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Distinct UV-B and UV-A/Blue Light Signal Transduction Pathways Induce Chalcone Synthase Gene Expression in Arabidopsis Cells

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UV and blue light control the expression of flavonoid biosynthesis genes in a range of higher plants. To investigate the signal transduction processes involved in the induction of chalcone synthase (*CHS*) gene expression by UV-B and UV-A/blue light, we examined the effects of specific agonists and inhibitors of known signaling components in mammalian systems in a photomixotrophic Arabidopsis cell suspension culture. *CHS* expression is induced specifically by these wavelengths in the cell culture, in a manner similar to that in mature Arabidopsis leaf tissue. Both the UV-B and UV-A/blue phototransduction processes involve calcium, although the elevation of cytosolic calcium is insufficient on its own to stimulate *CHS* expression. The UV-A/blue light induction of *CHS* expression does not appear to involve calmodulin, whereas the UV-B response does; this difference indicates that the signal transduction pathways are, at least in part, distinct. We provide evidence that both pathways involve reversible protein phosphorylation and require protein synthesis. The UV-B and UV-A/blue light signaling pathways are therefore different from the phytochrome signal transduction pathway regulating *CHS* expression in other species.

INTRODUCTION

Light is one of the most important environmental factors regulating plant development and the expression of plant genes. In recent years, considerable progress has been made in defining the photoreceptors that mediate the effects of light and the *cis* elements and transcription factors that are involved in the photoregulation of specific genes. However, our understanding of the signal transduction processes that couple photoreception to transcription remains fragmentary. Moreover, much more information is available regarding the phytochrome photoreceptors and signal transduction pathways than the corresponding UV-B, UV-A, and blue light perception and transduction components. Therefore, it is essential to develop systems in which the mechanisms coupling UV and blue photoreception to defined downstream responses, in particular gene expression, can be dissected.

Several components of phytochrome signal transduction pathways have been identified (Millar et al., 1994). There is evidence that G protein activation is an early event (Bossen et al., 1990; Romero et al., 1991; Neuhaus et al., 1993; Romero and Lam, 1993), and transient increases in cytosolic calcium ions have been reported (Roux et al., 1986; Chae et al., 1990; Shacklock et al., 1992). By using microinjection into hypocotyl cells of the tomato *aurea* mutant and pharmacological experiments with a soybean cell culture, Neuhaus et al. (1993) and Bowler et al. (1994a, 1994b) defined two distinct pathways of phytochrome signal transduction coupled to the transcription of specific genes. One of these involves cGMP and induces chalcone synthase (*CHS*) gene expression, whereas the other is calcium/calmodulin dependent and stimulates expression of genes encoding the type I chlorophyll *a/b* binding protein of light-harvesting complex II (*CAB*) and ribulose-1,5-bisphosphate carboxylase small subunit (*rbcS*). Both pathways are required to stimulate expression of the ferredoxin $NADP^+$ oxidoreductase (*fnr*) gene. Additional experiments have revealed that these pathways show reciprocal negative regulation.

By comparison, the published information on UV and blue light signal transduction is poorly related to specific photoreceptors and downstream responses (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995). The CRY1 (cryptochrome) photoreceptor is the only UV/blue photoreceptor for which molecular information is available (Ahmad and Cashmore, 1993; Lin et al., 1995a, 1995b). It mediates both extension growth and gene expression responses (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Jackson and Jenkins, 1995; Lin et al., 1995a, 1995b), presumably through separate or branching signal transduction pathways, but no specific signal transduction events have been identified for this photoreceptor. Because CRY1 contains a flavin chromophore (Lin et al., 1995a; Malhotra et al., 1995), it is possible that redox reactions and electron

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transport are early steps in signal transduction. There is no direct evidence for this possibility, but several studies report blue light–induced redox processes in plants (e.g., Raghavendra, 1990; Gautier et al., 1992; Berger and Brownlee, 1994).

Probably the best evidence for a signal transduction event coupled to both a specific photoreceptor and a particular photoresponse is the blue light–induced plasma membrane protein phosphorylation activity defined by Briggs and co-workers (Gallagher et al., 1988; Short et al., 1992; Short and Briggs, 1994). It is likely that this activity is either directly concerned with photoreception or is a closely coupled signal transduction event. The Arabidopsis *nph1* (non-phototropic hypocotyl) mutant impaired in phototropism lacks this protein phosphorylation activity (Liscum and Briggs, 1995). Other studies of blue light signal transduction have provided evidence for membrane potential changes (e.g., Assmann et al., 1985; Spalding and Cosgrove, 1989), G protein activity (Warpeha et al., 1991), proton and ion fluxes (Shimazaki et al., 1986; Spalding and Cosgrove, 1992; Nishizaki, 1994), and calcium/calmodulin–dependent protein kinase activity (Shimazaki et al., 1992) in particular systems; however, additional work is needed to relate these events to specific photoreceptors and particular downstream processes (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995). Little information is available specifically for UV-A and UV-B signal transduction in plants.

The single *CHS* gene in Arabidopsis provides an excellent system to investigate the signal transduction processes initiated by UV and blue light and the mechanisms coupling these processes to the regulation of transcription. In contrast to some other species (Batschauer et al., 1991; Frohnmeyer et al., 1992; Neuhaus et al., 1993; Bowler et al., 1994b), phytochrome appears to have little involvement in the regulation of *CHS* in Arabidopsis. Very young dark-grown seedlings show far-red light induction of *CHS* expression, but this is lost in seedlings more than 6 days old (Kaiser et al., 1995). Very little red induction is seen in dark-grown seedlings, whereas UV and blue light are very effective (Feinbaum et al., 1991; Kubasek et al., 1992; Kaiser et al., 1995). Furthermore, the UV and blue light regulation of *CHS* promoter activity in Arabidopsis is little altered in mutants deficient in phytochromes A and B (Batschauer et al., 1996). Light-grown leaf tissue also shows little evidence of phytochrome-mediated *CHS* expression, whereas expression is induced by UV-B, UV-A, and blue light (Li et al., 1993; Jackson et al., 1995; G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data). The CRY1-deficient long hypocotyl *hy4-2.23N* mutant is impaired in the induction of *CHS* expression in blue (Jackson and Jenkins, 1995) and UV-A light (G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data) but appears to be unaltered in UV-B induction (G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data). Therefore, distinct UV-B and UV-A/blue phototransduction pathways concerned with the induction of *CHS* expression can be identified in Arabidopsis.

In this study, we used a pharmacological approach with an Arabidopsis cell suspension culture to identify signal transduction processes concerned with the induction of *CHS* expression by UV-B and UV-A/blue light. We show that the UV-B and UV-A/blue light signaling pathways are distinct and demonstrate that the pathways are different from the presently defined phytochrome signal transduction pathway regulating *CHS* expression.

RESULTS

UV-B and UV-A/Blue Light Induce *CHS* Transcript Accumulation in an Arabidopsis Cell Culture

To investigate the signal transduction pathways concerned with the UV-B and UV-A/blue light induction of *CHS* in Arabidopsis, we required a cellular system that would permit biochemical experimentation. We used an Arabidopsis cell suspension culture introduced by May and Leaver (1993). This green cell culture was obtained from calli of Arabidopsis ecotype Landsberg *erecta* and requires both light and a carbon source for maximal growth.

Initially, it was important for us to establish whether the regulation of *CHS* expression in the Arabidopsis cell culture was similar to that in the intact plant. In Arabidopsis leaf tissue, *CHS* promoter activity and *CHS* transcript accumulation are induced by distinct UV-B and UV-A/blue phototransduction pathways, with the latter involving CRY1 (Jackson and Jenkins, 1995; G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data); phytochrome has little effect except in the youngest seedlings (Feinbaum et al., 1991; Kubasek et al., 1992; Jackson et al., 1995; Kaiser et al., 1995; Batschauer et al., 1996). The Arabidopsis cell culture was grown routinely in a low fluence rate of white light, which induces a very low level of *CHS* transcripts in mature leaf tissue (Feinbaum and Ausubel, 1988; Jackson et al., 1995). The cells were then transferred to different light qualities for 24 hr, total RNA was isolated, and *CHS* transcript levels were analyzed. A probe encoding the *H1* gene product (Lawton and Lamb, 1987), which appears to be constitutively expressed in plant cells, was used as a control.

As shown in Figure 1A, *CHS* transcripts are at a very low level in Arabidopsis cells grown in a low fluence rate of white light and show a large increase after transfer to a sixfold higher fluence rate; this response is similar to that of Arabidopsis leaf tissue (Feinbaum and Ausubel, 1988; Jackson et al., 1995). Transfer of the culture to UV-B or UV-A/blue light also induced *CHS* transcript accumulation, but transfer to red or far-red light did not, which is again consistent with the *CHS* expression pattern found in mature leaves (Li et al., 1993; Jackson et al., 1995; G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data). The effect of the UV-B source on *CHS* expression is specific to wavelengths below 320 nm, because removal of these wavelengths with an appropriate filter prevents transcript accumulation (Figure 1B). After exposure of the

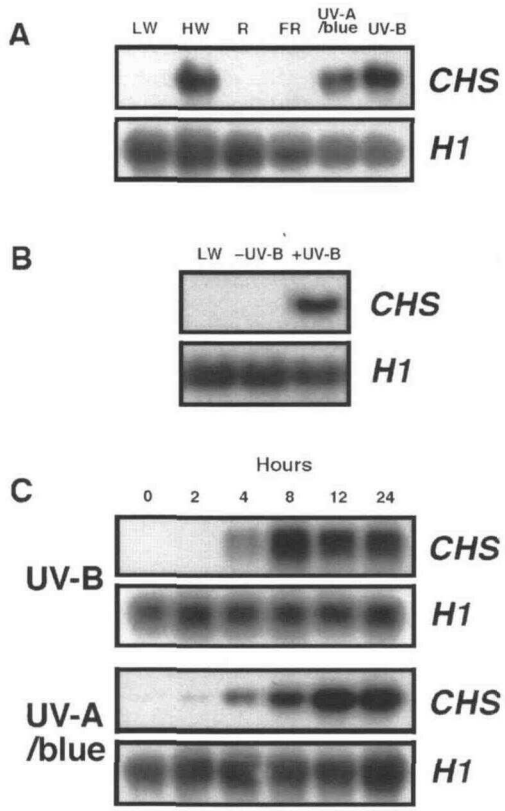

Figure 1. UV-B and UV-A/Blue Light Induce *CHS* Transcript Accumulation in the Arabidopsis Cell Culture.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were given the following light treatments for 24 hr: 20 μmol m^{-2} sec^{-1} white (LW), 120 μmol m^{-2} sec^{-1} white (HW), 80 μmol m^{-2} sec^{-1} red (R), 45 μmol m^{-2} sec^{-1} far-red (FR), 80 μmol m^{-2} sec^{-1} UV-A/blue, or 3 μmol m^{-2} sec^{-1} UV-B light. Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells grown in 20 μmol m^{-2} sec^{-1} white light (LW) were illuminated with the standard 3 μmol m^{-2} sec^{-1} UV-B source either with (−UV-B) or without (+UV-B) a filter that prevented transmission of wavelengths below 320 nm. Cells were harvested after 8 hr, and transcripts were analyzed as described in **(A)**.

(C) Cells grown in 20 μmol m^{-2} sec^{-1} white light were transferred to either 3 μmol m^{-2} sec^{-1} UV-B or 80 μmol m^{-2} sec^{-1} UV-A/blue light for the times indicated. Cells were then harvested, and transcripts were analyzed as described in **(A)**.

Arabidopsis cell culture to UV-B and UV-A/blue light, transcript accumulation was detectable within 4 hr and was not transient over a 24-hr period (Figure 1C).

Thus, the regulation of *CHS* gene expression by different light qualities in the Arabidopsis cell culture is very similar to that observed in mature Arabidopsis leaf tissue. Moreover, the kinetics of the response facilitates biochemical investigation of the signal transduction processes.

Requirement for Calcium in the UV-B and UV-A/Blue Light Regulation of *CHS*

Calcium is ubiquitously involved in a variety of plant responses (Johannes et al., 1991; Bush, 1993, 1995; Poovaiah and Reddy, 1993), including the phytochrome regulation of gene expression (Neuhaus et al., 1993; Bowler et al., 1994a, 1994b; Millar et al., 1994). Calcium channel blockers have been used to demonstrate the involvement of calcium in biochemical responses in several plant systems (e.g., Knight et al., 1992; Bowler et al., 1994b; Preisig and Moreau, 1994; Monroy and Dhinsda, 1995; Ohto et al., 1995; Reiss and Beale, 1995). Therefore, we investigated whether calcium was involved in the UV-B and UV-A/blue light signaling pathways regulating *CHS* by examining the effects of various calcium antagonists in the Arabidopsis cell culture. Several concentrations were used for each compound, based on those found to be effective in previous studies in mammalian and plant systems.

Figure 2A shows that the addition of the voltage-dependent calcium channel blocker nifedipine to the culture medium at 50 μM inhibited the accumulation of *CHS* transcripts in response to both UV-B and UV-A/blue light treatments. In contrast, incubation of Arabidopsis cells with 100 μM verapamil, a second class of voltage-dependent calcium channel blocker, had no inhibitory effect on *CHS* induction by UV-B and UV-A/blue light (Figure 2B). Similarly, *CHS* induction was unaffected when cells were treated with 10 mM lanthanum (Figure 2B), which competes externally with calcium for plasma membrane calcium channels (Tester, 1990). These concentrations of verapamil and lanthanum are the highest we employed, and equivalent or lower concentrations are reported to be inhibitory in other systems (e.g., Bossen et al., 1988; Bush, 1995; Monroy and Dhindsa, 1995; Suzuki et al., 1995; Knight et al., 1996).

The lack of effect of lanthanum suggests an internal source of calcium. Therefore, we examined the effect of ruthenium red, which is known to inhibit mitochondrial and endoplasmic reticulum calcium channels (Knight et al., 1992; Allen et al., 1995; Haley et al., 1995; Monroy and Dhinsda, 1995). Incubation of Arabidopsis cells with 50 μM ruthenium red substantially reduced *CHS* transcript accumulation in response to UV-B and UV-A/blue light treatments (Figure 2B). However, this does not necessarily imply that calcium is generated from an intracellular source because recent reports indicate that ruthenium red can also act on plasma membrane calcium channels in plants (Marshall et al., 1994). None of the above inhibitors had any effect on the level of *H1* transcripts, indicating that their effects on *CHS* expression were not the result of cell damage causing a general breakdown of transcripts.

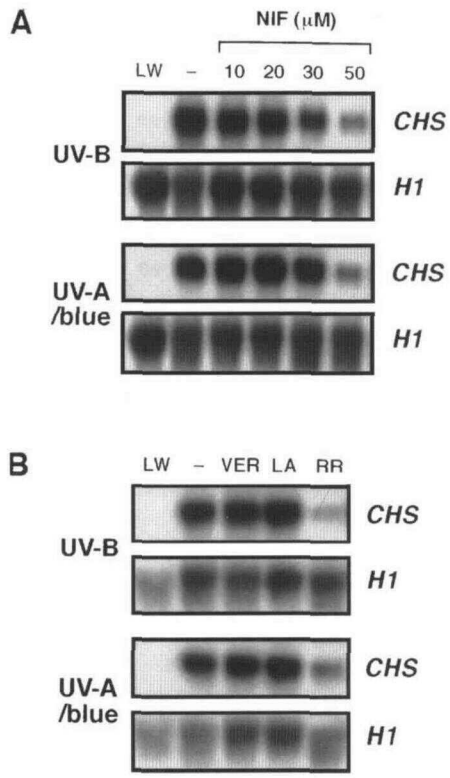

Figure 2. Effects of Calcium Channel Blockers on the UV-B and UV-A/Blue Light Induction of *CHS* Expression.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with increasing concentrations of nifedipine (NIF) before illumination for 6 hr with UV-B (3 μmol m^{-2} sec^{-1}) or UV-A/blue light (80 μmol m^{-2} sec^{-1}). Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells grown as described in **(A)** were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 100 μM verapamil (VER), 10 mM lanthanum chloride (LA), or 50 μM ruthenium red (RR) before illumination as in **(A)**. Cells were then harvested, and transcripts were analyzed as described in **(A)**.

An Increase in Cytosolic Calcium Is Not Sufficient to Stimulate *CHS*

Because the results provided above indicate an involvement of cellular calcium in the UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression, we investigated whether the artificial elevation of cytosolic calcium could stimulate *CHS* transcript accumulation. The calcium ionophore A23187, widely used to study the regulatory role of calcium in biological systems, was added to cells kept in a low fluence rate of white light. The culture medium was also supplemented with 10 mM $CaCl_2$ because studies have shown that treatment with A23187 alone is not sufficient to elevate cytosolic calcium levels in some plant cells (Suzuki et al., 1995). Braam (1992) reported that *TOUCH3* (*TCH3*) gene expression in Arabidopsis root cell cultures is stimulated by treatments that elevate cytosolic calcium. Therefore, we measured *TCH3* transcript levels as a control to show that an increase in cytosolic calcium, sufficient to affect gene expression, had occurred in our cells. As shown in Figure 3, the ionophore and calcium treatment caused an increase in *TCH3* transcripts in the Arabidopsis cell culture. In contrast to the root cell culture experiments (Braam, 1992), increasing external calcium up to 100 mM in the absence of ionophore failed to induce *TCH3* expression (data not shown). No increase in *CHS* transcripts, above the level normally present in low white light, was observed in the presence of the ionophore and calcium over a 12-hr period. Therefore, these results indicate that an increase in cytosolic calcium is insufficient on its own to stimulate *CHS* expression.

Calmodulin Is Involved in the UV-B but Not the UV-A/Blue Phototransduction Pathway Regulating *CHS*

Because the inhibitor experiments (Figure 2) indicated a requirement for calcium in the UV-B and UV-A/blue phototransduction pathways, we investigated the involvement of calmodulin. Calmodulin antagonists have been used to demonstrate an involvement of calmodulin in a number of plant responses (Lam et al., 1989a; Shimazaki et al., 1992; Bowler et al., 1994b; Preiseg and Moreau, 1994; Ohto et al., 1995). We tested the effects of the potent calmodulin antagonist W-7 (Hidaka et al., 1981) on the UV-B and UV-A/blue light induction of *CHS* in the Arabidopsis cell culture. As a control, we

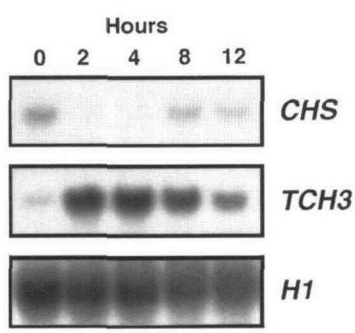

Figure 3. Elevating Cytosolic Calcium Increases *TCH3* but Not *CHS* Transcript Levels.

Cells grown in 20 μmol m^{-2} sec^{-1} white light were treated with 10 μM A23187 and 10 mM $CaCl_2$ and incubated in 20 μmol m^{-2} sec^{-1} white light for the times indicated. Cells were then harvested for RNA extraction. A gel blot of total RNA (20 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized sequentially with the *TCH3* and *H1* cDNAs.

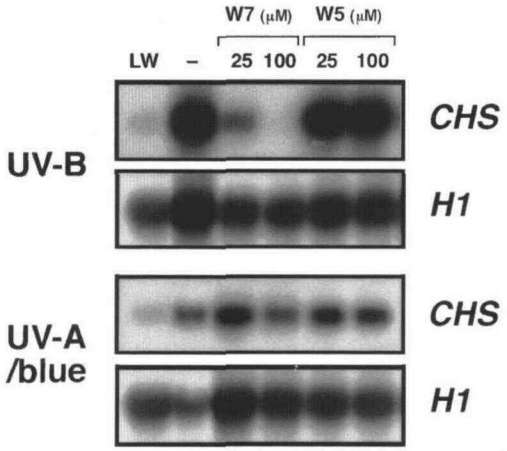

Figure 4. Differential Effect of the Calmodulin Antagonist W-7 on the UV-B and UV-A/Blue Light Induction of *CHS* Expression.

Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with increasing concentrations of W-7 or W-5 before illumination for 6 hr with UV-B (3 μmol m^{-2} sec^{-1}) or UV-A/blue light (80 μmol m^{-2} sec^{-1}). Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

also tested the effects of the relatively inactive analog W-5. Figure 4 shows that *CHS* induction in response to UV-B illumination was dramatically reduced at increasing concentrations of W-7, whereas treatment with W-5, at equivalent concentrations, had no effect. In contrast, we repeatedly observed that W-7 and W-5 had very little effect on the induction of *CHS* transcripts by UV-A/blue light, even at concentrations higher than those presented (up to 200 μM; data not shown). Because the UV-A/blue light induction of *CHS* was not significantly affected by the calmodulin antagonists, the effect of W-7 on the UV-B response could not have been due to a general inhibition of transcription or acceleration of mRNA turnover. Moreover, the differential effect of W-7 implies that the UV-B and UV-A/blue light signaling pathways are, at least in part, distinct. The quantitative differences observed in the effects of W-7 and W-5 are consistent with their known differential potencies as calmodulin antagonists (Hikada et al., 1981; Lam et al., 1989a; Ohto et al., 1995).

Protein Kinase and Phosphatase Inhibitors Prevent the UV-B and UV-A/Blue Light Induction of *CHS*

Several studies have demonstrated a role for protein phosphorylation in plant responses by using a variety of protein kinase and phosphatase inhibitors (e.g., Renelt et al., 1993; Sheen, 1993; Kamada and Muto, 1994; MacKintosh et al., 1994; Takeda et al., 1994; Suzuki et al., 1995). Some blue light responses have been shown to involve protein phosphorylation (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995). Therefore, we examined the effects of various protein kinase and phosphatase inhibitors on the UV-B and UV-A/blue light regulation of *CHS* in the Arabidopsis cell culture system, using concentrations found to be effective in previous studies.

Figures 5A and 5B show that incubation of cells with increasing concentrations of the general serine/threonine protein kinase inhibitors staurosporine and K252a completely inhibits *CHS* induction in response to both UV-B and UV-A/blue light. Levels of *H1* transcripts were unaffected at any of the inhibitor concentrations examined. In contrast, the tyrosine/histidine kinase inhibitor genistein had no inhibitory effect on the induction of *CHS* by either UV-B or UV-A/blue light (Figure 5C). At equivalent concentrations, genistein completely inhibits the phytochrome transduction pathway regulating *CHS* expression in soybean suspension cultures (Bowler et al., 1994b). These results indicate that the UV-B and UV-A/blue light signaling pathways regulating *CHS* in Arabidopsis cells are different from the phytochrome signaling pathway identified in soybean.

We examined the effect of okadaic acid, a potent inhibitor of protein phosphatases 1 and 2A (PP1 and PP2A), on *CHS* expression in the Arabidopsis cell culture. As shown in Figure

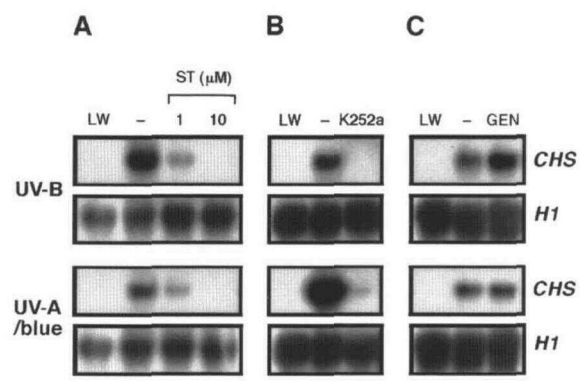

Figure 5. Effects of Protein Kinase Inhibitors on the UV-B and UV-A/Blue Light Induction of *CHS* Expression.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 1 or 10 μM staurosporine (ST) before illumination for 6 hr with UV-B (3 μmol m^{-2} sec^{-1}) or UV-A/blue light (80 μmol m^{-2} sec^{-1}). Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 5 μM K252a before illumination as described in **(A)**. Cells were then harvested, and transcripts were analyzed as given in **(A)**.

(C) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 75 μM genistein (GEN) before illumination as described in **(A)**. Cells were then harvested, and transcripts were analyzed as given in **(A)**.

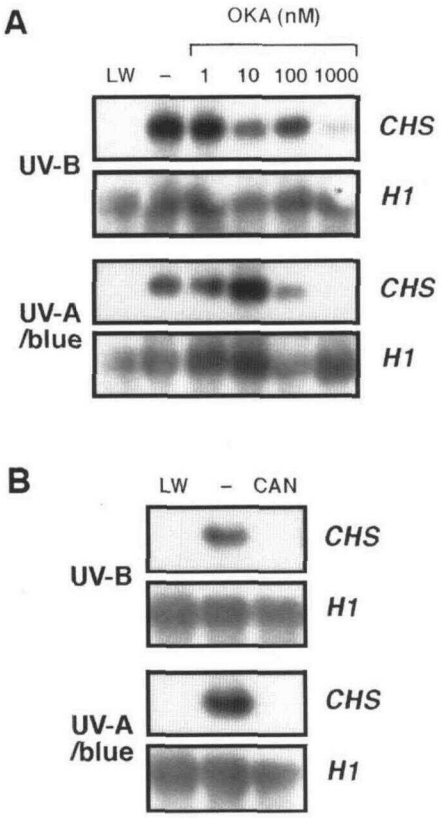

Figure 6. Effects of Protein Phosphatase Inhibitors on the UV-B and UV-A/Blue Light Induction of *CHS* Expression.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with increasing concentrations of okadaic acid (OKA) before illumination for 6 hr with UV-B (3 μmol m^{-2} sec^{-1}) or UV-A/blue light (80 μmol m^{-2} sec^{-1}). Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 100 μM cantharidin (CAN) before illumination as described in **(A)**. Cells were then harvested, and transcripts were analyzed as given in **(A)**.

6A, incubation of cells with 1 μM okadaic acid prevented *CHS* transcript accumulation in response to UV-B and UV-A/blue light treatments. In addition, *CHS* induction by UV-B and UV-A/blue light was abolished after treatment with 100 μM cantharidin, a less potent protein phosphatase inhibitor (Figure 6B). Cantharidin had no inhibitory effect at a concentration of 1 μM (data not shown). Neither inhibitor had any effect on the level of *H1* transcripts. Therefore, these observations indicate a requirement for protein phosphatase acivity, in addition to kinase activity, in the UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression.

Protein Phosphatase Inhibitors Induce Phenylalanine Ammonia-Lyase Gene Expression

Although we observed that cantharidin inhibited *CHS* expression in response to UV-B and UV-A/blue light, we also discovered, as shown in Figure 7A, that this protein phosphatase inhibitor, at equivalent concentrations, could stimulate phenylalanine ammonia-lyase (*PAL*) transcript accumulation in cells incubated in a low fluence rate of white light. Similarly, *PAL* transcripts were also induced by treatment with 1 μM okadaic acid (data not shown). These results are consistent with the findings of MacKintosh et al. (1994), who reported that cantharidin, okadaic acid, and other protein phosphatase inhibitors stimulate PAL activity and phytoalexin production in soybean cotyledons and suspension cultures. However, MacKintosh et al. (1994) did not investigate the effect of protein phosphatase inhibitors on *PAL* transcript levels. The observation that cantharidin and okadaic acid stimulate *PAL* expression in the Arabidopsis cell culture demonstrates that the effects of these compounds on *CHS* expression do not result from a general inhibition of transcription or an acceleration of mRNA turnover.

To satisfy ourselves that the other compounds used in this study were also not generally detrimental to gene expression, we investigated their effects on *PAL* induction by cantharidin.

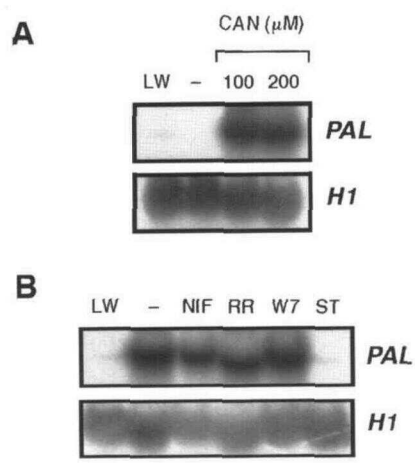

Figure 7. Effects of Various Signaling Antagonists on the Cantharidin-Induced Accumulation of *PAL* Transcripts.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 6 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 100 or 200 μM cantharidin (CAN) and then harvested for RNA extraction. A gel blot of total RNA (20 μg per lane) was hybridized with the *PAL* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells grown as described in **(A)** were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 50 μM nifedipine (NIF), 50 μM ruthenium red (RR), 25 μM W-7 (W7), or 1 μM staurosporine (ST) before the addition of 200 μM cantharidin. Cells were harvested after 6 hr, and transcripts were analyzed as described in **(A)**.

Figure 7B demonstrates that incubation of cells with nifedipine, ruthenium red, and W-7, at concentrations found to inhibit the induction of *CHS* by UV-B and/or UV-A/blue light, had no significant inhibitory effect on this response. However, the addition of 1 μM staurosporine prevented *PAL* induction by cantharidin. This result is consistent with the observation that the protein kinase inhibitor K252a, at similar concentrations, inhibits the induction of PAL activity in soybean cells (MacKintosh et al., 1994).

Cycloheximide Inhibits the UV-B and UV-A/Blue Light Induction of *CHS* Expression

The phytochrome-mediated light induction of *CAB* gene expression has been shown to require protein synthesis because it is inhibited by cycloheximide (Lam et al., 1989b). More recently, Green and Fluhr (1995) have reported that cytoplasmic protein synthesis is required for the UV-B–induced expression of the plant pathogenesis–related protein PR-1 in tobacco leaves. Therefore, we examined whether protein synthesis is required for the UV-B and UV-A/blue light induction of *CHS* expression. As shown in Figure 8A, the addition of cycloheximide to the cell culture medium abolished the increase in *CHS* transcripts in UV-B and UV-A/blue light. As a control, we found that cycloheximide did not affect *TCH3* gene expression in response to A23187 and calcium treatment (Figure 8B). Moreover, no effect on the level of *H1* transcripts was observed. Therefore, the inhibition of protein synthesis does not have a general, damaging effect on transcription and transcript accumulation in the Arabidopsis cells.

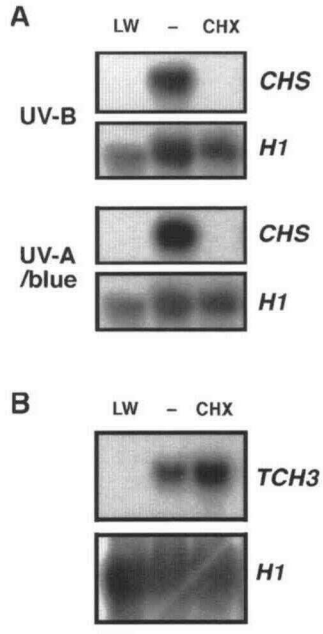

Figure 8. Effects of Cycloheximide on *CHS* and *TCH3* Gene Expression.

(A) Cells grown in 20 μmol m^{-2} sec^{-1} white light were incubated for 1 hr in 20 μmol m^{-2} sec^{-1} white light (LW) either without (–) or with 20 μM cycloheximide (CHX) before illumination for 6 hr with UV-B (3 μmol m^{-2} sec^{-1}) or UV-A/blue light (80 μmol m^{-2} sec^{-1}). Cells were then harvested for RNA extraction. A gel blot of total RNA (10 μg per lane) was hybridized with the *CHS* cDNA probe and rehybridized with the *H1* cDNA.

(B) Cells were grown and incubated either without (–) or with 20 μM cycloheximide (CHX) as described in **(A)**. A23187 (10 μM) and $CaCl_2$ (10 mM) were then added, and the cells were harvested after 6 hr. A gel blot of total RNA (20 μg per lane) was hybridized with the *TCH3* cDNA probe and rehybridized with the *H1* cDNA.

DISCUSSION

Our study demonstrates that a dissection of the signal transduction processes involved in the regulation of *CHS* gene expression by UV-B and UV-A/blue light is experimentally feasible with the Arabidopsis cell culture system. We show that the signaling pathways are complex and provide initial information on their components. Moreover, our findings indicate that the UV-B and UV-A/blue light signal transduction pathways are distinct at least in part and that they are different from the phytochrome signal transduction pathway coupled to *CHS* gene expression in other species.

An Arabidopsis Cell Culture Permits Biochemical Analysis of the UV-B and UV-A/Blue Light Regulation of *CHS*

The photomixotrophic Arabidopsis cell culture used here is similar to the soybean cell culture that has provided information on the signaling processes coupling phytochrome to *CAB*, *rbcS*, *fnr*, and *CHS* gene expression (Lam et al., 1989a; Romero and Lam, 1993; Bowler et al., 1994a, 1994b). However, in contrast to the soybean system, *CHS* is not regulated by phytochrome in the Arabidopsis cell culture, as shown by the lack of induction by either red or far-red light. This is consistent with the lack of phytochrome induction of *CHS* in all but the youngest Arabidopsis seedlings (Feinbaum et al., 1991; Kubasek et al., 1992; Kaiser et al., 1995). Furthermore, the regulation of *CHS* expression in the Arabidopsis cell culture by distinct UV-B and UV-A/blue phototransduction pathways is similar to the situation in mature, intact Arabidopsis leaf tissue. Only a low fluence rate of UV-B, specific to wavelengths below 320 nm and similar to the levels in sunlight, is required to induce *CHS*. Therefore, the Arabidopsis suspension culture provides an excellent homogeneous cellular system for the biochemical and molecular dissection of the UV-B and

UV-A/blue light signal transduction pathways regulating *CHS*. Although several putative signal transduction events initiated by UV and blue light in plants have been identified (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995), to date little progress has been made in relating these to the regulation of gene expression.

Involvement of Calcium in the UV-B and UV-A/Blue Phototransduction Pathways Regulating *CHS*

Several environmental signals have been shown to alter cytosolic levels of calcium in plant cells, and such changes are likely to be primary events in triggering cellular responses (Bush, 1993). However, only a few observations in the literature implicate calcium in UV and blue light signal transduction (Murphy, 1988; Shinkle and Jones, 1988; Russ et al., 1991). The experiments reported here, with well-established calcium antagonists, indicate that calcium is involved in both the UV-B and UV-A/blue phototransduction pathways regulating *CHS* gene expression in the Arabidopsis cell culture.

Increases in cytosolic calcium can occur by an influx of calcium from the extracellular space across the plasma membrane, by an efflux of calcium from intracellular stores, or by a combination of both pathways (Schroeder and Thuleau, 1991; Bush, 1995). Several types of calcium channels have been identified in plant cells that are located not only in the plasma membrane but also in the tonoplast (Johannes et al., 1991; Schroeder and Thuleau, 1991; Allen and Sanders, 1994; Bush, 1995). The voltage-dependent calcium channel blocker nifedipine strongly inhibited both the UV-B and UV-A/blue light induction of *CHS*. Bowler et al. (1994b) showed that an equivalent concentration of nifedipine completely inhibited the phytochrome induction of *CAB* in soybean cells, and Preisig and Moreau (1994) have used higher concentrations to implicate calcium in the synthesis of phytoalexins in tobacco cell suspensions.

Although lanthanum and verapamil are reported to inhibit calcium channels efficiently in higher plants (Schroeder and Thuleau, 1991), these reagents had no effect on the induction of *CHS* in response to UV-B and UV-A/blue light at concentrations found to be inhibitory in other systems. Similar differential effects of verapamil and nifedipine have been observed in studies of sugar-inducible gene expression in tobacco and sweet potato (Ohto et al., 1995). Therefore, a particular class of calcium channel that is sensitive to nifedipine but insensitive to verapamil appears to be involved in the UV-B and UV-A/blue light induction of *CHS* expression in the Arabidopsis cell culture. Because nifedipine is readily cell permeable, it is impossible to say whether its action is targeted to voltage-dependent calcium channels at the plasma membrane, internal membranes, or both. The lack of effect of lanthanum suggests that an influx of external calcium into the cells may not be required for the UV-B and UV-A/blue light responses. This hypothesis is supported by the observation that ruthenium red, which is known to inhibit the release of calcium from intracellular stores (Knight et al., 1992; Allen et al., 1995; Haley et al., 1995; Monroy and Dhindsa, 1995), severely attenuated the UV-B and UV-A/blue light responses (Figure 2B). However, recent studies have shown that ruthenium red blocks the activity of a voltage-dependent calcium channel in plasma membrane isolated from maize roots (Marshall et al., 1994). Therefore, it is unclear whether ruthenium red and nifedipine exert their effects on the same or different calcium channels in our system. More information is needed to determine whether the calcium requirement is extracellular, intracellular, or both.

The cantharidin stimulation of *PAL* expression was not affected by nifedipine or ruthenium red; this finding demonstrates that their effects on *CHS* expression did not result from a general inhibition of transcription or an acceleration of mRNA turnover (Figure 7B). Moreover, none of the compounds used in these experiments caused browning or bleaching of the cells. Some reagents, which have not been described here, do have such effects and are obviously toxic to the cells. Our results therefore indicate that calcium is involved in both the UV-B and UV-A/blue phototransduction pathways controlling *CHS* in the Arabidopsis cell culture and suggest that the most likely source of the calcium is from internal stores.

The artificial elevation of cytosolic calcium was insufficient to increase the *CHS* transcript level in low white light (Figure 3). The increase in *TCH3* transcripts in the same experiment provides good evidence that the ionophore and Ca^{2+} treatment did elevate cytosolic calcium. Similarly, although calcium has been implicated in phytochrome signal transduction, treatment of soybean cells with ionomycin only weakly induced *CAB* gene expression (Lam et al., 1989a).

It is possible that UV-B and UV-A/blue light trigger an increase in calcium that either is not cytosolic (e.g., nuclear) or occurs in a particular subcellular microdomain. For example, Knight et al. (1996) have measured a cold-induced increase in calcium in the microdomain associated with the vacuolar membrane by using transgenic Arabidopsis containing aequorin targeted to the cytosolic face of the vacuole. Consistent with the possibilities provided above, in preliminary experiments with transgenic Arabidopsis containing cytosolic aequorin (Knight et al., 1991), we have not observed any significant, rapid increase in calcium in response to UV-B or UV-A/blue light; as a control, low temperature elicited a large, immediate increase in the same plants (J.M. Christie, J.C. Long, M.R. Knight, and G.I. Jenkins, unpublished data). Additional experiments are required to determine whether UV and blue light induce an increase in calcium in a particular cellular location.

A further possible interpretation of the ionophore experiment (Figure 3) is that UV-B and UV-A/blue light each initiate more than one transduction process and that an increase in cytosolic calcium, although necessary, can only stimulate *CHS* expression by acting in conjunction with some other signaling process. Indeed, this may be the case, regardless of the location of the calcium pool. The complexity of the transduction processes, discussed below, supports this possibility.

The UV-B and UV-A/Blue Light Signal Transduction Pathways Regulating *CHS* Differ in the Involvement of Calmodulin

Calcium can regulate the activities of target proteins directly or via calcium binding proteins such as calmodulin. The inhibitory effect of the well-characterized antagonist W-7 on the UV-B induction of *CHS* indicates that calmodulin activation is required (Figure 4). Consistent with this conclusion is the observation that the less effective analog W-5 has no effect. It should be noted that inhibition by W-7 was observed at the same concentration as in animal cells (10 to 30 μM). This is important, because at higher concentrations calmodulin antagonists can inhibit other target proteins, such as calcium-dependent protein kinases (Roberts and Harmon, 1992). In contrast, the lack of effect of W-7 on the UV-A/blue light induction of *CHS* indicates that this response is not mediated by calmodulin in this system. The UV-A/blue pathway is likely to involve a different calcium-dependent or calcium binding component, but additional experiments are required to establish its identity. Our findings therefore demonstrate that the UV-B and UV-A/blue light signal transduction pathways differ in at least one component. That the pathways are distinct, at least in part, is in agreement with experiments with the *hy4-2.23N* mutant (G. Fuglevand, J.A. Jackson, and G.I. Jenkins, unpublished data), which show that the CRY1 photoreceptor does not mediate the UV-B response.

Protein Phosphorylation Appears to Be Involved in the Regulation of *CHS* and *PAL*

Several studies have implicated protein phosphorylation in UV-A/blue light signal transduction (Kaufman, 1993; Short and Briggs, 1994; Jenkins et al., 1995). Probably the best characterized example is the blue light–regulated protein kinase activity identified by Briggs and co-workers (Short and Briggs, 1994; Liscum and Briggs, 1995). This is an early event in the UV-A/blue phototransduction pathway mediating phototropism (Short and Briggs, 1994; Jenkins et al., 1995; Liscum and Briggs, 1995). Other studies, using inhibitors of animal protein kinases, provide evidence for the involvement of protein phosphorylation in the blue light regulation of stomatal opening (Shimazaki et al., 1992, 1993).

In our studies, the inhibitory effects of staurosporine and K252a indicate a requirement for serine/threonine protein kinase activity in the UV-B and UV-A/blue light induction of *CHS* (Figures 5A and 5B). The inhibitory effects of okadaic acid and the less potent protein phosphatase inhibitor cantharidin (MacKintosh et al., 1994) suggest that *CHS* induction requires a protein dephosphorylation event(s) in addition to protein kinase activity (Figure 6). Cantharidin is reported to be an inhibitor of PP2A (Li and Casida, 1992), whereas okadaic acid has been shown, in cell-free extracts, to inhibit PP2A (1 nM) and PP1 at higher concentrations (1 μM) (Cohen et al., 1990). Nevertheless, the present data do not allow us to conclude whether PP1 and/or PP2A are involved in the UV-B and UV-A/blue light induction of *CHS* because precise concentration-dependent effects of okadaic acid are more difficult to interpret in intact cells. Sheen (1993) has shown that the light-regulated expression of two photosynthetic genes requires a protein phosphatase activity, which is likely to be PP1.

The observation that these protein phosphatase inhibitors can stimulate *PAL* expression in the Arabidopsis cell culture demonstrates that their effects on *CHS* expression are not due to a general inhibition of transcription (Figure 7A). These data extend the observations of MacKintosh et al. (1994), who reported the stimulation of PAL activity by protein phosphatase inhibitors in soybean. The fact that *CHS* induction is prevented by both protein kinase and phosphatase inhibitors indicates the complexity of the transduction processes. In a simple system, these inhibitors would be expected to have opposing effects, as is the case with *PAL* expression. Therefore, it appears that *CHS* induction involves separate signaling processes in which these inhibitors have differential effects. This concept is consistent with the hypothesis developed in relation to the ionophore and calcium experiment that both UV-B and UV-A/blue light initiate more than one transduction process.

Protein Synthesis Is Required for the Induction of *CHS* by UV-B and UV-A/Blue Light

Our results (Figure 8) demonstrate that the induction of *CHS* by UV-B and UV-A/blue light in Arabidopsis cells requires cytoplasmic protein synthesis. It is most likely that one or more components, such as a transcription factor, that are essential for the stimulation of expression, are synthesized. Indeed, the expression of common plant regulatory factor 1, a transcription factor that binds to the parsley *CHS* promoter, is rapidly induced by light in parsley cells and plants (Weisshaar et al., 1991; Feldbrügge et al., 1994). However, not all blue light signal transduction processes coupled to gene expression require protein synthesis. Marrs and Kaufman (1991) observed that cycloheximide had no effect on the blue light regulation of two genes in pea. The fact that protein synthesis is required for the UV-B and UV-A/blue light induction of *CHS* in Arabidopsis cells illustrates the complexity of the transduction processes. The identification of the gene product(s) required for *CHS* expression is therefore a priority.

The UV-B and UV-A/Blue Phototransduction Pathways Appear Distinct from the Phytochrome Pathway Regulating *CHS* in Other Species

Microinjection experiments with tomato and pharmacological experiments with soybean cells have provided information on the phytochrome signal transduction pathway regulating *CHS*

expression (Neuhaus et al., 1993; Bowler et al., 1994a, 1994b). This pathway involves cGMP rather than calcium and calmodulin and is inhibited by genistein, an inhibitor of tyrosine/histidine kinases in animal cells. Our observations indicate that the UV-B and UV-A/blue light signaling pathways regulating *CHS* in Arabidopsis cells are distinct from the phytochrome pathway in tomato and soybean. First, both the UV-B and UV-A/blue light signaling pathways involve calcium, in contrast with the phytochrome pathway regulating *CHS*. Second, we have not found any stimulation of *CHS* transcript accumulation after the addition of cGMP (provided as cell-permeable dibutyryl-cGMP at the same concentration as in the soybean experiments) to Arabidopsis cells in a low fluence rate of white light (data not shown). Moreover, sodium nitroprusside, which dramatically induces *CHS* in soybean cells by presumably increasing intracellular cGMP concentrations through activation of guanylyl cyclase (Bowler et al., 1994b), does not stimulate *CHS* in the Arabidopsis system (data not shown). Finally, genistein does not inhibit *CHS* expression at a concentration that abolishes the phytochrome induction of *CHS* in soybean cells (Figure 5C; Bowler et al., 1994b). Given that genistein is effective in soybean cells, it seems very unlikely that its lack of effect in Arabidopsis cells was due to a lack of uptake. Taken together, these observations indicate a real difference between the phytochrome and UV-B and UV-A/blue phototransduction pathways regulating *CHS* expression. Consistent with this, the UV-B and UV-A/blue light induction of *CHS* transcript accumulation in Arabidopsis cells does not show the rapid transient increase characteristic of the phytochrome induction of *CHS* in soybean cells (Bowler et al., 1994a, 1994b).

In conclusion, we believe that the combined application of biochemical, cell physiological, molecular, and genetic approaches in Arabidopsis will permit the detailed functional characterization of the components of the UV-B and UV-A/blue phototransduction pathways regulating gene expression. The Arabidopsis cell culture will be a very useful tool in this research, and the initial characterization of the pathways regulating *CHS* expression presented here provides a starting point for further experimentation.

METHODS

Arabidopsis thaliana Cell Culture

The Arabidopsis cell suspension culture, described previously by May and Leaver (1993), was grown photomixotrophically in 200 mL of sterile culture medium containing Murashige and Skoog salts (Sigma), 0.5 mg L^{-1} α-naphthaleneacetic acid, 0.05 mg L^{-1} kinetin, and 3% (w/v) sucrose in 500-mL conical flasks. Suspension cultures were grown at 20°C in a continuous low fluence rate of white light (20 μmol m^{-2} sec^{-1}) with constant shaking (110 rpm). Cells were subcultured every week by a 1:10 dilution. On the third day after subculture, 10-mL aliquots of cells were transferred aseptically to sterile 50-mL tissue culture flasks (Nunclon; Life Technologies, Paisley, Scotland) and illuminated, with the appropriate light regimen, for the times indicated, with constant shaking (80 rpm). Samples were collected on filter paper by vacuum filtration and immediately frozen in liquid nitrogen.

Light Treatments

Illuminations were performed in controlled-environment rooms at 21°C. White light was provided by warm-white fluorescent tubes (Osram, Munich, Germany). UV-A/blue light was provided by 40 W T12 blue fluorescent tubes (GTE Sylvania, Shipley, UK) with a λ_{max} of 430 nm and no emission below 330 nm (Sawbridge et al., 1994). UV-B was obtained from TL 40W/12 UV fluorescent tubes (Philips, London, UK) covered with cellulose acetate, which was changed every 24 hr, to remove UV-C wavelengths. The UV-B source emits very low levels of UV-A and blue light, which we found are insufficient to induce *CHS* gene expression in Arabidopsis plants and cells. In the experiment described in Figure 1B, wavelengths below 320 nm were removed by covering the tubes with a clear polyester 130 filter (Lee Filters, Andover, UK). Red light was obtained by covering the white fluorescent tubes with orange cinemoid (Sawbridge et al., 1994), and far-red light was provided by FL20S FR-74 tubes (Toshiba, Tokyo, Japan). Fluence rates were measured with a spectroradiometer (model SR9910; Macam Photometrics, Livingston, UK), and the values stated are those at the surface of the cells, taking into account light absorption by the culture flasks. The fluence rate from the UV-B source was 3 μmol m^{-2} sec^{-1} (280 to 320 nm) and that from the UV-A/blue source was 80 μmol m^{-2} sec^{-1}.

Treatments with Chemical Agonists/Antagonists

Nifedipine, verapamil, lanthanum chloride, ruthenium red, W-7, W-5, and cycloheximide were obtained from Sigma. Okadaic acid was purchased from Calbiochem-Novabiochem (Nottingham, UK). Cantharidin, staurosporine, K252a, genistein, and A23187 were obtained from Calbiochem-Novabiochem. Okadaic acid, staurosporine, and K252a were dissolved in dimethyl sulfoxide (DMSO) at 1 mM to yield stock solutions. Nifedipine, W-7, W-5, and A23187 were dissolved in DMSO at 10 mM. Cantharidin and genistein were dissolved in DMSO at 100 mM. Verapamil, lanthanum chloride, ruthenium red, and cycloheximide were dissolved in distilled water at 10 mM (ruthenium red, verapamil, and cycloheximide) or 1 M (lanthanum chloride). After the addition of the selected reagents, the 10-mL cell aliquots were returned to low white light (20 μmol m^{-2} sec^{-1}) for 1 hr with constant shaking (80 rpm) before further light treatment. For controls, cells were treated with equivalent amounts of distilled water or DMSO; these treatments did not affect transcript levels.

RNA Isolation and Hybridization Analysis

Frozen cell samples were ground with a mortar and pestle in liquid nitrogen, and RNA was extracted using guanidinine thiocyanate as described by Jackson et al. (1995). RNA (equal amounts per lane; usually 10 or 20 μg) was fractionated in 1.3% agarose–formaldehyde gels and blotted onto a nylon membrane (Hybond-N; Amersham), using standard procedures (Sambrook et al., 1989). Radiolabeled DNA probes were prepared with an appropriate deoxynucleotide triphosphate, using the Rediprime system (Amersham). Hybridization analysis was conducted as described by Jackson et al. (1995). Arabidopsis cDNA fragments encoding *CHS* (Feinbaum and Ausubel, 1988; Trezzini et

al., 1993), *PAL* (Trezzini et al., 1993), *TCH3* (Braam and Davis, 1990), and a *Phaseolus vulgaris* cDNA fragment encoding *H1* (Lawton and Lamb, 1987) were used as probes. After autoradiography, filters were washed to remove radioactivity before rehybridization.

Reproducibility of Experiments

All experiments were repeated three to six times, and in each case, similar trends were observed. The data presented are from individual experiments that are representative of the results obtained.

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REFERENCES

Ahmad, M., and Cashmore, A.R. (1993). *HY4* gene of *A. thaliana* encodes a protein with the characteristics of a blue-light photoreceptor. Nature **366,** 162–166.

Allen, G.J., and Sanders, D. (1994). Two voltage-gated, calcium release channels coreside in the vacuolar membrane of broad bean guard cells. Plant Cell **6,** 685–694.

Allen, G.J., Muir, S.R., and Sanders, D. (1995). Release of Ca^{2+} from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. Science **268,** 735–737.

Assmann, S.M., Simoncini, L., and Schroeder, J.I. (1985). Blue light activates electrogenic ion pumping in guard cell protoplasts of *Vicia faba*. Nature **318,** 285–287.

Batschauer, A., Ehmann, B., and Schäfer, E. (1991). Cloning and characterization of a chalcone synthase gene from mustard and its light-dependent expression. Plant Mol. Biol. **16,** 175–185.

Batschauer, A., Rocholl, M., Kaiser, T., Nagatani, A., Furuya, M., and Schäfer, E. (1996). Blue and UV-A light-regulated *CHS* expression in *Arabidopsis* independent of phytochrome A and phytochrome B. Plant J. **9,** 63–69.

Berger, F., and Brownlee, C. (1994). Photopolarization of the *Fucus* sp. zygote by blue light involves a plasma membrane redox chain. Plant Physiol. **105,** 519–527.

Bossen, M.E., Dassen, H.A., Kendrick, R.E., and Vredenberg, W.J. (1988). The role of calcium ions in phytochrome-controlled swelling of etiolated wheat (*Triticum aestivum* L.) protoplasts. Planta **174,** 94–100.

Bossen, M.E., Kendrick, R.E., and Vredenberg, W.J. (1990). The involvement of a G-protein in phytochrome-regulated, Ca^{2+}-dependent swelling of etiolated wheat protoplasts. Physiol. Plant. **80,** 55–62.

Bowler, C., Neuhaus, G., Yamagata, H., and Chua, N.-H. (1994a). Cyclic GMP and calcium mediate phytochrome phototransduction. Cell **77,** 73–81.

Bowler, C., Yamagata, H., Neuhaus, G., and Chua, N.-H. (1994b). Phytochrome signal transduction pathways are regulated by reciprocal control mechanisms. Genes Dev. **8,** 2188–2202.

Braam, J. (1992). Regulated expression of the calmodulin-related *TCH* genes in cultured Arabidopsis cells: Induction by calcium and heat shock. Proc. Natl. Acad. Sci. USA **89,** 3213–3216.

Braam, J., and Davis, R.W. (1990). Rain-, wind-, touch-induced expression of calmodulin and calmodulin-related genes in Arabidopsis. Cell **60,** 357–364.

Bush, D.S. (1993). Regulation of cytosolic calcium in plants. Plant Physiol. **103,** 7–13.

Bush, D.S. (1995). Calcium regulation in plant cells and its role in signaling. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 95–122.

Chae, Q., Park, H.J., and Hong, S.D. (1990). Loading of quin2 into protoplasts and measurement of cytosolic calcium ion concentration changes by phytochrome action. Biochem. Biophys. Acta **1051,** 115–122.

Cohen, P., Holmes, C.F.B., and Tsukitani, Y. (1990). Okadaic acid: A new probe for the study of cellular regulation. Trends Biol. Sci. **15,** 98–102.

Feinbaum, R.L., and Ausubel, F.M. (1988). Transcriptional regulation of the *Arabidopsis thaliana* chalcone synthase gene. Mol. Cell. Biol. **8,** 1985–1992.

Feinbaum, R.L., Storz, G., and Ausubel, F.M. (1991). High intensity and blue light regulated expression of chimeric chalcone synthase genes in transgenic *Arabidopsis thaliana* plants. Mol. Gen. Genet. **226,** 449–456.

Feldbrügge, M., Sprenger, M., Dinkelbach, M., Yazaki, K., Harter, K., and Weisshaar, B. (1994). Functional analysis of a light-responsive plant bZIP transcriptional regulator. Plant Cell **6,** 1607–1621.

Frohnmeyer, H., Ehmann, B., Kretsch, T., Rocholl, M., Harter, K., Nagatani, A., Furuya, M., Batschauer, A., Hahlbrock, K., and Schäfer, E. (1992). Differential usage of photoreceptors for chalcone synthase gene expression during plant development. Plant J. **2,** 899–906.

Gallagher, S., Short, T.W., Ray, P.M., Pratt, L.H., and Briggs, W.R. (1988). Light-mediated changes in two proteins found associated with plasma membrane fractions from pea stem sections. Proc. Natl. Acad. Sci. USA **85,** 8003–8007.

Gautier, H., Vavasseur, A., Lascève, G., and Boudet, A. (1992). Redox processes in the blue light responses of guard cell protoplasts of *Commelina communis* L. Plant Physiol. **98,** 34–38.

Green, R., and Fluhr, R. (1995). UV-B–induced PR-1 accumulation is mediated by active oxygen species. Plant Cell **7,** 203–212.

Haley, A., Russel, A.J., Wood, N., Allan, A.C., Knight, M., Campbell, A.K., and Trewavas, A.J. (1995). Effects of mechanical signaling on plant cell cytosolic calcium. Proc. Natl. Acad. Sci. USA **92,** 4124–4128.

Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fuji, Y., and Nagata, T. (1981). *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, a calmodulin antagonist, inhibits cell proliferation. Proc. Natl. Acad. Sci. USA **78,** 4354–4357.

Jackson, J.A., and Jenkins, G.I. (1995). Extension growth responses and flavonoid biosynthesis gene expression in the *Arabidopsis hy4* mutant. Planta **197,** 233–239.

Jackson, J.A., Fuglevand, G., Brown, B.A., Shaw, M.J., and Jenkins, G.I. (1995). Isolation of *Arabidopsis* mutants altered in the light-regulation of chalcone synthase gene expression using a transgenic screening approach. Plant J. **8,** 369–380.

Jenkins, G.I., Christie, J.M., Fuglevand, G., Long, J.C., and Jackson, J.A. (1995). Plant responses to UV and blue light: Biochemical and genetic approaches. Plant Sci. **112,** 117–138.

Johannes, E., Brosnan, J.M., and Sanders, D. (1991). Calcium channels and signal transduction in plant cells. Bioessays **13,** 331–336.

Kaiser, T., Emmler, K., Kretsch, T., Weisshaar, B., Schäfer, E., and Batschauer, A. (1995). Promoter elements of the mustard *CHS1* gene are sufficient for light-regulation in transgenic plants. Plant Mol. Biol. **28,** 219–229.

Kamada, Y., and Muto, S. (1994). Protein kinase inhibitors inhibit stimulation of inositol phospholipid turnover and induction of phenylalanine ammonia-lyase in fungal elicitor-treated tobacco suspension culture cells. Plant Cell Physiol. **35,** 405–409.

Kaufman, L.S. (1993). Transduction of blue light signals. Plant Physiol. **102,** 333–337.

Knight, H., Trewavas, A.J., and Knight, M.R. (1996). Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell **8,** 489–503.

Knight, M.R., Campbell, A.K., Smith, S.M., and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature **352,** 524–526.

Knight, M.R., Smith, S.M., and Trewavas, A.J. (1992). Wind-induced plant motion immediately increases cytosolic calcium. Proc. Natl. Acad. Sci. USA **89,** 4967–4971.

Koornneef, M., Rolff, E., and Spruit, C.J.P. (1980). Genetic control of light-inhibited hypocotyl elongation in *Arabidopsis thaliana*. Z. Pflanzenphysiol. **100,** 147–160.

Kubasek, W.I., Shirley, B.W., McKillop, A., Goodman, H.M., Briggs, W., and Ausubel, F.M. (1992). Regulation of flavonoid biosynthetic genes in germinating Arabidopsis seedlings. Plant Cell **4,** 1229–1236.

Lam, E., Benedyck, M., and Chua, N.-H. (1989a). Characterisation of phytochrome-regulated gene expression in a photoautotrophic cell suspension: Possible role for calmodulin. Mol. Cell. Biol. **9,** 4819–4823.

Lam, E., Green, P.J., Wong, M., and Chua, N.-H. (1989b). Phytochrome activation of two nuclear genes requires cytoplasmic protein synthesis. EMBO J. **8,** 2777–2783.

Lawton, M.A., and Lamb, C.J. (1987). Transcriptional activation of plant defense genes by fungal elicitor, wounding, and infection. Mol. Cell. Biol. **7,** 335–341.

Li, J., Ou-Lee, T.-M., Raba, R., Amundson, R.G., and Last, R.L. (1993). Arabidopsis flavonoid mutants are hypersensitive to UV-B irradiation. Plant Cell **5,** 171–179.

Li, Y.-M., and Casida, J.E. (1992). Cantharidin binding protein: Identification as protein phosphatase 2A. Proc. Natl. Acad. Sci. USA **89,** 11867–11870.

Lin, C., Ahmad, M., Gordon, D., and Cashmore, A.R. (1995a). Expression of an *Arabidopsis* cryptochrome gene in transgenic tobacco results in hypersensitivity to blue, UV-A, and green light. Proc. Natl. Acad. Sci. USA **92,** 8423–8427.

Lin, C., Robertson, D.E., Ahmad, M., Raibekas, R.A., Jorns, S., Dutton, L., and Cashmore, A.R. (1995b). Association of flavin adenine dinucleotide with the *Arabidopsis* blue light receptor CRY1. Science **269,** 968–970.

Liscum, E., and Briggs, W.R. (1995). Mutations in the *NPH1* locus of Arabidopsis disrupt the perception of phototropic stimuli. Plant Cell **7,** 473–485.

MacKintosh, C., Lyon, G.D., and MacKintosh, R.W. (1994). Protein phosphatase inhibitors activate anti-fungal defence responses of soybean cotyledons and cell cultures. Plant J. **5,** 137–147.

Malhotra, K., Kim, S.-T., Batschauer, A., Dawut, L., and Sancar, A. (1995). Putative blue-light photoreceptors from *Arabidopsis thaliana* and *Sinapis alba* with a high degree of sequence homology to DNA photolyase contain the two photolyase cofactors but lack DNA repair activity. Biochemistry **34,** 6892–6899.

Marrs, K.A., and Kaufman, L.S. (1991). Rapid transcriptional regulation of the *Cab* and *pEA207* gene families in peas by blue light in the absence of cytoplasmic protein synthesis. Planta **183,** 327–333.

Marshall, J., Corzo, A., Leigh, R.A., and Sanders, D. (1994). Membrane potential-dependent calcium transport in right-side-out plasma membrane vesicles from *Zea mays* L. roots. Plant J. **5,** 683–694.

May, M.J., and Leaver, C.J. (1993). Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. Plant Physiol. **103,** 621–627.

Millar, A.J., McGrath, R.B., and Chua, N.-H. (1994). Phytochrome phototransduction pathways. Annu. Rev. Genet. **28,** 325–349.

Monroy, A.F., and Dhindsa, R.S. (1995). Low-temperature signal transduction: Induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell **7,** 321–331.

Murphy, T.M. (1988). Ca^{2+} dependence and La^{3+} interference of ultraviolet radiation–induced K^+ efflux from rose cells. Physiol. Plant. **74,** 537–543.

Neuhaus, G., Bowler, C., Kern, R., and Chua, N.-H. (1993). Calcium/calmodulin-dependent and -independent phytochrome signal transduction pathways. Cell **73,** 937–952.

Nishizaki, Y. (1994). Vanadate and dicyclohexylcarbodiimide inhibit the blue light–induced depolarisation of the membrane in pulvinar motor cells of *Phaseolus*. Plant Cell Physiol. **35,** 841–844.

Ohto, M.-A., Hayashi, K., Isobe, M., and Nakamura, K. (1995). Involvement of Ca^{2+} signaling in the sugar-inducible expression of genes coding for sporamin and β-amylase of sweet potato. Plant J. **7,** 297–307.

Poovaiah, B.W., and Reddy, A.S.N. (1993). Calcium and signal transduction in plants. Crit. Rev. Plant Sci. **12,** 185–211.

Preisig, C.L., and Moreau, R.A. (1994). Effects of potential signal transduction antagonists on phytoalexin accumulation in tobacco. Phytochemistry **36,** 857–863.

Raghavendra, A.S. (1990). Blue light effects on stomata are mediated by the guard cell plasma membrane redox system distinct from the proton translocating ATPase. Plant Cell Environ. **13,** 105–110.

Reiss, C., and Beale, S.I. (1995). External calcium requirements for light-induction of chlorophyll accumulation and its enhancement by red light and cytokinin pretreatments in excised etiolated cucumber cotyledons. Planta **196,** 635–641.

Renelt, A., Colling, C., Hahlbrock, K., Nürnberger, T., Parker, J.E., Sacks, W.R., and Scheel, D. (1993). Studies on elicitor recognition and signal transduction in plant defence. J. Exp. Bot. **44,** 257–268.

Roberts, D.M., and Harmon, A.C. (1992). Calcium-modulated proteins: Targets of intracellular calcium signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **43,** 375–414.

Romero, L.C., and Lam, E. (1993). Guanine nucleotide binding protein involvement in early steps of phytochrome-regulated gene expression. Proc. Natl. Acad. Sci. USA **90,** 1465–1469.

Romero, L.C., Sommer, D., Gotor, C., and Song, P.-S. (1991). G-proteins in *Avena* seedlings: Possible phytochrome regulation. FEBS Lett. **282,** 341–346.

Roux, S.J., Wayne, R.O., and Datta, N. (1986). Role of calcium ions in phytochrome responses: An update. Physiol. Plant. **66,** 344–348.

Russ, U., Grolig, F., and Wagner, G. (1991). Changes of cytoplasmic free Ca^{2+} in the green alga *Mougeotia scalaris* as monitored with indo-1, and their effect on the velocity of chloroplast movements. Planta **184,** 105–112.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory).

Sawbridge, T.I., López-Juez, E., Knight, M.R., and Jenkins, G.I. (1994). A blue-light photoreceptor mediates the fluence-rate-dependent expression of genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase in light-grown *Phaseolus vulgaris* primary leaves. Planta **192,** 1–8.

Schroeder, J.I., and Thuleau, P. (1991). Ca^{2+} channels in higher plant cells. Plant Cell **3,** 555–559.

Shacklock, P.S., Read, N.D., and Trewavas, A.J. (1992). Cytosolic free calcium mediates red light–induced photomorphogenesis. Nature **358,** 753–755.

Sheen, J. (1993). Protein phosphatase activity is required for light-inducible gene expression in maize. EMBO J. **12,** 3497–3505.

Shimazaki, K., Ino, M., and Zeiger, E. (1986). Blue light–dependent proton extrusion by guard-cell protoplasts of *Vicia faba.* Nature **319,** 324–326.

Shimazaki, K.-I., Kinoshita, T., and Nishimura, M. (1992). Involvement of calmodulin and calmodulin-dependent myosin light chain kinase in blue light–dependent H^+ pumping by guard cell protoplasts from *Vicia faba* L. Plant Physiol. **99,** 1416–1421.

Shimazaki, K.-I., Omasa, K., Kinoshita, T., and Nishimura, M. (1993). Properties of the signal transduction pathways in the blue light response of stomatal guard cells of *Vicia faba* and *Commelina benghalensis.* Plant Cell Physiol. **34,** 1321–1327.

Shinkle, J.R., and Jones, R.L. (1988). Inhibition of stem elongation in *Cucumis* seedlings by blue light requires calcium. Plant Physiol. **86,** 960–966.

Short, T.W., and Briggs, W.R. (1994). The transduction of blue light signals in higher plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **45,** 143–171.

Short, T.W., Porst, M., and Briggs, W.R. (1992). A photoreceptor system regulating *in vivo* and *in vitro* phosphorylation of pea plasma membrane protein. Photochem. Photobiol. **55,** 773–781.

Spalding, E.P., and Cosgrove, D.J. (1989). Large plasma-membrane depolarization precedes rapid blue-light-induced growth inhibition in cucumber. Planta **178,** 407–410.

Spalding, E.P., and Cosgrove, D.J. (1992). Mechanism of blue-light-induced plasma-membrane depolarisation in etiolated cucumber hypocotyls. Planta **188,** 199–205.

Suzuki, K., Fukuda, Y., and Shishi, H. (1995). Studies on elicitor-signal transduction leading to differential expression of defense genes in cultured tobacco cells. Plant Cell Physiol. **36,** 281–289.

Takeda, S., Mano, S., Ohto, M.-A., and Nakamura, K. (1994). Inhibitors of protein phosphatases 1 and 2A block the sugar-inducible gene expression in plants. Plant Physiol. **106,** 567–574.

Tester, M. (1990). Plant ion channels: Whole-cell and single-channel studies. New Phytol. **114,** 305–340.

Trezzini, G.F., Horrichs, A., and Somssich, I.E. (1993). Isolation of putative defense-related genes from *Arabidopsis thaliana* and expression in fungal elicitor-treated cells. Plant Mol. Biol. **21,** 385–389.

Warpeha, K.M.F., Hamm, H.E., Rasenick, M.M., and Kaufman, L.S. (1991). A blue-light-activated GTP-binding protein in the plasma membranes of etiolated peas. Proc. Natl. Acad. Sci. USA **88,** 8925–8929.

Weisshaar, B., Armstrong, G.A., Block, A., da Costa e Silva, O., and Hahlbrock, K. (1991). Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light-responsiveness. EMBO J. **10,** 1777–1786.