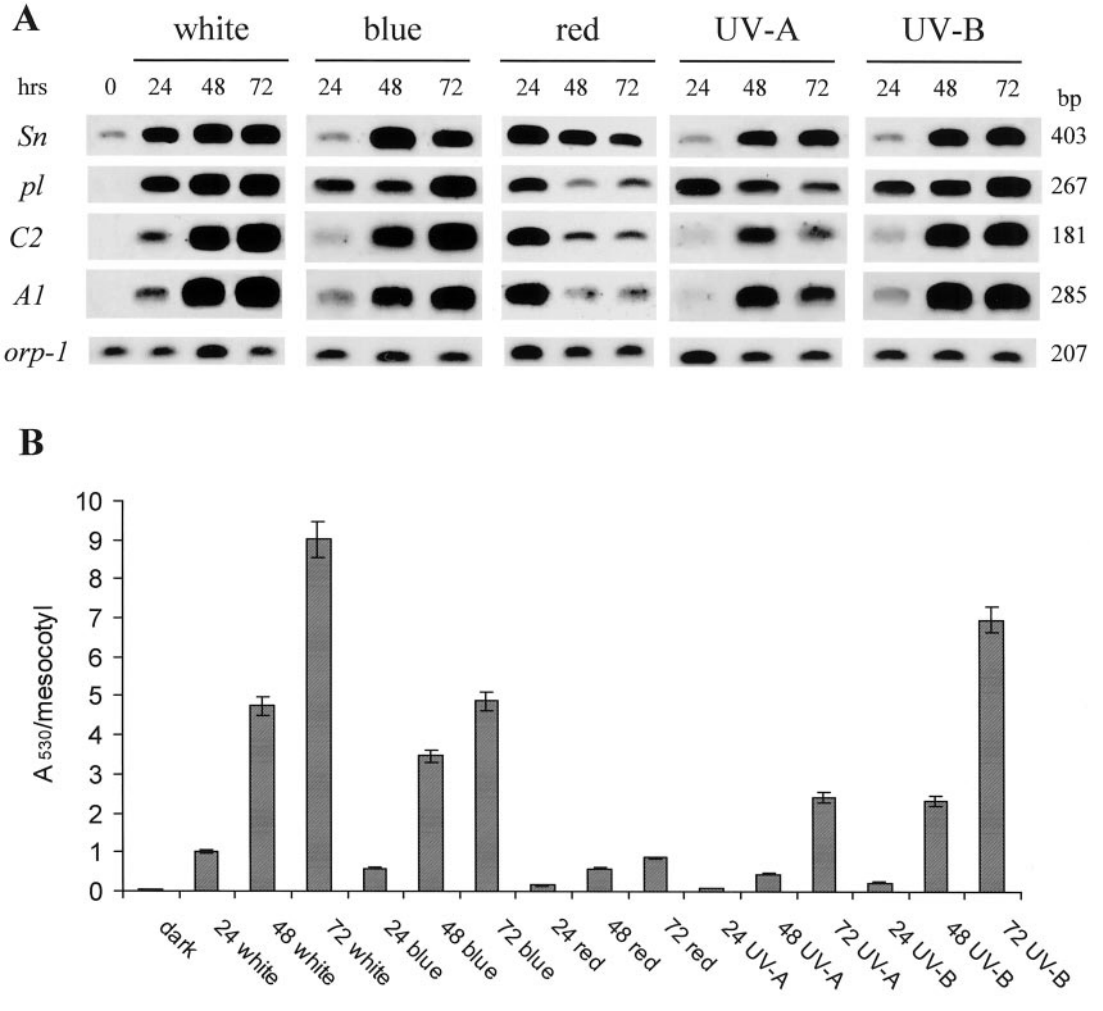

Figure 1. A, RT-PCR analysis of mRNA accumulation of anthocyanin structural and regulatory genes in *r-Δ Sn1-bol3 pl-bol3* seedlings exposed to continuous white, blue, red, UV-A, and UV-B light for 0, 24, 48, or 72 h. cDNA was made from total RNA extracted from mesocotyls. Specific primers for the *Sn*, *pl*, *C2*, and *A1* genes were used to amplify the cDNA (see "Materials and Methods"). Amplifications were carried out for 20 cycles. The amplification of the *orp-1* transcript was used as an internal control. The blots were hybridized with the different probes (see "Materials and Methods"). B, Anthocyanin accumulation in mesocotyl was measured in each treatment as described in A. Values are expressed as A_{530} per mesocotyl. Mean values represent 10 independent replicates. Mean SEs are below 5%.

up to 72 h of white and blue illumination. On the contrary, after red treatment, the initial induction was followed by a strong subsequent decrease in transcript level. Analysis of *Sn1-bol3* expression showed that it was expressed in the dark at a low level. After exposure to white light, its transcript increased up to 72 h of treatment. Treatment with blue light did not have any effect at 24 h, whereas 48 and 72 h of illumination induced mRNA accumulation. In contrast, red-light treatment strongly induced *Sn1-bol3* expression after 24 h, followed by a subsequent decrease in mRNA level. In this case, the decrease in transcript level was less pronounced compared with *pl-bol3*. Therefore, from this RT-PCR analysis it appeared that, even if the *bHLH*-like *Sn1-bol3* gene is necessary for the full transactivation of the *A1* and *C2* structural genes, the ability of the mesocotyl to respond to different light qualities was most closely correlated with the expression of the *MYB*-related regulatory gene. This result is strengthened by the observation that in *r-Δ Sn1-bol3 pl-W22* mesocotyl, the *pl-W22* allele is less induced by white light than *pl-bol3* and this lower expression is well correlated to the structural genes transcript levels (data not shown).

To discover whether this observation was also valid for other members of the *bHLH* and *MYB* regulatory gene families, the same analysis was performed in the aleurone of *R-sc C1* homozygous seeds. Pigmentation of the external layer of the endosperm is due to the concomitant expression of the *r1* and *c1* genes. Figure 2A shows that different results were obtained for the aleurone compared with the mesocotyl. White-, blue-, and red-light treatments were all able to induce anthocyanin accumulation, although to slightly different extents. It is interesting that red light-induced anthocyanin accumulation almost as much as white light. Weak but detectable anthocyanin accumulation was observed in darkness. The expression pattern of the structural gene *A1* was well correlated with anthocyanin accumulation (Fig. 2B). *A1* was feebly expressed in the absence of light but was strongly active after white- and red-light treatments. Peaks of expression were observed after 48 h of white-light exposure and after 72 h of red-light treatment. In contrast, blue light induced only a moderate expression of the structural gene. Analysis of regulatory gene activity showed that expression of the *R-sc* gene was constitutive in all treatments performed. In contrast, the *C1* gene expression pattern was very similar to that of *A1*, being weak in darkness and strongest in white light. Red light stimulated expression over 72 h, whereas blue light had only a slight effect on *C1* expression after 48 and 72 h.

We emphasise that red light was able to induce anthocyanin accumulation in aleurone but much less so in mesocotyl. Both structural and regulatory genes were strongly expressed in red light in the seed, whereas their expression was transient in the young plant, decreasing to a very low level after the initial induction.

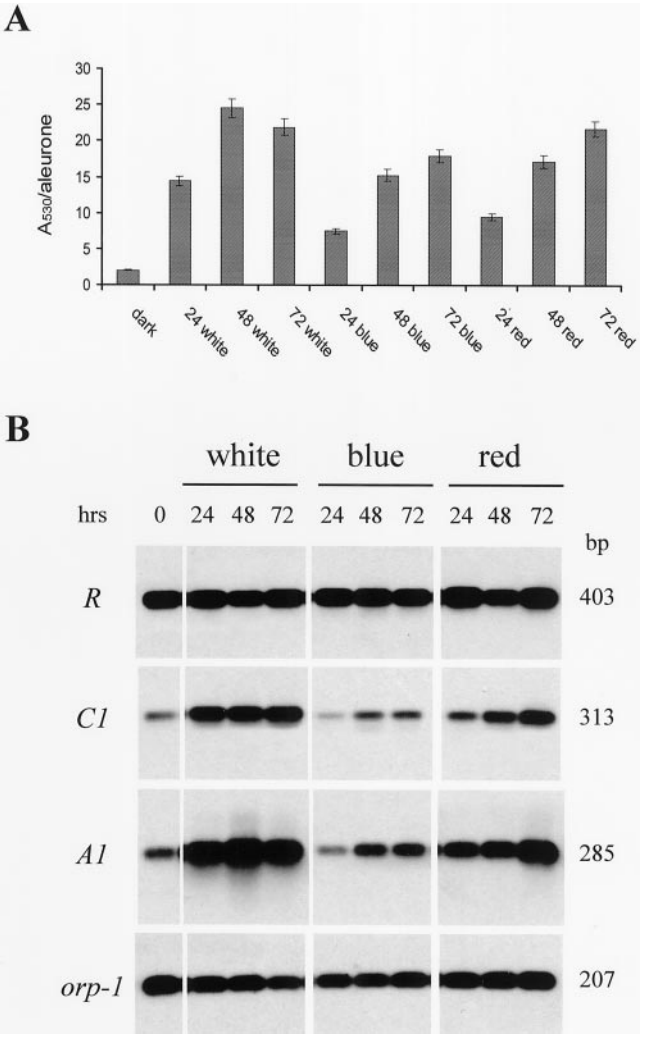

Figure 2. A, RT-PCR analysis of mRNA accumulation of anthocyanin structural and regulatory genes in *R-sc sn C1* aleurones of 30-d after pollination seeds exposed to continuous white, blu,e and red light for 0, 24, 48, or 72 h. cDNA was made from total RNA extracted from aleurones. Specific primers for the *R*, *C1*, and *A1* genes were used to amplify the cDNA (see "Materials and Methods"). Amplifications were carried out for 20 cycles. The amplification of the *orp-1* transcript was used as an internal control. The blots were hybridized with the different probes (see "Materials and Methods"). B, Anthocyanin accumulation in aleurones was measured in each treatment as described in A. Values are expressed as A_{530} per aleurone. Mean SEs are below 5%.

UV-B Light Greatly Induces Anthocyanin Accumulation in Mesocotyls

One of the most important abiotic stresses that plants experience is UV irradiation. With the aim to understand how different light qualities can modulate anthocyanin biosynthesis, we analyzed the response of the mesocotyl to UV-A and UV-B light treatments. As shown in Table I and Figure 1B, UV

light is able to induce anthocyanin accumulation in homozygous *r-Δ Sn1-bol3 pl-bol3* mesocotyls; in particular, UV-B light was very effective. The UV-B light induction was, in fact, almost comparable with that in white light. However, UV-A light induced a moderate response only after 72 h of treatment (Fig. 1B). Also, in *r-Δ Sn1-bol3 pl-W22* mesocotyls, the UV-B light is more effective than UV-A light in inducing anthocyanin accumulation, although the levels are still much lower than in *r-Δ Sn1-bol3 pl-bol3* as already observed for the other light treatments (Table I).

Analysis of mRNA accumulation of the structural *A1* and *C2* genes revealed very low expression after 24 h of exposure to UV-A or UV-B light (Fig. 1A). Considerable transcript accumulation was observed only after 48 and 72 h of illumination. Moreover, UV-B light treatment was able to induce stronger *A1* and *C2* expression compared with UV-A at each time analyzed. The *MYB*-related gene *pl-bol3* was expressed at a high level even after 24 h of treatment with both light qualities. Subsequently, the *pl-bol3* transcript level slightly increased after exposure to UV-B light, whereas it decreased after exposure to UV-A light. In contrast, *Sn1-bol3* was only feebly expressed after 24 h, whereas its transcript level increased after longer exposure. This increase was to a similar extent in both light qualities. We conclude that pigment accumulation and structural gene mRNA levels in the mesocotyl are most closely correlated with expression of *pl-bol3*.

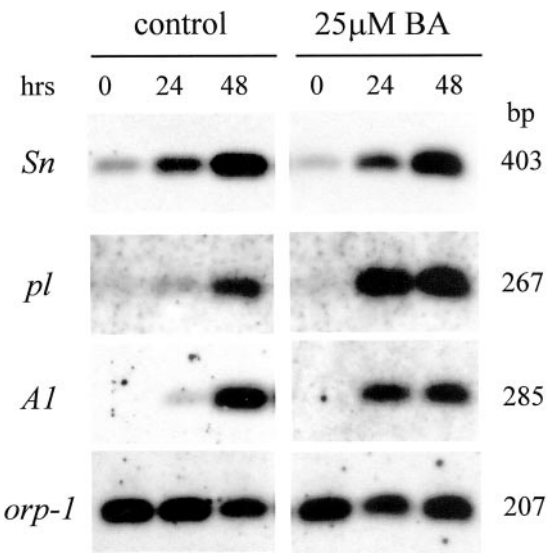

Figure 3. RT-PCR analysis of mRNA accumulation of anthocyanin structural and regulatory genes in *r-Δ Sn1-bol3 pl-bol3* seedlings treated with a 25 μM BA solution for 7 d in darkness and subsequently exposed to continuous white light for 0, 24, and 48 h. Control plants were maintained in the same conditions. cDNA was made from total RNA extracted from mesocotyls. Specific primers for the *Sn*, *pl*, and *A1* genes were used to amplify the cDNA (see "Materials and Methods"). Amplifications were carried out for 20 cycles. The amplification of the *orp-1* transcript was used as an internal control. The blots were hybridized with the different probes (see "Materials and Methods").

MYB-Related Genes Mediate the Effects of Cytokinin on Anthocyanin Accumulation in Maize

To test the effects of cytokinin on maize anthocyanin accumulation, homozygous *r-Δ Sn1-bol3 pl-bol3* and homozygous *r-Δ Sn1-bol3 pl-W22* plantlets were grown in the presence of different concentrations (0.5 and 25 μM) of the synthetic cytokinin benzyladenine (BA) for 10 d in darkness and then exposed to continuous white light for 48 h. Even at the lower concentration of BA tested, pigments accumulated more in *r-Δ Sn1-bol3 pl-bol3*-treated plants than in the controls (Table II). At 25 μM BA, the anthocyanin amount in treated plants was 5-fold greater than in the controls. In contrast, both treatments were unable to induce a detectable response in homozygous *r-Δ Sn1-bol3 pl-W22* mesocotyls. Therefore, the *pl* gene seems to be the key factor for pigment accumulation in response to cytokinin application.

To analyze anthocyanin gene expression in response to cytokinin treatment, we performed a RT-PCR analysis (Fig. 3) on mesocotyls maintained in darkness for 7 d, watered with BA solution (25 μM), and then exposed for increasing periods of time to continuous white light (0, 24, and 48 h). First, we found that no expression of the *A1* structural gene was observed in the absence of light in control and cytokinin-treated plants. Therefore, cytokinin alone is insufficient to induce the response. Second, BA treatment induced an increase in the steady-state level of the transcripts of both structural (*A1*) and *MYB*-related regulatory (*pl-bol3*) genes. This effect was particularly evident after 24 h of exposure to light. An effect of cytokinin was not observed for *Sn1-bol3*. A similar increase in *Sn1-bol3* transcripts was observed in treated and control plants after exposure to white light. Therefore, cytokinin acts to enhance the effect of the light stimulus in maize mesocotyls and this effect is correlated with the ability of the hormone to affect the level of the *MYB*-related regulatory gene transcript.

Table II. *Anthocyanin accumulation in the mesocotyl of r-Δ Sn1-bol3 pl-bol3 and r-Δ Sn1-bol3 pl-W22 plantlets grown for 10 d in the dark with BA at 0, 0.5, and 25 μM, followed by 2 d in the light*

Mean values are expressed as A_{530} per mesocotyl. Mean SEs are below 5%. For hormone treatments, see "Materials and Methods."

Line	BA Treatment		
	Control	0.5	25
		μM	
pl-bol3	0.47	0.78	2.32
pl-W22	0.11	0.11	0.12

DISCUSSION

Our aim was to investigate the role of specific transcription factors in the regulation of anthocyanin biosynthesis by different light qualities and by cytokinin in maize. Different maize tissues are characterized by the expression of different combinations of *r1/b1* and *c1/pl1* regulatory genes, and the corresponding transcription factors mediate the tissue-specific regulation of anthocyanin biosynthesis (Cone et al., 1993b; Consonni et al., 1993). Moreover, several studies have indicated a role for specific transcription factors in light-induced anthocyanin accumulation in maize (Scheffler et al., 1994; Procissi et al., 1997). However, the function of these transcription factors in responses to different light qualities and to cytokinin was unknown. Therefore, we analyzed anthocyanin accumulation, anthocyanin structural and regulatory gene expression in different tissues, a seed tissue (the aleurone), and a plant shoot tissue (the mesocotyl) after light and hormone treatments.

In the mesocotyl, light strongly induces anthocyanin accumulation and expression of two key structural genes, *C2* and *A1*. There is no detectable accumulation in darkness. The induction is strongest in white, blue, and UV-B light. This is similar to the regulation of anthocyanin biosynthesis in several species; for instance, mature Arabidopsis leaf tissue (Jackson et al., 1995; Fuglevand et al., 1996). Blue light is likely to be detected by a cryptochrome photoreceptor in maize, whereas UV-B is not detected by cryptochromes, at least in Arabidopsis (Fuglevand et al., 1996; Wade et al., 2001). The relatively small effect of red light on anthocyanin accumulation in the mesocotyl indicates a low level of responsiveness to light-stable phytochrome. Although not examined here, it is possible that far-red light, detected by a light-labile phytochrome, would have been effective. In contrast, the aleurone has detectable anthocyanin accumulation and structural gene expression in darkness and a strong response to red light, presumably mediated by phytochrome. A similar observation was reported by Mereghetti et al. (1991). The response to blue light in the aleurone could be mediated by cryptochrome, or phytochrome, but this is unknown at present. It is interesting that 24 h of illumination with red light strongly induces *A1* and *C2* transcript accumulation in the mesocotyl, but these accumulations are transient and no significant anthocyanin accumulation ensues. The possibility of a translational or posttranslational control merits investigation. Similarly, Noh and Spalding (1998) reported that an anion channel blocker inhibited anthocyanin accumulation in response to blue light in Arabidopsis, but did not prevent light induction of transcripts or protein of several biosynthetic enzymes, suggesting a posttranslational control.

The *pl-bol3* allele encoding the MYB-related transcription factor is strongly light induced in the mesocotyl. The pattern of *pl-bol3* regulation by different light qualities, for instance in blue versus red light and UV-B versus UV-A, closely correlates with that of the structural genes and anthocyanin accumulation. A sustained increase in *pl-bol3* transcript accumulation is seen in white, blue, and UV-B light, in parallel with continued anthocyanin accumulation, whereas in red light *pl-bol3* transcript accumulation is transient. The much weaker white, blue, and UV-B light response of plants possessing the *pl-W22* allele demonstrates the importance of the MYB transcription factor in mediating light induction. We hypothesize that cryptochrome and UV-B light signal transduction pathways promote rapid induction of the *pl-bol3* gene and that the encoded MYB-related factor mediates expression of the anthocyanin biosynthetic genes. An MYB-related transcription factor also has a key role in regulating anthocyanin structural gene expression in other species, such as parsley (*Petroselinum crispum*; Feldbrugge et al., 1997) and Arabidopsis (Hartmann et al., 1998; Borevitz et al., 2000; Harmer et al., 2000). In these species, the identities of the specific *MYB*-related genes that mediate light induction are not yet clear. MYBs are encoded by large gene families and light is reported to induce the expression of several *MYB*-related genes (e.g. in Arabidopsis; Kranz et al., 1998), so identification of the genes mediating specific responses is difficult. The effect of cytokinin is to enhance the response to light. Exogenous cytokinin is insufficient to stimulate *A1* gene expression and anthocyanin accumulation in darkness in the mesocotyl. Similar results were found with Arabidopsis seedlings grown in a light/dark cycle: Cytokinin enhanced anthocyanin accumulation and biosynthetic gene expression during the photoperiod (Deikman and Hammer, 1995). However, in contrast, the addition of cytokinin to dark-grown Arabidopsis seedlings stimulated activity of the chalcone synthase gene promoter (Chory et al., 1991). The effect of cytokinin in the maize mesocotyl appears to be mediated by the *pl1* allele. We observed a hyper-stimulation of *pl-bol3* expression in the light in the presence of cytokinin. In contrast, there was no effect of cytokinin on light induced expression of the *Sn1-bol3* allele. Moreover, the much reduced cytokinin response of plants possessing the *pl-W22* allele highlights the importance of the *MYB*-related gene in mediating the response to cytokinin.

In the aleurone, the *C1* gene is important in mediating the effects of light (Scheffler et al., 1994; Petroni et al., 2000). In darkness, *C1* shows a low level of expression and there is a small amount of anthocyanin accumulation. White and red light promote a strong, sustained increase in *C1* transcripts, and these treatments produce the highest levels of structural gene expression and anthocyanin accumulation. Blue light elicits the least response in terms of *C1* expression and anthocyanin biosynthesis. Nevertheless, substantial levels of anthocyanin are formed despite the small increase in *C1* expression. In fact, after 24 h

of blue light, anthocyanin accumulates in the absence of any increase in *C1*, or *A1*, expression. This observation suggests that the response to blue light in this tissue may involve additional factors, such as the posttranslational control of preexisting enzymes.

Although the importance of the *pl1/c1* regulatory gene family in responses to light and cytokinin is highlighted by our findings, the role of the *r1/sn1* genes should not be diminished because at least one member of this family must be expressed to activate the structural genes. In aleurone tissue, expression of the *R* allele is not affected by light, in contrast to *C1*. Previous studies have shown that *Sn1-bol3* gene expression is modulated by light in several tissues (Tonelli et al., 1991, 1994; Procissi et al., 1997). Here, we show that *Sn1-bol3* gene expression is light induced in mesocotyls somewhat differently to *pl-bol3*. Moreover, *Sn1-bol3* expression is less well correlated with structural gene expression and anthocyanin accumulation than *pl-bol3* expression; this is evident, for example, in red versus blue light, UV-B versus UV-A light, and in the response to cytokinin. Furthermore, the presence of *Sn1-bol3* transcripts in darkness in the mesocotyl is insufficient to induce anthocyanin accumulation.

In summary, our research extends previous studies in maize of the effects of light on anthocyanin accumulation and the light regulation of transcription factors controlling anthocyanin biosynthesis. We report the effects of different light qualities on the expression of specific transcription factors and correlate these with the biosynthesis of anthocyanin in both the mesocotyl and aleurone. Our findings include the first data on the mechanisms underlying the UV-B induction and cytokinin regulation of anthocyanin accumulation in maize. The results point to a key role for MYB-related transcription factors in mediating the responses.

MATERIALS AND METHODS

Plant Materials

All maize (*Zea mays*) seed stocks used in this study were in the W22 background and were homozygous dominant for the color factors *a1*, *a2*, *c1*, *c2*, *bz1*, and *bz2*, and homozygous recessive for the *b1* gene. However, they differed in *r1*, *sn1*, and *pl1* gene constitution. *r1*, *sn1*, and *pl1* were collected from diverse sources and incorporated by backcrossing into the background of inbred W22. *R-sc* is self-colored aleurone from green plants, a germinal derivative of the *R-st*, composed of (*Sc*)(*I-R*)(*Nc*) and obtained by loss of the (*I-R*) component (Kermicle, 1984; Ronchi et al., 1995). *sn1* is a factor lying two map units distal to *r1* conferring specific pigmentation, after light exposure, to the scutellar node, mesocotyl tissue, leaf base, midrib, and to seed integuments (glumes and pericarp). Three independent accessions (*bol1*, *bol2*, and *bol3*) have been identified in separate Bolivian populations. *Sn1-bol3* differs from the others in that it confers, after light exposure, a higher pigmentation level to mesocotyls. Detailed descriptions of the origin, phenotypes, and structural characteristics of the *pl1* locus can be found in Cone et al. (1993a, 1993b). The genetic stocks used are as follows: (a) *R-sc Sn1-bol3 C1* is a line homozygous for *R-sc* and *C1* and devoid of the *Sn1-bol3* gene. This line allows the detection of pigment in the aleurone. *R-sc* and *C1* are expressed in the aleurone where they lead to homogeneous pigmentation. (b) *r-*Δ *Sn1-bol3 pl-bol3* is a line homozygous for *r-*Δ, *Sn1-bol3*, and *pl-bol3* genes. *r-*Δ indicates an interstitial deletion involving a region of the long arm of chromosome 10 containing the *r1* locus. Plant and seed tissues homozygous for the deficiency are totally devoid of pigment (Alleman and Kermicle, 1993) unless they contain a functional *sn1* allele. *r-*Δ *Sn1-bol3* plants have been obtained by crossing heterozygous *r-r Sn1-bol3/r-*Δ females to *r-*Δ*/r-*Δ males (Consonni et al., 1997). *pl-bol3* is a *pl1* allele conferring high mesocotyl pigmentation upon light treatment and recessive to *Pl-Rh* (Ronchi et al., 1998). (c) *r-*Δ *Sn1-bol3 pl-W22* is a homozygous line bearing the *pl-W22* allele. *pl-W22* is the recessive *pl1* allele residing in the W22 line.

Light Treatment

Immature ears at 30 d after pollination were cut longitudinally into two halves and placed in plastic boxes layered with 0.9% (w/v) agar. They were then exposed to continuous white, blue, and red light for 0, 24, 48, or 72 h at 22°C. At the end of the light treatments, seeds were excised and anthocyanin or total RNA was extracted.

For mesocotyl analysis, seeds were allowed to germinate in darkness for 5 d at 25°C until a mesocotyl approximately 3 cm long had developed. Seedlings were then exposed to continuous light for 0, 24, 48, and 72 h at 21°C. At the end of the treatment with white, blue, red, UV-A, and UV-B light, mesocotyls were sampled and anthocyanin and total RNA was extracted. Illumination was performed in controlled-environment rooms at 21°C.

White light was provided by cool-white (F36T12/CW/HO) fluorescent tubes (21 W m^{-2}) from GTE Sylvania (Lighting Products Group, Danvers, MA). Red light was obtained by covering the special phosphor red (F36T12/236/HO) fluorescent lamps from GTE Sylvania with one layer of RESCOLUX number 27 red filter (Rosco, Port Chester, NY), which emit light between 610 and 690 nm with a λ_{max} of 660 nm. The fluence rate was 125 μmol m^{-2} s^{-1}. Blue light was obtained by covering the special phosphor blue (F36T12/246/HO) fluorescent lamps from GTE Sylvania with one layer of RESCOLUX N° 83 blue filter (Rosco), which emit light between 400 and 490 nm with a λ_{max} of 434 nm. The fluence rate was 88 μmol m^{-2} s^{-1}. UV-A light was provided by TLK 40W/10R UV-A lamps (Philips, London), which emit light between 350 and 400 nm with a λ_{max} of 370 nm. The fluence rate was 21 μmol m^{-2} s^{-1}. UV-B light was provided by TL 20W/12RS UV-B lamp (Philips), covered with a cellulose acetate filter, and changed each 24 h to remove UV-C wavelenghts. The fluence rate was 5 μmol m^{-2} s^{-1} (280–320 nm).

Cytokinin Treatment

For cytokinin treatment, the synthetic hormone BA was dissolved initially in a small volume of 1 N KOH and then diluted to the final concentration with water. Plants were grown on 3 M filter paper (Whatman, Clifton, NJ) and watered with different BA solutions (0.5 and 25 μM) for 10 d in darkness at 25°C. Plantlets were then exposed to continuous white light for 48 h and anthocyanins were extracted. Control plants were watered with the same final concentration of KOH and maintained in the same conditions.

For kinetic experiments, plants were grown for 7 d in darkness at 25°C in the presence of the BA solution (25 μM) and then exposed to continuous white light for 0, 24, and 48 h. Control plants were maintained in the same conditions. At the end of the treatment, mesocotyls were sampled and total RNA was extracted.

Anthocyanin Determination

Anthocyanins were extracted by grinding a single seed or mesocotyl in a precooled mortar with 1 mL of cold ethanol containing 1% (v/v) HCl. Extracts were centrifuged twice and absorption determined spectrophotometrically at 530 nm. Anthocyanin concentration is expressed as absorbance value at 530 nm per seed or per mesocotyl. Mean values represent 10 independent replicates. SES of means are below 5%.

RNA Isolation and RT-PCR Analysis

Total RNA was isolated from mesocotyls and aleurones as previously described (van Tunen et al., 1988). All RNA samples were treated with DNaseI (Boehringer, Mannheim, Germany) before cDNA synthesis. First strand cDNA synthesis was carried out from 5 μg of total RNA with an oligo(dT) and RT SuperscriptII as recommended by the manufacturers (Life Technologies, Gaithersburg, MD). The primer used was a 35-base oligonucleotide with 17dT residues and a sequence adapter (5′-GGGAATTCGTCGACAAGC-3′; Frohman, 1990). First strand cDNA was used as a template for PCR amplification. Amplification reactions containing an aliquot of cDNA; 1× Promega (Madison, WI) polymerase buffer; 2.5 mM $MgCl_2$; 200 μM each of dATP, dCTP, dGTP, and dTTP; 0.1 μM of each primer; and 1 unit of *Taq* DNA polymerase (Promega) were performed in a final volume of 50 μL. After the first denaturation step (5 min at 94°C), the reaction mix underwent 20 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 1 min and extension at 72°C for 2 min. A final extension at 72°C for 5 min was performed to complete the reaction.

A set of primers specific for the *orp-1* (*orange pericarp-1*) gene, which encodes the β-subunit of Trp synthase (Wright et al., 1992), were used to standardize the concentration of different samples. An *orp-1*-specific sequence was amplified using the following primers: upstream primer, 5′-AAGGACGTGCACACCGC-3′; and downstream primer, 5′-CAGATACAGAACAACAACTC-3′. The length of the amplified product was 207 bp. Several cycles of successive cDNA dilution and *orp1* amplification and hybridization were done to obtain a similar signal of amplification among the different samples. To ensure that amplification reactions were within linear range, the reactions were carried out for 20 cycles.

PCR products were fractionated on 1.2% (w/v) agarose gels, transferred onto Hybond N^+ nylon membranes (Amersham, Buckinghamshire, UK), and hybridized with random primed fluorescein fragments (Amersham) according to the manufacturer's protocols.

For mRNA detection of the genes under analysis, the following specific primer sets were used: for *R-sc* and *Sn1-bol3*, OR31 (upstream primer 5′-ATGGCTTCATGGGGCTTAGATAC-3′) and OR32 (downstream primer 5′-GAATGCAACCAAACACCTTATGCC-3′); for *C1*, PL6 (upstream primer 5′-TCGGACGACTGCAGCTCGGC-3′) and AC1 (downstream primer 5′-CACCGTGCCTAATTTCCTGTCCGA-3′); for *pl-bol3*, PL6 (upstream primer 5′-TCGGACGACTGCAGCTCGGC-3′) and PL8 (downstream primer 5′-GATTATATTGTTTACACGATGAAG-3′); for *A1*, A1 (upstream primer 5′-TTCTCGTCCAAGAAGCTCCAGGA-3′) and A2 (downstream primer 5′-CAATTCGTTGAACATGGAAGTAAG-3′); and for *C2*, CHS1 (upstream primer 5′-TCGACGAGATGCGCAAGCGCT-3′) and CHS2 (downstream primer 5′-GAATTTGATCGTTGATGAATC-3′).

The sizes of the amplified products were 403 bp for *R-sc* and *Sn1-bol3*, 313 bp for *C1*, 267 bp for *pl-bol3*, 285 bp for *A1*, and 181 bp for *C2*. The *R-sc* and *Sn1-bol3* PCR products were hybridized with the 1.4-kb *Pst*I-*Eco*RI fragment of *Sn1-bol3* cDNA (Tonelli et al., 1991), the *C1* products with the 1.2-kb *Eco*RI fragment of *Pl-Rh* cDNA (Cone et al., 1993b), the *pl-bol3* products with the *Xho*I-*Dra*I fragment of *Pl-Rh* cDNA (Cone et al., 1993b), the *A1* products with a 700-bp *Bam*HI fragment of the *A1* gene (Schwarz-Sommer et al., 1987), and the *C2* products with the PCR fragment obtained by amplification of a *C2* genomic clone using the CHS1 and CHS2 primers.

ACKNOWLEDGMENT

The authors are very grateful to Cristina Bandera for help in this project.

Received August 31, 2001; returned for revision November 8, 2001; accepted December 11, 2001.

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