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UV-B, UV-A, and Blue Light Signal Transduction Pathways Interact Synergistically to Regulate Chalcone Synthase Gene Expression in Arabidopsis

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UV and blue light stimulate transcription of key flavonoid biosynthesis genes in a range of higher plants. Here, we provide evidence that several distinct "inductive" and "synergistic" UV/blue phototransduction pathways regulate chalcone synthase (*CHS*) gene transcription and transcript accumulation in Arabidopsis leaf tissue. Experiments with the long-hypocotyl *hy4-2.23N* mutant showed that separate inductive pathways mediate responses to UV-B and UV-A/blue light. Only the UV-A/blue light induction of *CHS* expression involved the CRY1 photoreceptor. In addition, UV-A and blue light each act synergistically with UV-B to stimulate *CHS* transcript accumulation and β-glucuronidase activity driven by a *CHS* promoter in transgenic leaf tissue. The UV-A and blue phototransduction pathways responsible for synergism are distinct because they produce transient and relatively stable signals, respectively, and can function additively to stimulate *CHS* promoter function. The *hy4-2.23N* mutant retains the synergistic interactions between UV-B and both UV-A and blue light, indicating that neither synergism pathway involves the CRY1 photoreceptor. Our findings reveal considerable complexity in both photoreception and signal transduction in regulating *CHS* gene expression by UV and blue light.

INTRODUCTION

Plants detect and respond to a wide range of endogenous and environmental signals that control their metabolism and development. Information about the cellular and molecular mechanisms of signal perception and transduction is gradually accumulating (Bowler and Chua, 1994), and it is becoming clear that plant cells contain networks of signal transduction pathways with the potential for interaction. One example of interaction is the reciprocal negative regulation between the cGMP- and Ca^{2+}/calmodulin–dependent phytochrome signal transduction pathways (Bowler et al., 1994a). Signal transduction pathways may also interact synergistically, as illustrated by the hyperstimulation of the osmotin promoter in the presence of ethylene and methyl jasmonate (Xu et al., 1994). Hence, to gain a full understanding of signal transduction in plant cells, it is important to define the interactions between signaling pathways as well as to identify their primary components.

Light is one of the most important environmental signals regulating plant growth and development. The phytochrome photoreceptors, which mediate responses to red and far-red light, are well characterized (Quail, 1994), and as indicated above, details are now emerging about the signal transduction processes coupling phytochrome to downstream responses (Neuhaus et al., 1993; Bowler et al., 1994a, 1994b; Millar et al., 1994). However, much less is known about the photoreceptors and signal transduction processes that mediate responses specifically to UV and blue light (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995). These photoreceptors initiate a range of important responses in plants, such as phototropism, stomatal opening, suppression of stem extension, and expression of several genes.

Various lines of evidence indicate that there must be several photoreceptors absorbing in the UV-B (280 to 320 nm), UV-A (320 to 390 nm), and blue (390 to 500 nm) regions of the spectrum (Briggs and Iino, 1983; Liscum and Hangarter, 1994; Short and Briggs, 1994; Jenkins et al., 1995), but only recently has the molecular characterization of the first UV/blue photoreceptor been reported. The Arabidopsis *HY4* gene encodes a protein with sequence similarity to microbial DNA photolyases that binds flavin and pterin chromophores and has the expected properties of a UV-A/blue photoreceptor (Ahmad and Cashmore, 1993, 1996; Lin et al., 1995a; Malhotra et al., 1995). Cashmore and co-workers have named this photoreceptor CRY1 (for cryptochrome). *hy4* mutants are impaired in several extension growth responses and in the expression of genes concerned with flavonoid biosynthesis in blue light (Koornneef et al., 1980; Chory, 1992; Ahmad and Cashmore, 1993; Ahmad et al., 1995; Jackson and Jenkins, 1995). Transgenic tobacco plants overexpressing CRY1 show a

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hyperresponsive phenotype, with shorter hypocotyls in blue, UV-A, and green light (Lin et al., 1995b). Much less information is available about other photoreceptors that absorb specifically UV and blue light.

Genes encoding chalcone synthase (CHS), the first committed step in the flavonoid biosynthesis pathway, offer an excellent experimental system for investigating the signal transduction processes involved in the control of gene expression by UV and blue light. *CHS* expression is regulated by light in a complex manner. In parsley cell cultures, maximal *CHS* expression requires both blue and UV light (Ohl et al., 1989). In *Sinapis alba* (white mustard) and parsley plants, there is a developmental switch in photoreceptor usage in that *CHS* expression in dark-grown seedlings is controlled by phytochrome, whereas in mature leaves, it is mediated by UV-B and UV-A/blue photoreceptors (Batschauer et al., 1991; Frohnmeyer et al., 1992). In Arabidopsis, UV and blue photoreceptors control *CHS* expression in seedlings (Feinbaum et al., 1991; Kubasek et al., 1992; Kaiser et al., 1995) and mature leaf tissue (Li et al., 1993; Jackson et al., 1995), although there is some phytochrome regulation of expression in very young dark-grown seedlings (Kaiser et al., 1995). Studies with several species have shown that specific DNA sequence elements in *CHS* promoters are required for UV and blue light regulation (Schulze-Lefert et al., 1989; Feinbaum et al., 1991; Fritze et al., 1991; Weisshaar et al., 1991a; Rocholl et al., 1994; Kaiser et al., 1995), and transcription factors that interact with specific elements have been identified (Weisshaar et al., 1991b; Feldbrügge et al., 1994; Mol et al., 1996).

Recently, we obtained information about the signal transduction processes involved in the induction of *CHS* expression by UV-B and UV-A/blue light in Arabidopsis (Christie and Jenkins, 1996). Pharmacological experiments using an Arabidopsis cell culture indicate that the UV-B and UV-A/blue phototransduction pathways involve calcium and reversible protein phosphorylation and require protein synthesis. However, the pathways are distinct, at least in part, because experiments with the calmodulin antagonist W-7 indicate that calmodulin is involved in UV-B but not UV-A/blue light signal transduction. Both pathways are different from the cGMP-dependent phytochrome signaling pathway regulating *CHS* that has been identified in other species (Neuhaus et al., 1993; Bowler et al., 1994a, 1994b).

In this study, we show that the regulation of *CHS* promoter function and transcript accumulation in Arabidopsis leaf tissue involves both "inductive" and "synergistic" phototransduction pathways. Maximal levels of *CHS* expression are the product of complex interactions between UV-B, UV-A, and blue light signal transduction pathways. We show that the UV-A/blue but not the UV-B inductive pathway involves the CRY1 photoreceptor and provide evidence that further, distinct blue and UV-A pathways interact synergistically with the UV-B pathway to stimulate *CHS* expression. The phototransduction pathways mediating these synergistic interactions do not involve the CRY1 photoreceptor.

RESULTS

CHS Expression Is Induced by Distinct UV-B and UV-A/Blue Phototransduction Pathways in Arabidopsis

We investigated the effects of different light qualities on *CHS* transcript levels in wild-type and *hy4* leaf tissue and on expression of β-glucuronidase (GUS) driven by the white mustard *CHS-1* promoter in the transgenic Arabidopsis line NM4. We previously reported that the light regulation of *CHS–GUS* expression in this line is very similar to that of the endogenous *CHS* gene (Jackson et al., 1995). In the experiments reported here, plants were grown routinely in a low fluence rate (20 μmol m^{-2} sec^{-1}) of white light for 21 days before transferring them to specific light conditions for up to 24 hr. Plants grown in 20 μmol m^{-2} sec^{-1} white light have a very low level of *CHS* transcripts and *CHS–GUS* expression (Jackson et al., 1995).

Transfer of plants from low white light to a very low fluence rate (3 μmol m^{-2} sec^{-1}) of UV-B irradiation induces approximately a five- to 10-fold increase in *CHS–GUS* expression in leaf tissue over 16 hr (Jackson et al., 1995). Removal of wavelengths <320 nm from this UV-B source prevents the increase in expression, indicating that the induction is caused specifically by UV-B wavelengths (data not shown; see Christie and Jenkins, 1996). Transfer of plants from low white light to 75 μmol m^{-2} sec^{-1} blue (Jackson et al., 1995) or UV-A light (see below) induces *CHS–GUS* expression to a similar extent to UV-B. Red light does not induce a significant increase over low white light (Jackson et al., 1995). None of the light treatments has much effect on GUS activity in control plants containing the cauliflower mosaic virus *35S–GUS* transgene (data not shown).

Consistent with the above-mentioned results, Figure 1 (first to fourth lanes) shows that UV-B, UV-A, and blue light each induce an increase in *CHS* transcript levels in light-grown wild-type leaf tissue, although the induction by UV-A and UV-B is usually less than that with blue light. Higher induction of GUS activity in blue light relative to UV-B and UV-A was not consistently observed; this could be explained by a differential effect of light quality on *CHS* transcript stability versus transcription. Alternatively, there may have been subtle differences in the responses of the white mustard and endogenous *CHS* promoters and possibly some developmental influence, because all the leaves of the plant were used to prepare RNA, whereas the GUS assays were undertaken with the expanding third pair of leaves.

We investigated which of the inductive responses were mediated by the CRY1 photoreceptor by examining *CHS* transcript levels in the *hy4-2.23N* mutant. *hy4-2.23N* is the original *hy4* allele and was generated by fast neutron bombardment (Koornneef et al., 1980). Because of a deletion starting in the third intron, *hy4-2.23N* produces an aberrant transcript that accumulates in reduced amounts (Ahmad and Cashmore,

1993). There is strong evidence that *hy4-2.23N* is a null mutant: Cashmore and co-workers did not detect CRY1 when immunoblots of *hy4-2.23N* leaf proteins were probed with an antibody raised against CRY1 expressed in *Escherichia coli* (A.R. Cashmore, personal communication; Lin et al., 1996). Similar results were reported for several other *hy4* null mutants (Ahmad et al., 1995). Furthermore, the mutant phenotype of *hy4-2.23N* is as strong as that of other *hy4* null alleles (Ahmad and Cashmore, 1993).

We have shown previously that *hy4-2.23N* has a lower level of *CHS* transcripts than does the wild type in blue light (Jackson and Jenkins, 1995). Figure 1 (fourth lane) confirms this observation and further shows that *hy4-2.23N* is strongly impaired in the induction of *CHS* transcripts in UV-A light (third lane). Under these UV-A conditions, 6-day-old *hy4-2.23N* seedlings have significantly longer hypocotyls than does the wild type (Jenkins et al., 1995), in agreement with published findings (Koornneef et al., 1980; Ahmad and Cashmore, 1993). In contrast, we have not observed any consistent difference between *hy4-2.23N* and the wild type in the level of *CHS* transcripts induced by UV-B (Figure 1, second lane). To investigate this further, we introduced the *CHS–GUS* transgene into the *hy4-2.23N* mutant background by crossing with NM4. As shown in Figure 2, the fold induction of *CHS–GUS* expression does not differ significantly between the wild type and mutant in UV-B, but it is altered in UV-A and blue light, consistent with Figure 1.

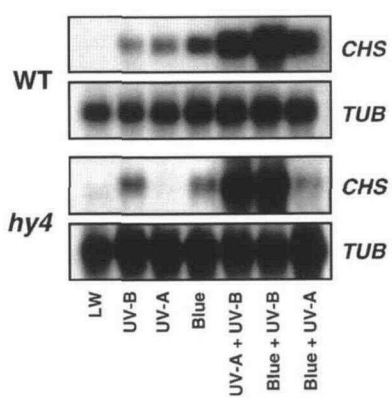

Figure 1. *CHS* Transcript Levels in the Wild Type and *hy4-2.23N*.

Wild-type (WT) and *hy4-2.23N* (*hy4*) plants were grown in 20 μmol m^{-2} sec^{-1} white light for 3 weeks (LW, first lane) and then transferred for 16 hr to 3 μmol m^{-2} sec^{-1} UV-B (second lane), 75 μmol m^{-2} sec^{-1} UV-A (third lane), 75 μmol m^{-2} sec^{-1} blue (fourth lane), 75 μmol m^{-2} sec^{-1} UV-A plus 3 μmol m^{-2} sec^{-1} UV-B (fifth lane), 75 μmol m^{-2} sec^{-1} blue plus 3 μmol m^{-2} sec^{-1} UV-B (sixth lane), or 75 μmol m^{-2} sec^{-1} UV-A plus 75 μmol m^{-2} sec^{-1} blue (seventh lane) light treatments. *CHS* and α-tubulin (*TUB*) transcript levels in total leaf RNA (10 μg per lane) were measured by hybridization of the appropriate probes to RNA gel blots.

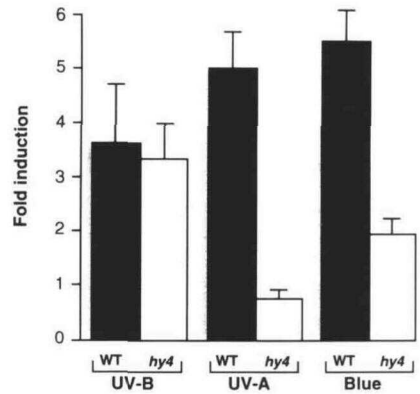

Figure 2. *CHS–GUS* Expression in the Wild Type and *hy4-2.23N*.

Wild-type (WT) and *hy4-2.23N* (*hy4*) plants containing the *CHS–GUS* transgene were grown in 20 μmol m^{-2} sec^{-1} white light for 3 weeks and then transferred for 8 hr to 3 μmol m^{-2} sec^{-1} UV-B, or for 16 hr to 75 μmol m^{-2} sec^{-1} UV-A or 75 μmol m^{-2} sec^{-1} blue light. GUS activity was measured as described in Figure 3 and is expressed as fold induction relative to that in plants kept in 20 μmol m^{-2} sec^{-1} white light. Bars indicate standard error (n = 10).

UV-B and Blue Light Interact Synergistically to Stimulate *CHS-GUS* Expression in Transgenic Arabidopsis

Figure 3 shows that when plants are exposed to both UV-B and blue light, the magnitude of *CHS–GUS* expression is much greater than it is when plants are exposed to either blue or UV-B alone. Moreover, the level of expression is much greater than the effects of UV-B and blue light added together. In repeated experiments, we found that the level of expression under simultaneous irradiation is four- to eightfold higher than with either treatment alone. Furthermore, the initial rate of increase is greater (Figure 3). These observations cannot be explained by a fluence threshold phenomenon, because the effect is seen at different fluence rates and increasing either UV-B or blue light alone does not give a comparable response (data not shown). We therefore conclude that there is a strong synergistic interaction between UV-B and blue light in the stimulation of *CHS–GUS* expression. This implies that UV-B and blue light regulate expression through separate, interacting phototransduction pathways.

We investigated whether the order of illumination with UV-B and blue light was important in this synergistic interaction. Plants were exposed to varying durations of blue light followed by 8 hr of UV-B exposure or varying UV-B treatments followed by 8 hr of blue light. As shown in Figure 4, the level of *CHS–GUS* expression was much greater when blue light treatment preceded UV-B treatment than vice versa. A 5-hr blue light pretreatment was sufficient for maximal GUS activity. As a control, we included plants given a 24-hr red light pretreatment

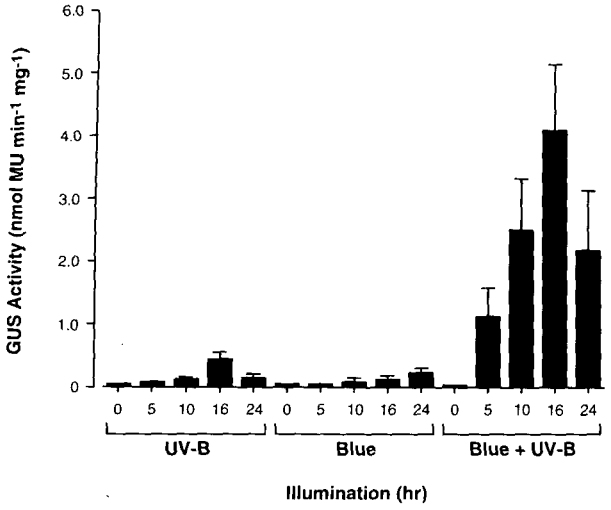

Figure 3. Synergistic Interaction between UV-B and Blue Light in *CHS–GUS* Expression.

Wild-type plants containing the *CHS–GUS* transgene were grown in 20 μmol m^{-2} sec^{-1} white light for 3 weeks. They were then transferred to 3 μmol m^{-2} sec^{-1} UV-B, 75 μmol m^{-2} sec^{-1} blue light, or 3 μmol m^{-2} sec^{-1} UV-B plus 75 μmol m^{-2} sec^{-1} blue light for the durations indicated. GUS activity was measured in the third leaf as the amount of 4-methylumbelliferone (MU) formed per unit of protein. Bars indicate standard error (n = 8).

before exposure to UV-B, and this resulted in a much lower level of expression than did the equivalent blue pretreatment.

The results given above indicate that blue light produces a signal that enhances the subsequent response to UV-B. We therefore investigated the stability of this blue light–induced signal. We introduced a dark period between an initial blue and a subsequent UV-B irradiation. We measured both *CHS–GUS* expression and *CHS* transcript levels to counter the possibility that the stability of GUS may influence the results. As shown in Figures 5A and 5B, the blue light signal is present for several hours during the dark period and is available to enhance the subsequent response to UV-B. Although the signal started to decline immediately in darkness, sufficient signal remained after 5 hr to give an increased response in subsequent UV-B irradiations for both *CHS–GUS* expression and *CHS* transcript accumulation. The stability of the signal varied between experiments. In some experiments, we found that even after a 15-hr dark period, the level of expression in subsequent UV-B exposure was significantly higher than that seen without any blue light pretreatment.

UV-A Acts Synergistically with UV-B through a Distinct Pathway to Blue Light

UV-A is able to induce *CHS–GUS* expression to levels similar to UV-B. Figure 6 shows that a synergistic effect was observed when UV-B and UV-A light treatments were given together. The kinetics of the response increased, and the maximum levels of GUS activity observed in repeated experiments were similar to those seen with blue light plus UV-B. Experiments with different fluence rates of UV-B and UV-A indicated that the increased expression was not due to a fluence threshold phenomenon (data not shown). The interaction between UV-A and UV-B is therefore similar to that seen between UV-B and blue light, and indicates that the effects of UV-A and UV-B are mediated by separate, interacting pathways.

However, in contrast to the result with blue light pretreatment (Figure 4), the data in Figure 7 show that UV-A given before UV-B does not produce a synergistic response and neither does UV-B before UV-A. Thus, UV-A and UV-B treatments must be given together to see the synergistic effect. This indicates that the signal generated by UV-A is not stable, in contrast to the blue signal, and therefore implies that the UV-A and blue phototransduction pathways for the synergistic response are distinct.

Blue and UV-A Synergism Pathways Together Stimulate Maximal Levels of *CHS-GUS* Expression

We investigated whether the distinct blue and UV-A phototransduction pathways that interact synergistically with the UV-B

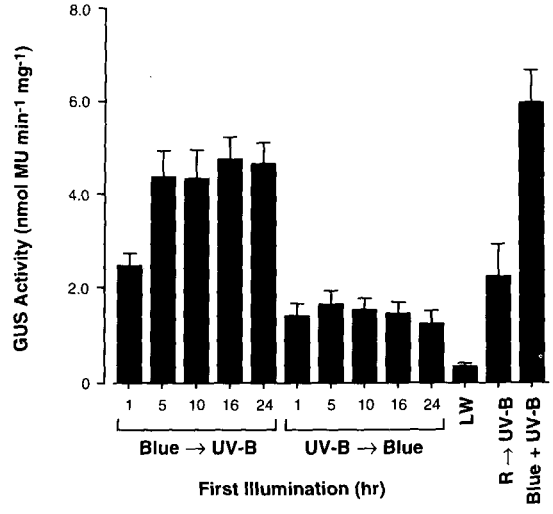

Figure 4. Synergistic Interaction between UV-B and Blue Light Depends on the Order of Illumination.

Plants were grown as described in Figure 3 and transferred to either 75 μmol m^{-2} sec^{-1} blue light for the times indicated followed by 8 hr of 3 μmol m^{-2} sec^{-1} UV-B (Blue → UV-B) or were given varying durations of UV-B before an 8-hr blue light treatment (UV-B → Blue). Control plants were given no treatment (LW), 24 hr of 75 μmol m^{-2} sec^{-1} red light followed by 8 hr of 3 μmol m^{-2} sec^{-1} UV-B (R → UV-B), or 8 hr of UV-B and blue light together at the above fluence rates (Blue + UV-B). GUS activity was assayed as given in Figure 3. Bars indicate standard error (n = 10).

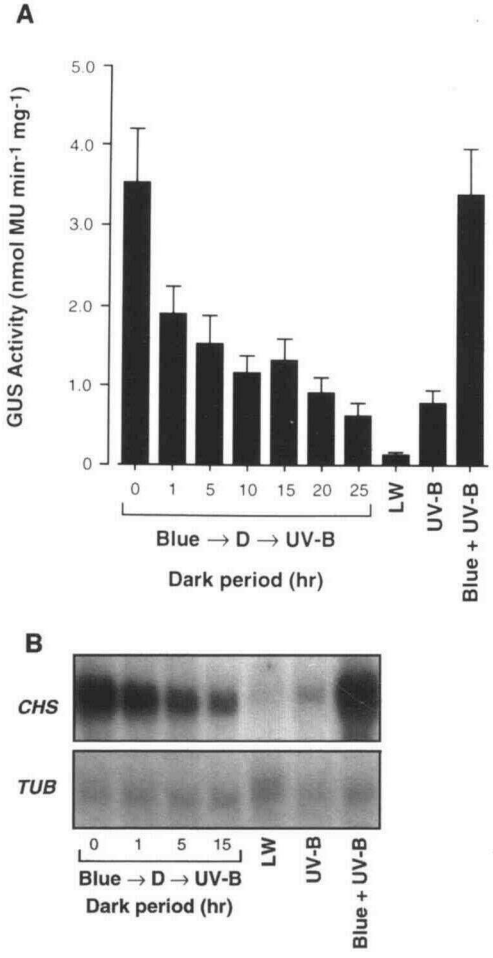

Figure 5. Stability of the Blue Light–Derived Signal.

(A) Plants were grown as described in Figure 3 and transferred to 8 hr of 3 μmol m^{-2} sec^{-1} UV-B with either no preillumination (UV-B) or preillumination for 8 hr with 75 μmol m^{-2} sec^{-1} blue light followed by varying dark periods (D) of the duration indicated (Blue → D → UV-B). Control plants were given either no treatment (LW), no dark period (0 hr D), or 8 hr UV-B and blue light together at the above fluence rates (Blue + UV-B). GUS activity was assayed as given in Figure 3. Bars indicate standard error from the combined results of three experiments (n = 30 plants in total).

(B) Wild-type plants were grown and illuminated as described in **(A)**, and *CHS* and *TUB* transcript levels were measured as given in Figure 1.

pathway were capable of acting together to stimulate *CHS–GUS* expression. Plants were preilluminated with blue light and then transferred to UV-B plus UV-A. As shown in Figure 8 (fourth bar), the levels of *CHS–GUS* expression produced in these plants were approximately double those in plants given either blue then UV-B (first bar) or UV-A plus UV-B (third bar), which is what is expected if both synergistic pathways were operating. The combination of synergistic interactions gave the highest-fold stimulation (150-fold) by any light treatment we have used. The effects of the two synergistic responses appear slightly more than additive; however, this may not be significant, given the variability in the expression levels. These results provide further evidence that the synergistic interactions involve distinct blue and UV-A phototransduction pathways that separately enhance the response to UV-B. It can also be seen from Figure 8 (second bar) that exposure to blue and UV-A light, without UV-B, produced much less expression than did the synergistic combinations. In all of our experiments, the combined effects of UV-A and blue light, whether given together or consecutively, were additive rather than synergistic.

hy4-2.23N Mutant Shows That the Inductive and Synergistic Pathways Involving UV-A and Blue Light Are Distinct

Consistent with the *CHS–GUS* expression data, Figure 1 shows that illumination of the wild type with either UV-B and UV-A together or UV-B and blue light together gave synergistic rather than additive increases in the *CHS* transcript levels (Figure 1, compare the fifth lane with the second and third lanes and the sixth lane with the second and fourth lanes, respectively). Moreover, blue light together with UV-A gave an additive rather than a synergistic response (Figure 1, compare the seventh lane with the third and fourth lanes). Densitometric scans of autoradiographs from several experiments, normalized for RNA loading differences, support this interpretation (data not shown).

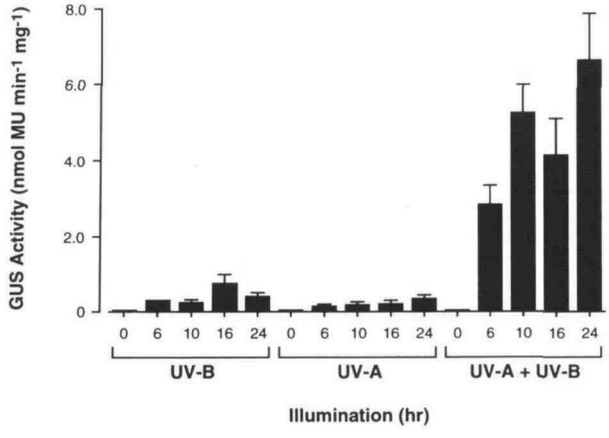

Figure 6. Synergistic Interaction between UV-B and UV-A Light in *CHS–GUS* Expression.

Plants were grown as described in Figure 3 and then transferred to 3 μmol m^{-2} sec^{-1} UV-B, 75 μmol m^{-2} sec^{-1} UV-A, or 3 μmol m^{-2} sec^{-1} UV-B plus 75 μmol m^{-2} sec^{-1} UV-A for the durations indicated. GUS activity was assayed as given in Figure 3. Bars indicate standard error (n = 10).

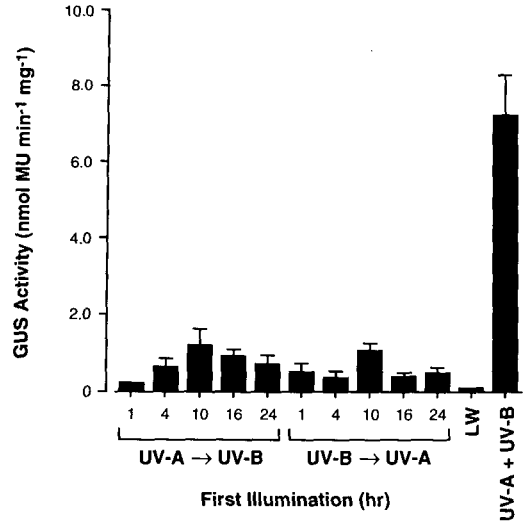

Figure 7. UV-A Does Not Produce a Stable Signal.

Plants were grown as described in Figure 3 and then transferred to either 75 μmol m^{-2} sec^{-1} UV-A for the times indicated followed by 8 hr of 3 μmol m^{-2} sec^{-1} UV-B (UV-A → UV-B) or were given varying durations of UV-B treatment before an 8-hr UV-A illumination (UV-B → UV-A). Control plants were given either no treatment (LW) or 16 hr of UV-B and UV-A together at the above fluence rates (UV-A + UV-B). GUS activity was assayed as in Figure 3. Bars indicate standard error (n = 10).

Significantly, although *hy4-2.23N* is impaired in the induction of *CHS* transcripts by UV-A or blue light, it retains the synergistic interactions between both UV-A and UV-B and blue and UV-B in the accumulation of *CHS* transcripts (fifth and sixth lanes of Figure 1). In several experiments, the mutant and wild type were indistinguishable in the levels of *CHS* transcripts produced in each synergistic response. In contrast, the levels in UV-A plus blue light, which are not synergistic (Figure 8), were very much reduced in the mutant (lane 7 of Figure 1). To satisfy ourselves that the results obtained were not the consequence of selecting a particular time point (16 hr) for RNA isolation, we monitored *CHS* transcript levels over a time course for each of the treatments shown in Figure 1. No consistent differences were observed between the wild type and mutant in the extent of the synergistic response at any time point from 0 to 24 hr (data not shown).

These observations therefore indicate that the CRY1 photoreceptor is not involved in the perception of blue and UV-A light that interacts synergistically with UV-B, whereas it is involved in the perception of blue and UV-A light in the inductive response.

DISCUSSION

In this study, we provide evidence for separate inductive and synergistic UV/blue phototransduction pathways regulating *CHS* promoter function and transcript accumulation in Arabidopsis leaf tissue. At least two distinct pathways, a UV-A/blue light pathway involving CRY1 and a UV-B pathway, induce *CHS* expression. In addition, distinct blue and UV-A pathways interact synergistically with the UV-B pathway to provide maximal levels of expression, and neither of these pathways appears to involve CRY1. This study, therefore, provides evidence of considerable complexity, both in photoreception and signal transduction, in the UV/blue light regulation of *CHS* promoter function.

UV/Blue Light Induction of *CHS* Involves at Least Two Distinct Phototransduction Pathways

Previous studies have shown that light regulation of *CHS* expression may involve several different photoreceptors, depending on the species and the stage of development. In Arabidopsis, phytochrome regulation of *CHS* is confined to young dark-grown seedlings, and UV/blue light regulation

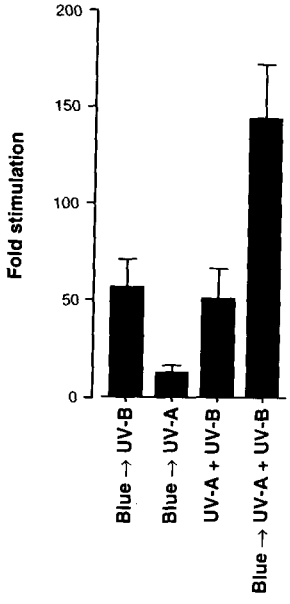

Figure 8. Synergism Pathways Act Together to Stimulate *CHS–GUS* Expression.

Plants grown under a low fluence rate of white light as described in Figure 3 were given 8 hr of 75 μmol m^{-2} sec^{-1} blue light then 16 hr of 3 μmol m^{-2} sec^{-1} UV-B (Blue → UV-B), 8 hr of 75 μmol m^{-2} sec^{-1} blue light then 16 hr of 75 μmol m^{-2} sec^{-1} UV-A (Blue → UV-A), 16 hr of 75 μmol m^{-2} sec^{-1} UV-A and 3 μmol m^{-2} sec^{-1} UV-B light together (UV-A + UV-B), or 8 hr of 75 μmol m^{-2} sec^{-1} blue light then 16 hr of 75 μmol m^{-2} sec^{-1} UV-A and 3 μmol m^{-2} sec^{-1} UV-B together (Blue → UV-A + UV-B). Fold stimulation of specific GUS activity, assayed as given in Figure 3, is shown relative to that in 20 μmol m^{-2} sec^{-1} white light. Bars indicate standard error (n = 10).

predominates (Feinbaum et al., 1991; Kubasek et al., 1992; Kaiser et al., 1995). In mature leaf tissue, *CHS–GUS* expression is stimulated by increasing the fluence rate of white light (Feinbaum et al., 1991; Jackson et al., 1995) and by transfer of plants grown in low white light to UV-B or blue light but not to red light (Jackson et al., 1995). Our current data show that UV-B, UV-A, and blue light each separately induce *CHS–GUS* expression and *CHS* transcript accumulation in mature leaves of Arabidopsis. Only a low fluence rate of UV-B is required for induction, and the level we have used is similar to that measured in sunlight (data not shown). Li et al. (1993) reported previously that UV-B stimulates *CHS* expression in Arabidopsis leaf tissue, but UV-B was provided as a supplement to white light, which, according to our findings, results in a synergistic response through the combined actions of UV-B, UV-A, and blue light.

The experiments with the *hy4-2.23N* mutant (Figures 1 and 2) indicate that there are at least two distinct UV/blue phototransduction pathways involved in *CHS* induction in Arabidopsis. UV-B induction is unaltered in the *hy4-2.23N* mutant and therefore does not involve the CRY1 photoreceptor. The UV-B light detection system remains to be characterized. In contrast, the induction of *CHS* expression by both UV-A and blue light is reduced in the *hy4-2.23N* mutant, demonstrating that CRY1 initiates a UV-A/blue inductive pathway. Whereas *hy4-2.23N* was initially considered to be a blue light response mutant, based on the hypocotyl extension response (Koornneef et al., 1980), it is evident that it is also significantly altered in responses to UV-A and, to some extent, green light (Ahmad and Cashmore, 1993; Lin et al., 1995a). Under our UV-A conditions, the mutant has much reduced induction of *CHS* (Figures 1 and 2) and significantly longer hypocotyls than does the wild type (Jenkins et al., 1995). The altered response to blue and UV-A light is consistent with the absorption properties of the chromophores bound to CRY1 (Lin et al., 1995a; Malhotra et al., 1995).

Additional evidence that the UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression are distinct is provided by experiments with an Arabidopsis cell culture (Christie and Jenkins, 1996). The calmodulin antagonist W-7 strongly inhibits the UV-B induction of *CHS* in these cells but has little effect on the UV-A/blue light induction, indicating that the pathways differ in at least one component.

The fact that some *CHS* expression is observed in the *hy4-2.23N* mutant, particularly in blue light, requires explanation. Because CRY1 is undetectable in the leaves (A.R. Cashmore, personal communication; Lin et al., 1996), some other photoreceptor must be involved. It is conceivable that the blue light response is mediated by phytochrome, but mature Arabidopsis leaves appear to have negligible phytochrome induction of *CHS*. Therefore, the most likely explanations are either that the UV-B light detection system has some action in blue light, but not UV-A, or that there is an additional, blue-absorbing photoreceptor that initiates *CHS* induction. Until the nature of the UV-B light detection system is known, it is impossible to exclude the first possibility; it is not yet clear whether there is a specific UV-B photoreceptor. If there is another UV-A/blue photoreceptor, it may account for the residual UV-A suppression of hypocotyl extension observed in the *hy4-2.23N* mutant as well as the residual *CHS* induction in blue light. Cashmore and co-workers have identified another protein, CRY2, which is related to CRY1 (Ahmad and Cashmore, 1996), but the function of this putative photoreceptor has not been reported.

UV-B, UV-A, and Blue Phototransduction Pathways Interact Synergistically to Regulate *CHS*

The observation that UV-B and blue light act synergistically to stimulate *CHS–GUS* expression and *CHS* transcript accumulation indicates that their effects are mediated by distinct phototransduction pathways that interact at some level. Similarly, UV-B and UV-A must be perceived by separate, interacting pathways to account for their synergistic effect on *CHS* expression. The combined effects of UV-A and blue light are additive rather than synergistic. The blue phototransduction pathway that promotes the synergistic interaction with UV-B appears to be distinct from the corresponding UV-A synergism pathway because the blue-induced signal is stable in darkness for several hours, whereas that generated by UV-A is not able to interact with UV-B unless given at the same time. Because the pathways that produce these different signals are stimulated by different light qualities, they cannot be initiated by the same photoreceptor (or at least not by the same chromophore; see Liscum and Briggs, 1995). The hypothesis that there are two separate phototransduction pathways generating signals that interact synergistically with the UV-B pathway is supported by the experiment (Figure 8) in which plants were illuminated with blue light followed by UV-A and UV-B together. The level of *CHS–GUS* expression observed under these conditions was approximately double that obtained with either synergistic combination and was the level expected if the blue/UV-B and UV-A/UV-B synergistic interactions were operating in an additive manner.

hy4-2.23N Mutant Shows That the Inductive and Synergistic Pathways Are Distinct

Although the *hy4-2.23N* mutant is impaired in the induction of *CHS* transcripts by blue and UV-A light, it retains the synergistic interactions between both UV-B and blue light and UV-B and UV-A. This finding implies that neither the UV-A nor blue light pathway that interacts synergistically with UV-B involves the CRY1 photoreceptor and that both pathways are therefore distinct from the UV-A/blue light inductive pathway. Because, as discussed above, there is strong evidence that *hy4-2.23N* is a null mutant, it is very unlikely that residual CRY1 in the mutant could generate sufficient signal for an unimpaired synergistic response while not being able to effect an inductive response (Figures 1 and 2). Hence, there is no evidence that CRY1 is involved in either the blue or UV-A synergism pathways.

Our data are therefore consistent with a model, shown in Figure 9, in which separate inductive and synergistic signal

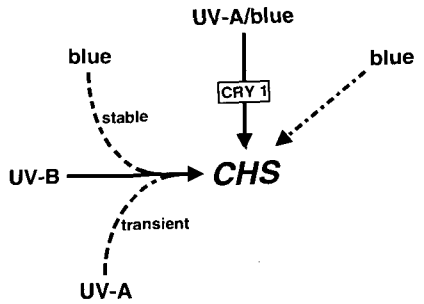

Figure 9. Model Summarizing the Different UV/Blue Phototransduction Pathways Regulating *CHS* Expression in Light-Grown Arabidopsis Leaf Tissue.

Two inductive pathways, involving either CRY1 or the UV-B light detection system, are shown by solid lines, and an additional, hypothetical, inductive blue light pathway is represented by a dot–dash line. The distinct UV-A and blue pathways that interact synergistically with the UV-B pathway and produce transient and relatively stable signals, respectively, are indicated by dashed lines. They are proposed to interact at separate points in the pathway, but there is no information on the order or sites of interaction.

transduction pathways can be defined in the regulation of *CHS* expression. Induction is achieved by distinct UV-B and UV-A/blue phototransduction pathways, the latter initiated by CRY1 photoreception. The UV-A and blue synergism pathways do not involve CRY1 and are distinct in that the UV-A pathway produces a transient signal, whereas the blue pathway generates a more stable signal. In our working hypothesis, both of these synergism signals can interact with the UV-B inductive pathway to regulate the *CHS* promoter but do not interact with the UV-A/blue inductive pathway. The model assumes that there is a single UV-B pathway. The two synergistic interactions appear to function largely independently because their effects are essentially additive (Figure 8). Together they give the maximal level of *CHS* expression. The observation that the two synergism pathways act together in an additive rather than a synergistic manner suggests that the transient signal generated by UV-A light does not interact with the stable component produced by blue light. Although we find that UV-A does not generate a stable signal, we cannot exclude the possibility that blue light produces a transient signal as well as a stable signal. However, we do not observe any consistent increase in the level of *CHS–GUS* expression when blue and UV-B light are given together, compared with when blue light precedes UV-B.

The above model extends previous hypotheses on UV/blue phototransduction pathways regulating *CHS*. Evidence that blue light produces a relatively stable signal that interacts synergistically with UV-B in the regulation of *CHS* expression was reported previously for cultured parsley cells (Ohl et al., 1989). Our experiments extend these findings in that they reveal distinct UV-A and blue synergism pathways that produce transient and relatively stable signals, respectively. Moreover, we have presented evidence that the inductive and synergistic pathways are separate and have identified which pathway involves CRY1.

Significance of the Synergistic Interactions

Evidence of synergistic interactions between different signal transduction pathways has been provided in other studies. For example, methyl jasmonate interacts synergistically with soluble sugars to stimulate expression of the soybean vegetative storage protein genes (Mason et al., 1992) and with ethylene to enhance osmotin gene transcription (Xu et al., 1994). Abscisic acid and NaCl interact synergistically in the regulation of *Em* gene expression (Bostock and Quatrano, 1992). However, we are not aware of any instances in which two separate synergism pathways have been shown to enhance the response to an inductive pathway in the manner described here for *CHS*. Details of the cellular and molecular bases of synergism are lacking in all of the systems described to date.

Synergistic interactions between signal transduction pathways allow for a greater overall amplification and thus a greater sensitivity in the extent of the response in relation to the stimulus. Moreover, synergistic interaction produces a more rapid response (Figures 3 and 6). The ability of signal transduction pathways to amplify rapidly the response to a stimulus is obviously of great importance if plants are to protect themselves effectively against potentially damaging conditions such as increased UV-B irradiation or pathogen attack. We have found that the survival of plants after a 24-hr UV-B treatment is nearly 100% if UV-B is given together with UV-A or blue light, whereas many of the plants exposed to 24 hr of UV-B alone die within 3 to 4 days. In their natural environment, plants are exposed to UV-B at the same time as, or at least not before, UV-A and blue light. Hence, the evolution of this mechanism appears to confer some selective advantage.

METHODS

Plant Material

Seeds of wild-type *Arabidopsis thaliana* ecotype Landsberg *erecta* and the *hy4-2.23N* mutant (Koornneef et al., 1980) were obtained from C. Dean (John Innes Centre, Norwich, UK) and the Arabidopsis Stock Centre (Nottingham, UK), respectively. The transgenic line NM4, described in detail by Jackson et al. (1995), contains the *uidA* (*GUS*) coding sequence fused to the *Sinapis alba SA–CHS1* gene promoter sequences from positions −907 to +26 (Batschauer et al., 1991; Frohnmeyer et al., 1992). NM4 is diploid and homozygous for the transgene at a single heritable locus (Jackson et al., 1995). *hy4-2.23N* plants containing the *CHS–GUS* transgene were obtained by crossing *hy4-2.23N* and NM4 plants and selecting F_2 progeny that were homozygous for both GUS activity and the long hypocotyl phenotype.

Seeds were sown in 9-cm pots containing compost. The pots were covered with clingfilm and placed in the dark at 6°C for 3 to 4 days

before transfer to continuous white light at a temperature of 21°C. Plants were routinely grown in a low fluence rate (20 μmol m^{-2} sec^{-1}) of white light for 21 days before transfer to different light qualities.

Light Sources

Illumination was performed in controlled environment rooms at 21°C. White light was provided by warm-white fluorescent tubes (Osram, Munich, Germany). UV-B was obtained from TL 40W/12 UV fluorescent tubes (Philips, London, UK) covered with cellulose acetate, which was changed every 24 hr. This source emits very low levels of UV-A and blue light, which we have found are insufficient to induce *CHS* expression. One tube was employed, and the fluence rate between 280 and 320 nm was 3 μmol m^{-2} sec^{-1}. UV-A light was provided by F35W/BI-2B blacklight-blue fluorescent tubes (GTE Sylvania, Shipley, UK), which emit light between 350 and 400 nm with a λ_{max} of 370 nm. Blue light was provided by 40W T12 blue fluorescent tubes (GTE Sylvania), λ_{max} 430 nm, covered with a UV226 filter (Lee Filters, Andover, UK), which removes wavelengths <400 nm. Red light was obtained by covering the white fluorescent tubes with orange cinemoid; there was no emission <500 nm (Sawbridge et al., 1994). Fluence rates were measured with a spectroradiometer (model SR9910; Macam Photometrics, Livingston, UK).

Quantitative GUS Assays

In each experiment, eight to 10 plants were harvested for each treatment or time point. GUS activity was assayed quantitatively (Jefferson et al., 1987) for one of the expanding third pair of leaves from each plant. These separate values were used to calculate the mean and standard error for each treatment. Each leaf was ground in extraction buffer and then incubated with extraction buffer containing 1 mM 4-methylumbelliferyl glucuronide at 37°C. Samples were removed after 15 and 60 min for measuring the fluorescent product 4-methylumbelliferone. The fluorescence was measured in a Perkin-Elmer LS5 luminometer (excitation 365 nm, emission 455 nm) against a series of standards, and GUS activity is expressed per milligram of protein in the extract, as determined by the Bradford (1976) protein assay.

RNA Isolation and Hybridization Analysis

Plants grown for 21 days in compost in low (20 μmol m^{-2} sec^{-1}) white light were transferred to the appropriate light treatment before harvest. Samples of leaf tissue (0.5 g) were harvested into liquid nitrogen and ground with a mortar and pestle. RNA was extracted using guanidine thiocyanate, as described by Jackson et al. (1995). RNA (10 mg per lane) was fractionated in 1.5% agarose–formaldehyde gels and blotted onto nylon membrane by using standard techniques (Sambrook et al., 1989). To measure *CHS* transcript levels, RNA was hybridized with a 1059-bp EcoRI-EcoRI genomic DNA fragment containing most of the second exon of the Arabidopsis *CHS* gene (Feinbaum and Ausubel, 1988; Trezzini et al., 1993). After washing, filters were rehybridized with the cDNA insert from pcf4-2 encoding *Chlamydomonas reinhardtii* α-tubulin (Silflow et al., 1985). DNA probes were labeled with an appropriate deoxynucleotide triphosphate by using the Rediprime system (Amersham International, Amersham, UK). Filters were prehybridized at 55°C for 2 hr in 0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 10 mg mL^{-1} BSA (Church and Gilbert, 1984) and hybridized for 24 hr at up to 55°C in the same solution. After hybridization, filters were washed in 0.1 to 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1 to 1% SDS at up to 60°C, depending on the probe, before autoradiography.

Reproducibility of Experiments

All of the experiments were repeated at least three times using replicated samples. The results obtained in repeated experiments followed the same trend, but absolute levels of GUS activity differed between experiments. Hence, except for the data shown in Figure 5A, representative results from individual experiments are presented with standard errors calculated for the samples within the experiment.

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