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Phytochrome-regulated repression of gene expression requires calcium and cGMP

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The plant photoreceptor phytochrome A utilizes three signal transduction pathways, dependent upon calcium and/or cGMP, to activate genes in the light. In this report, we have studied the phytochrome A regulation of a gene that is down-regulated by light, asparagine synthetase (*AS1*). We show that *AS1* is expressed in the dark and repressed in the light. Repression of *AS1* in the light is likely controlled by the same calcium/cGMP-dependent pathway that is used to activate other light responses. The use of the same signal transduction pathway for both activating and repressing different responses provides an interesting mechanism for phytochrome action. Using complementary loss- and gain-of-function experiments we have identified a 17 bp *cis*-element within the *AS1* promoter that is both necessary and sufficient for this regulation. This sequence is likely to be the target for a highly conserved phytochrome-generated repressor whose activity is regulated by both calcium and cGMP.

Keywords: calcium/cGMP/photomorphogenesis/phytochrome

Introduction

Light is perceived in plants by three major classes of photoreceptors: the phytochromes, the blue/UVA receptors (cryptochromes) and the UVB receptors (Quail *et al.*, 1995). Of these, the most intensively studied are the phytochromes, which exist in two photo-reversible forms: the red light absorbing form, Pr, generally considered to be physiologically inactive, and the far-red absorbing form, Pfr, known to mediate a broad range of plant responses to light (Quail *et al.*, 1995; Smith, 1995; von Arnim and Deng, 1996). Some responses mediated by Pfr can be reversed by far-red light, which converts Pfr back to Pr.

In higher plants, the phytochromes are encoded by multigene families (Quail *et al.*, 1995). Each phytochrome is thought to have a different physiological role and the recent availability of mutants deficient in individual phytochromes is allowing further definition of these specificities (reviewed in Millar *et al.*, 1994; Quail *et al.*, 1995; von Arnim and Deng, 1996). Some responses have now been linked to particular phytochromes, although there nonetheless appears to be some overlap between the functions of individual phytochromes within any given plant species (Reed *et al.*, 1994).

The different phytochromes make up two distinct classes, known as type I and type II (Quail *et al.*, 1995; Smith, 1995). Type I phytochromes are the most abundant in dark-grown plants, but they are light labile due to the rapid degradation and/or sequestration of the Pfr form in the light. In contrast, the type II phytochromes are present in much lower amounts, but their stability in the Pfr form ensures that they are predominant in light-grown plants. Hence, type I phytochrome is thought to play a specific role during the initial de-etiolation process, whereas type II may be more important for mediating phytochrome responses in mature plants. Phytochrome A (PHYA) is the only type I phytochrome to have been identified and it may in fact be the only molecular species within the type I pool (see Clack *et al.*, 1994). Like the PHYA apoprotein, *PHYA* mRNA abundance also decreases in the light (see Sharrock and Quail, 1989, and references therein), particularly in monocotyledons, where down-regulation of *PHYA* gene expression has been found to be mediated by an autoregulatory mechanism involving phytochrome itself (Lissemore and Quail, 1988).

In addition to *PHYA*, several other genes have been found to be down-regulated by light. These include genes encoding NADPH protochlorophyllide oxidoreductase (Mosinger *et al.*, 1985), β-tubulin (Colbert *et al.*, 1990; Tonoike *et al.*, 1994; Leu *et al.*, 1995), asparagine synthetase (AS1) (Tsai and Coruzzi, 1990, 1991), the homeodomain proteins Athb-2 and Athb-4 (Carabelli *et al.*, 1993) and two genes denoted *NPR1* and *NPR2* in *Lemna* (Okubara *et al.*, 1993). Phytochrome regulates these responses and two formal possibilities can be considered to account for how it does so (Bruce *et al.*, 1991): (i) Pfr generates a repressor in the light; (ii) Pr generates an activator in the dark. Current knowledge of phytochrome function would tend to favour Pfr repression as the most likely mechanism, because much evidence implicates Pfr, and not Pr, in controlling many other responses. However, it has proved extremely difficult to design physiological experiments that could definitively distinguish between the two possibilities.

In this report, we present the results of experiments that can discriminate between Pfr repression and Pr activation as possible mechanisms controlling the down-regulation of gene expression in the light. Specifically, we have

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studied the signal transduction events stimulated by PHYA to regulate expression of one of these negatively light regulated genes, *AS1*, using microinjection to deliver individual molecules into the cells of wild-type and *aurea* mutant tomato seedlings, as previously described (Neuhaus *et al*., 1993). PHYA is present in etiolated seedlings of the *aurea* mutant at 20% wild-type levels and is spectrally inactive, whereas PHYB (a type II phytochrome) is present and active at normal levels (Sharma *et al*., 1993). In contrast to the behaviour of wild-type seedlings, chloroplasts and anthocyanin pigments fail to develop within the hypocotyl cells of etiolated *aurea* seedlings in response to light. However, a wild-type phenotype can be restored to *aurea* hypocotyl cells by injection of exogenous PHYA (Neuhaus *et al*., 1993). This system therefore allows the manipulation and subsequent dissection of the signal transduction pathways used by PHYA by identifying agonists or antagonists of these responses. In this way, we have previously reported that the Pfr form of PHYA (PfrA) acts through heterotrimeric G proteins to stimulate gene expression that results in chloroplast development and anthocyanin biosynthesis (Neuhaus *et al*., 1993). Three different signal transduction pathways downstream of the G protein were subsequently identified that require cGMP and calcium (Bowler and Chua, 1994; Bowler *et al*., 1994a). cGMP can stimulate genes such as chalcone synthase (*CHS*) that are required for anthocyanin biosynthesis, whereas calcium and calcium-activated calmodulin (CaM) can stimulate other genes (e.g. chlorophyll a,b binding protein genes, *CAB*) necessary for partial chloroplast development. A third pathway, that requires both calcium and cGMP, is utilized to stimulate genes encoding the photosystem I (PSI) and cytochrome b_6f (cyt. b_6f) complexes (e.g. the gene encoding ferredoxin $NADP^+$ oxidoreductase, *FNR*). The combination of these three pathways therefore leads to full chloroplast development and anthocyanin biosynthesis.

Using similar experiments we wanted, specifically: (i) to address whether PfrA, PrA or both control *AS1* regulation, (ii) to determine whether *AS1* regulation requires calcium and/or cGMP or whether other signalling molecules are utilized and (iii) to identify specific *cis*-elements within the *AS1* promoter which are targets of PHYA regulation. Our results show that PfrA represses *AS1* expression in the light and that it does so via the calcium/cGMP-dependent pathway used to activate other responses, such as *FNR* gene expression. Hence, probably the same signal transduction pathway is used to simultaneously ‘turn on’ and ‘turn off’ different events. One *cis*-element within the *AS1* promoter, which in our assay system displays all the properties of the intact promoter, is highly homologous to the RE1 element within the oat *PHYA* gene, previously proposed to be a target for phytochrome autoregulation (Bruce *et al*., 1991).

Results

AS1–GUS is negatively regulated by PfrA

To examine the regulation of *AS1* by phytochrome, a plasmid containing 559 bp of the pea *AS1* promoter (Tsai, 1991) fused upstream of the gene encoding the reporter β-glucuronidase (*AS1–GUS)* was injected into subepidermal hypocotyl cells of 7- to 10-day dark-grown wild-type and *aurea* mutant tomato seedlings. For comparison, equivalent experiments were also performed with a *CAB–GUS* reporter gene (Neuhaus *et al*., 1993). Following injection (under green safelight conditions where necessary) the seedlings were exposed to different light irradiations. As we would predict from expression of the endogenous *AS1* and *CAB* genes, *AS1–GUS* was expressed in injected cells of wild-type seedlings maintained in the dark but not in the light, whereas *CAB–GUS* was only expressed in the light (Table I). Furthermore, expression of *AS1–GUS* in the dark could be down-regulated by a pulse of red light, but reactivated by 10 min of far-red irradiation subsequent to the red light pulse. In contrast, *CAB–GUS* expression could be stimulated in the dark by a pulse of red light and could be down-regulated by a far-red light pulse given immediately after the red light irradiation (Table I). These results thus demonstrate that in wild-type seedlings both *AS1–GUS* and *CAB–GUS* expression are regulated by phytochrome, but that this regulation acts in opposite ways, in one case down-regulating and in the other case up-regulating expression. Furthermore, the behaviour of the *AS1–GUS* gene in these injection experiments clearly reflects endogenous *AS1* expression in pea, which has been previously shown to be down-regulated at the level of transcription by white and red light (Tsai and Coruzzi, 1990, 1991).

In injected cells of *aurea* seedlings both reporter genes were insensitive to the light conditions: *AS1–GUS* was expressed both in the light and in the dark, whereas *CAB–GUS* was never expressed (Figure 1 and Table I). The lack of expression in *aurea* of *CAB–GUS*, even in the light or after a red light pulse, is consistent with its known requirement for Pfr, because, unlike in the wild-type, etiolated *aurea* seedlings are largely deficient in phytochrome (Sharma *et al*., 1993). Furthermore, the fact that in *aurea AS1–GUS* is expressed under all conditions implies that Pfr normally represses *AS1–GUS* expression in the light but that in phytochrome-deficient cells it is expressed constitutively.

We have previously found that injection of PHYA into hypocotyl cells of etiolated *aurea* seedlings in the light can restore chloroplast development and anthocyanin biosynthesis and can activate expression of *CAB–GUS*, *CHS–GUS* and *FNR–GUS* reporter genes (Figure 1 and Table II; Neuhaus *et al*., 1993; Bowler *et al*., 1994a). To determine whether PHYA could also regulate *AS1–GUS* expression, we co-injected *AS1–GUS* together with PHYA into *aurea* hypocotyl cells. We found that injection of PfrA (i.e. injection of PHYA in white light conditions) was able to down-regulate *AS1–GUS* expression in *aurea*, whereas injection of the Pr form (PrA) (i.e. injection of PHYA in green safelight conditions) could not (Figure 1 and Table II). Furthermore injection of PrA, followed by its conversion *in situ* to PfrA by a red light pulse could also inhibit expression. This down-regulation by red light could, however, be relieved by subsequent irradiation with far-red light (Table II). These results thus demonstrate that PHYA can control *AS1–GUS* expression and that it does so in an opposite way compared with *CAB–GUS*, *CHS–GUS* and *FNR–GUS* (Neuhaus *et al*., 1993; Bowler *et al*., 1994a).

Table I. Photoregulated expression of *AS1–GUS* and *CAB–GUS* in the wild-type and *aurea* mutant

Genotype	Reporter gene	Light conditions	No. injections	No. activations	Efficiency (%)
wt	*AS1–GUS*	D	132	12	9.0
wt	*AS1–GUS*	L	210	0	
wt	*AS1–GUS*	R	85	0	
wt	*AS1–GUS*	R/FR	89	9	10.1
wt	*CAB–GUS*	D	79	0	
wt	*CAB–GUS*	L	132	10	7.6
wt	*CAB–GUS*	R	55	6	10.9
wt	*CAB–GUS*	R/FR	57	0	
au	*AS1–GUS*	D	76	8	10.5
au	*AS1–GUS*	L	131	13	9.9
au	*AS1–GUS*	R	95	7	7.4
au	*AS1–GUS*	R/FR	71	8	11.3
au	*CAB–GUS*	D	68	0	
au	*CAB–GUS*	L	122	0	
au	*CAB–GUS*	R	62	0	
au	*CAB–GUS*	R/FR	64	0	

A summary of *AS1–GUS* and *CAB–GUS* expression in response to different light conditions in hypocotyl cells of etiolated wild-type (*wt*) and *aurea* (*au*) seedlings. Following injection of the reporter genes, the seedlings were either transferred to the dark (D) or to white light (L) for 48 h. Seedlings transferred to the dark were injected under green safelight conditions. For phytochrome photoreversibility experiments, seedlings injected under green safelight were irradiated with red light (R) or red light followed by far-red light (R/FR) prior to incubation in the dark for 48 h, as described in Materials and methods. The total number of injections is shown, together with the number of GUS-positive cells observed in each experiment. The efficiency of GUS activation is expressed as a percentage.

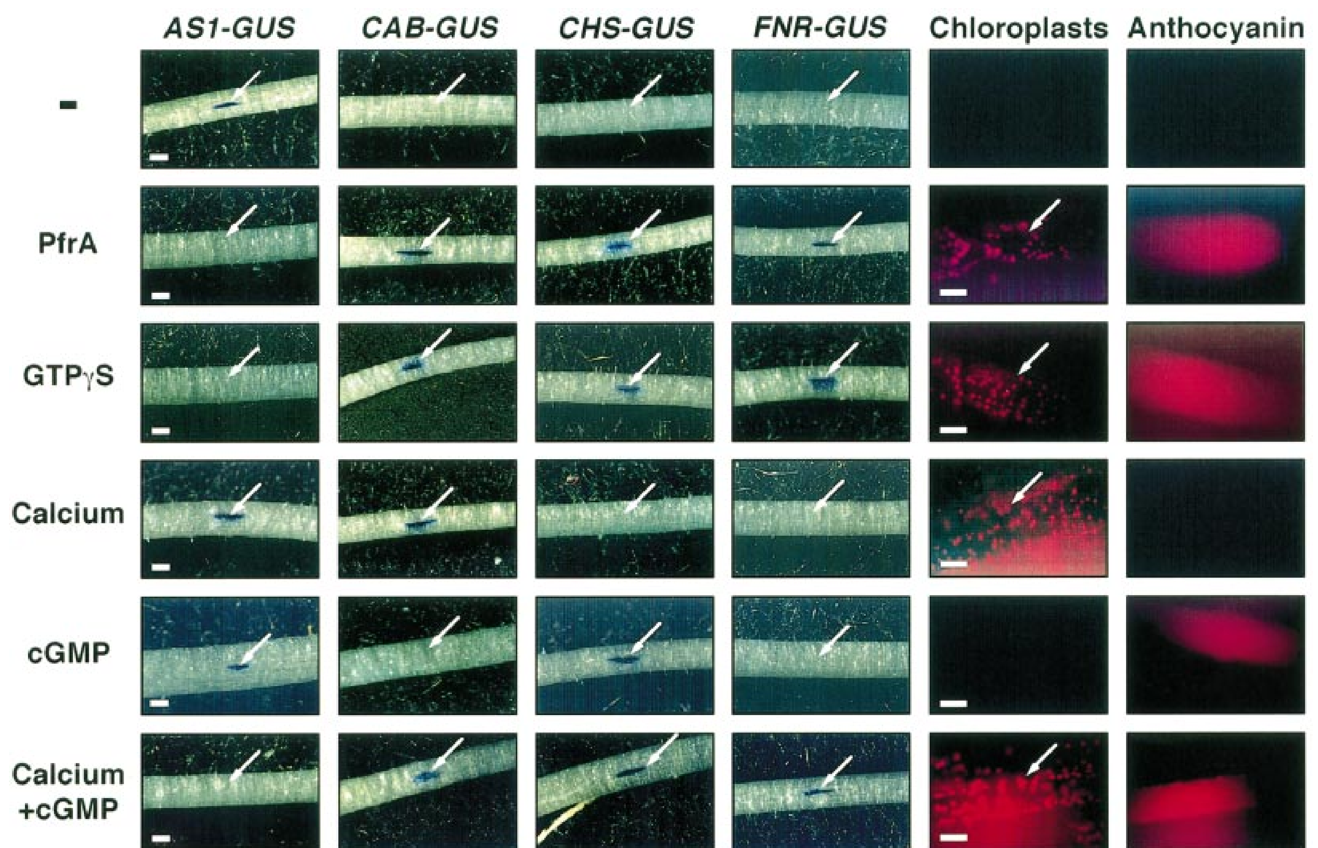

Fig. 1. Phenotypes of injected *aurea* hypocotyl cells after microinjection with signalling intermediates. The *AS1–GUS*, *CAB–GUS*, *CHS–GUS* and *FNR–GUS* panels show images of cells injected with the reporter genes alone (–) or co-injected with PfrA, GTPγS, calcium, cGMP or calcium plus cGMP. GUS activity was examined as previously described (Neuhaus *et al*., 1993) following incubation of injected material for 48 h in white light. Images of *CAB–GUS*, *CHS–GUS* and *FNR–GUS* expression patterns are derived from repetitions of previous experiments (Neuhaus *et al*., 1993; Bowler *et al*., 1994a). Actual experimental data are shown in Table II. The chloroplasts and anthocyanin panels show representative images of chlorophyll and anthocyanin fluorescence (visualized as described; Neuhaus *et al*., 1993; Bowler *et al*., 1994a) observed prior to GUS staining in cells injected with the different signalling intermediates. Chloroplasts generated by PfrA, GTPγS and calcium plus cGMP contain all the photosynthetic machinery, whereas those generated by calcium lack cyt. b_6f and PSI (Neuhaus *et al*., 1993; Bowler *et al*., 1994a). All images were taken from hand cut sections made through the injected regions of hypocotyls and are derived from independent injections into different seedlings. Approximate intracellular concentrations: PfrA, 20 000 molecules; GTPγS, 50 μM; calcium, 2 μM; cGMP, 50 μM. Arrows indicate the injected cells. Scale bars in bright field micrographs represent 500 μm, those in fluorescent micrographs 10 μm.

Table II. Down-regulation of *AS1–GUS* expression in the *aurea* mutant by PfrA and its signalling intermediates

Co-injected material	Light conditions	Efficiency (%) (No. activations/No. injections)				
		AS1–GUS	*CAB–GUS*	*CHS–GUS*	*FNR–GUS*	C and A
	D	9.2 (12/130)	n.d.	n.d.	n.d.	
	L	10.0 (37/372)	(0/110)	(0/113)	(0/123)	
PHYA (20 000)	D	7.8 (10/128)	n.d.	n.d.	n.d.	
PHYA (20 000)	L	(0/251)	10.8 (11/102)	9.9 (10/101)	8.8 (9/102)	C and A
PHYA (20 000)	R	(0/135)	n.d.	n.d.	n.d.	
PHYA (20 000)	R/FR	6.2 (8/129)	n.d.	n.d.	n.d.	
GTPγS (50 μM)	D	(0/85)	n.d.	n.d.	n.d.	
GTPγS (50 μM)	L	(0/300)	12.4 (13/105)	13.0 (15/115)	11.8 (13/110)	C and A
GTPγS (50 μM)	R	(0/72)	n.d.	n.d.	n.d.	
GTPγS (50 μM)	R/FR	(0/59)	n.d.	n.d.	n.d.	
CTX (5000) + GTPγS (1 μM)	L	(0/129)	n.d.	n.d.	n.d.	C and A
Ca^{2+} (2 μM)	L	9.3 (20/215)	13.2 (14/106)	(0/110)	(0/117)	C
CaM (10 000)	L	11.5 (15/131)	n.d.	n.d.	n.d.	C
cGMP (50 μM)	L	9.4 (23/244)	(0/113)	13.7 (16/117)	(0/109)	A
Ca^{2+} (2 μM) + cGMP (50 μM)	L	(0/296)	8.3 (9/109)	10.0 (11/110)	8.9 (10/113)	C and A
CaM (10 000) + cGMP (50 μM)	L	(0/176)	n.d.	n.d.	n.d.	C and A
CaM (3000) + cGMP (110 μM)	L	10.1 (15/148)	n.d.	n.d.	n.d.	A
CaM (100 000) + cGMP (50 μM)	L	(0/152)	n.d.	n.d.	n.d.	C
CaM (10 000) + cGMP (3.5 μM)	L	(0/152)	n.d.	n.d.	n.d.	C

PHYA and other compounds were co-injected with *AS1–GUS*, *CAB–GUS*, *CHS–GUS* or *FNR–GUS* into *aurea* hypocotyl cells at the concentrations given (expressed as estimated final intracellular concentrations in number of molecules, unless stated otherwise) as described (Neuhaus *et al*., 1993; Bowler *et al*., 1994a,b). Efficiency of GUS activation (expressed as %) following different treatments is shown, together with actual experimental data in parantheses (showing the total number of injections and the number of activations). GUS activity was examined 48 h post-injection. Phytochrome photoreversibility experiments were performed as described in the Table I legend and in Materials and methods. For white light experiments, PHYA injections were carried out in white light, whereas for dark, red and far-red experiments, injections were performed under green safelight conditions. Hence, in the former experiments, PHYA was in the PfrA form, while in the latter it was injected in the PrA form. Calmodulin was activated by calcium (CaM) as previously (Neuhaus *et al*., 1993). A subset of injected cells were examined for chlorophyll (C) and anthocyanin (A) fluorescence in order to confirm previous results (see Figure 1) (Neuhaus *et al*., 1993; Bowler *et al*., 1994a,b). In injected cells kept in the dark and in cells treated with red and/or far-red light pulses, no fluorescence was observed (Neuhaus *et al*., 1993). n.d., not done.

Down-regulation of AS1–GUS by PfrA requires calcium and cGMP

Previous microinjection experiments in *aurea*, together with pharmacological studies in soybean SB-P cells, have led to the identification of three major signal transduction pathways used by PfrA to control chloroplast development and anthocyanin biosynthesis (Neuhaus *et al*., 1993; Bowler and Chua, 1994; Bowler *et al*., 1994a,b). It was, therefore, of interest to determine whether these pathways are not only used for activation of these responses but also for down-regulation of other responses, e.g. negative regulation of *AS1* expression. To test this, we co-injected a range of previously characterized molecules known to stimulate various PfrA responses. Activation of heterotrimeric G proteins, by injection of GTPγS and cholera toxin (CTX), has been shown to stimulate full chloroplast development and anthocyanin biosynthesis in *aurea* hypocotyl cells (Neuhaus *et al*., 1993) and to activate the reporter genes *CAB–GUS*, *FNR–GUS* and *CHS–GUS* (Figure 1 and Table II; Bowler *et al*., 1994a). In contrast, co-injection of GTPγS and CTX with *AS1-GUS* in *aurea* led to down-regulation of *AS1–GUS* and, unlike with PfrA, this response was now unaffected by the light conditions (Figure 1 and Table II). Hence, the response was now light-independent, i.e. it had been uncoupled from the normal stimulus. These data therefore demonstrate that, as for *CAB–GUS*, *FNR–GUS* and *CHS–GUS* activation (Neuhaus *et al*., 1993; Bowler *et al*., 1994a), the PfrA-mediated down-regulation of *AS1–GUS* requires G protein activation and also reveal that there are no light-requiring steps downstream of G protein activation for *AS1–GUS* down-regulation. This has also been shown for *CAB–GUS* activation, indicating that the only light-dependent step between PfrA and nuclear gene regulation is likely to be photoreceptor activation (Neuhaus *et al*., 1993).

Injection of calcium and activated calmodulin (CaM) have been found to stimulate *CAB–GUS* expression and partial chloroplast development in etiolated *aurea* hypocotyl cells (Figure 1 and Table II; Neuhaus *et al*., 1993). Conversely, injection of cGMP can stimulate *CHS–GUS* expression and anthocyanin biosynthesis (Figure 1 and Table II; Bowler *et al*., 1994a). These molecules therefore control distinct subsets of PfrA responses and act downstream of G protein activation (Neuhaus *et al*., 1993; Bowler *et al*., 1994a). To determine if these previously characterized PfrA signalling intermediates also regulate *AS1* expression, they were co-injected with *AS1–GUS* into *aurea*. Interestingly, neither calcium, activated CaM nor cGMP alone (at concentrations previously found to be effective, 2 μM, 10 000 molecules, and 50 μM, respectively, estimated final intracellular concentrations; Neuhaus *et al*., 1993; Bowler *et al*., 1994a,b) could down-regulate *AS1–GUS* expression in the light in *aurea* cells (Figure 1 and Table II). However, a combination of calcium or activated CaM together with cGMP was able to effectively block *AS1–GUS* expression (Figure 1 and Table II), suggesting that the down-regulation of *AS1–GUS* by PfrA is controlled by the same signalling molecules that it uses to activate other responses. Specifically, it appeared that *AS1–GUS* down-regulation may be controlled via the same calcium/cGMP-dependent pathway we have found to activate expression of genes encoding PSI and cyt. b_6f

Table III. Effects of PfrA signal transduction inhibitors on reporter gene expression in wild-type cells

Inhibitor	Efficiency (%) (No. activations/No. injections)			
	AS1–GUS	*CAB–GUS*	*CHS–GUS*	*FNR–GUS*
	(0/132)	9.1 (10/110)	9.8 (9/92)	13.5 (12/89)
Genistein	(0/139)	14.0 (17/121)	(0/92)	18.8 (13/69)
Trifluoperazine	13.2 (16/121)	(0/83)	18.7 (14/75)	(0/122)
Staurosporine	14.4 (18/125)	(0/92)	24.7 (20/81)	(0/119)

Efficiency of GUS activation (expressed as %) following different treatments is shown, together with actual experimental data in parentheses (showing the total number of injections and the number of activations). Etiolated wild-type seedlings were injected and subsequently incubated for 48 h in white light. Treatment with inhibitors was as described (Bowler *et al.*, 1994b). Concentrations of inhibitors: genistein, 100 μM; trifluoperazine, 200 μM; staurosporine, 60 nM.

components, such as *FNR* (Figure 1 and Table II; Bowler *et al.*, 1994a).

PfrA signal transduction pathways have been found to be subject to cross-talk regulation, which has been termed reciprocal control (Bowler *et al.*, 1994b). For example, activity of the calcium/cGMP-dependent pathway has been found to be inhibited by high concentrations of cGMP, but not activated CaM, and to be able to function with significantly lower amounts of cGMP (at least 6-fold) than does the cGMP-dependent pathway. To examine whether regulation of *AS1–GUS* expression was also modulated by these phenomena, we co-injected different concentrations of activated CaM and cGMP. Indeed, high concentrations of cGMP (110 μM) injected with activated CaM (3000 molecules) were no longer effective in down-regulating *AS1–GUS*, whereas, in the presence of high concentrations of activated CaM (100 000 molecules) and normal amounts of cGMP (50 μM), down-regulation was still observed, as it was when co-injecting low levels of cGMP (3.5 μM) with activated CaM (10 000 molecules) (Table II). Again, these results indicated that *AS1–GUS* down-regulation by PHYA was likely mediated by the same calcium/cGMP-dependent pathway that has been previously characterized as activating other responses (Bowler *et al.*, 1994b).

It was interesting to observe that in these experiments with PfrA signalling intermediates, phenotypes characteristic of both dark- and light-exposed material were manifested concurrently in the same cell, e.g. although injection of calcium or activated CaM alone in the light resulted in *CAB–GUS* activation and biogenesis of partially developed chloroplasts and injection of cGMP alone resulted in *CHS–GUS* activation and anthocyanin pigment biosynthesis, in both cases these cells could not down-regulate *AS1–GUS* (Figure 1 and Table II).

As further evidence that *AS1–GUS* down-regulation was mediated by the previously characterized calcium/cGMP-dependent pathway, we tested the effect on *AS1–GUS* expression of previously characterized pharmacological agents. Genistein (an inhibitor of tyrosine and histidine protein kinases; Huang *et al.*, 1992) is known to inhibit the cGMP-dependent pathway, whereas trifluoperazine (a calmodulin antagonist; Massom *et al.*, 1990) and staurosporine (a non-specific protein kinase inhibitor; Ruegg and Burgess, 1989) both inhibit the two calcium-dependent pathways (Bowler *et al.*, 1994b). For these experiments, we injected dark-grown wild-type seedlings and then incubated them in the light in the presence of these different compounds. For comparison, we also examined the expression of *CAB–GUS*, *CHS–GUS* and *FNR–GUS* under the same conditions. As predicted from previous experiments in *aurea* (Bowler *et al.*, 1994b), *CAB–GUS*, *CHS–GUS* and *FNR–GUS* were expressed in the light in these wild-type seedlings (Table III). Furthermore, as already observed in *aurea*, *CHS–GUS* expression was inhibited by genistein, whereas *CAB–GUS* and *FNR–GUS* expression were inhibited by trifluoperazine and staurosporine (Table III). These results reveal the consistency of data obtained from *aurea* and wild-type seedlings.

AS1–GUS, however, was not expressed in the light, as previously observed (Table I), and this down-regulation by light was found to be sensitive to trifluoperazine and staurosporine, but not to genistein (Table III). Based on these data, together with that presented in Table II, it is therefore highly likely that the same signal transduction pathway (i.e. the calcium/cGMP-dependent pathway) is used by PfrA to control both up-regulation of some genes (e.g. *FNR–GUS*) and down-regulation of others (e.g. *AS1–GUS*).

The target of calcium and cGMP regulation within the AS1 promoter

The above data imply that there is a target(s) within the *AS1* promoter for PfrA-mediated down-regulation by calcium and cGMP. Most simply, PfrA may act via calcium and cGMP to activate a repressor that binds to such a sequence. To date, the best characterized *cis*-acting element found to be important for phytochrome-mediated down-regulation is RE1, an 11 bp GC-rich sequence centered at $^{-}75$ bp within the oat *PHYA* promoter (Bruce *et al.*, 1991). When the RE1 sequence is mutated by linker scanning mutagenesis, this promoter retains maximal expression following a far-red light pulse but is no longer down-regulated by a red light pulse (Bruce *et al.*, 1991). Interestingly, the RE1 core sequence, TGGG, is present within other *PHYA* promoters and can also be found in the promoters of all genes so far characterized as being down-regulated by light (Figure 2). Examination of the *AS1* promoter sequence revealed the presence of two such sequences, albeit on the opposite DNA strand with respect to monocotyledon *PHYA* promoters, showing significant homology with the RE1 core sequence, one centered at $^{-}43$ and the other centered at $^{-}160$ (Figure 2). Thus, it appeared possible that these elements may be the targets for PfrA-mediated repression within the *AS1* promoter. To

determine whether these sequences were required for down-regulation of *AS1–GUS* by light, we performed competition experiments using a tetramer of the most proximal RE1-related element within the *AS1* promoter (denoted RE3, centered at $^{-}43$) (Figure 2). Similar competition experiments have recently been performed in tobacco cotyledon cells to study regulation of the cauliflower mosaic virus (CaMV) $^{-}90$ 35S promoter (Neuhaus *et al.*, 1994).

Co-injection of *AS1–GUS* (5000 molecules) with a plasmid containing the RE3 tetramer sequence into wild-type cells indeed resulted in inhibition of the down-regulation of *AS1–GUS* normally observed in the light (Table IV). A 4-fold molar excess of the competitor (i.e. 5000 molecules) was sufficient to cause this effect, although higher concentrations were more effective (Table IV). In contrast, a tetramer of an RE3 element containing a mutated core sequence ($RE3_m$) (Figure 2) was not able to inhibit down-regulation by light, even when injected at an 80-fold molar excess (i.e. 100 000 molecules) per cell (Table IV). The sensitivity of *AS1–GUS* down-regulation to competition specifically by the RE3 tetramer therefore strongly implies that a light-activated repressor indeed interacts with the RE3 element and that its removal (by competition) results in release of repression of the *AS1* promoter in light. Conversely, we have found that introduction of a large excess of *AS1–GUS* molecules by microprojectile bombardment also results in de-regulated expression (data not shown), suggesting again that a repressor is being titrated out. When co-injected into *aurea* cells, neither RE3 nor $RE3_m$ had any effect on *AS1–GUS* expression, i.e. the reporter gene was always expressed (Table IV). This is consistent with the notion that in *aurea* this repressor is either not present or not active, due to the phytochrome deficiency in mutant seedlings.

To relate the activity of the repressor to PfrA and to the PfrA signalling intermediates, we performed experiments in *aurea* co-injecting *AS1–GUS* with RE3 or $RE3_m$, together with various signalling intermediates. Normal repression of *AS1–GUS* by PfrA co-injection into *aurea* cells in light could indeed be inhibited in the presence of sufficient amounts of the RE3 tetramer (an 8-fold molar excess), although $RE3_m$ was not able to inhibit *AS1–GUS* repression by PfrA (even at an 80-fold molar excess) (Table V). Hence, we can conclude that down-regulation of *AS1–GUS* via RE3 in light is due to PfrA repression rather than PrA activation. Furthermore, repression of *AS1–GUS* expression by co-injection of calcium and cGMP could be similarly competed by the RE3 tetramer, but not by $RE3_m$ (Table V). These data thus indicate that the RE3 element within the *AS1* promoter is necessary for PfrA-mediated repression of *AS1–GUS* and requires either calcium, cGMP or both.

		↓
Oat *PHYA*	(-75)	C A **T G G G** C G C G G
Rice *PHYA*	(-132)	G A **T G G G** G G A A G
Maize *PHYA*	(-141)	C A **T G G G** C A C C G
Pea *PHYA*	(-48 rev.)	A G **T G G G** A G A C C
Arabidopsis *PHYA*	(-67 rev.)	A A **T G G G** A C C A C
Pea *AS1*	(-43 rev.)	G G **T G G G** A G C T A
Pea *AS1*	(-160 rev.)	T A **T G G G** A T G T T
RE3		G A T C T G G **T G G G** A G C T A G
$RE3_m$		G A T C T G G A C C G A G C T A G

Fig. 2. RE1-related elements within *PHYA* promoters and the pea *AS1* promoter. Sequences were derived from the following sources: oat *PHYA*, Hershey *et al*., 1985; rice *PHYA*, Kay *et al*., 1989; maize *PHYA*, Christensen and Quail, 1989; pea *PHYA*, Sato, 1988; *Arabidopsis PHYA*, Dehesh *et al*., 1994; pea *AS1*, Tsai, 1991. The conserved core sequence TGGG is shown in bold and is underlined. The central nucleotide within the sequences (from which the numbering is based) is indicated by an arrow. The TGGG sequence can also be found within the promoters of other light down-regulated genes: *Lemna NPR1* and *NPR2* (Okubara *et al*., 1993) and soybean *tubB1* and *tubB2* (Guiltinan *et al*., 1987; Tonoike *et al*., 1994). The sequence of the RE3 element used in competition experiments is based on the RE1-related element within the pea *AS1* promoter centered at $^{-}43$. The control oligonucleotide sequence $RE3_m$ contains a mutated core sequence. For more information see Materials and methods.

The RE3 repressor is a target for calcium and cGMP

The experiments described above do not demonstrate unequivocally that RE3 is a negative element regulated

Table IV. Summary of competition experiments in wild-type and *aurea* hypocotyl cells

Genotype	Target (conc.)	Competitor (conc.)	No. injections	No. activations	Efficiency (%)
wt	*AS1–GUS* (5000)		145	0	
wt	*AS1–GUS* (10 000)		150	0	
wt	*AS1–GUS* (5000)	RE3 (1000)	123	0	
wt	*AS1–GUS* (5000)	RE3 (5000)	145	7	4.8
wt	*AS1–GUS* (5000)	RE3 (10 000)	130	12	9.2
wt	*AS1–GUS* (5000)	RE3 (100 000)	130	20	15.4
wt	*AS1–GUS* (5000)	RE3 m (1000)	115	0	
wt	*AS1–GUS* (5000)	RE3 m (10 000)	149	0	
wt	*AS1–GUS* (5000)	RE3 m (100 000)	140	0	
au	*AS1–GUS* (5000)		161	17	10.6
au	*AS1–GUS* (5000)	RE3 (1000)	109	11	10.1
au	*AS1–GUS* (5000)	RE3 (10 000)	121	14	11.6
au	*AS1–GUS* (5000)	RE3 (100 000)	120	13	10.8
au	*AS1–GUS* (5000)	RE3 m (10 000)	125	14	11.2
au	*AS1–GUS* (5000)	RE3 m (100 000)	119	13	10.9

Injected etiolated seedlings were maintained in white light for 48 h prior to analysis of GUS activity. Concentrations of target and competitor DNA injected into cells is shown in number of molecules as estimated final intracellular concentration. For further details see Materials and methods.

Table V. Competition experiments in *aurea* cells using PfrA and signalling intermediates

Co-injected material	Competitor (conc.)	No. injections	No. activations	Efficiency (%)	C and A
–	–	85	11	12.9	–
PHYA (20 000)		125	0		C and A
PHYA (20 000)	RE3 (1000)	124	0		C and A
PHYA (20 000)	RE3 (10 000)	126	9	7.1	C and A
PHYA (20 000)	RE3 (100 000)	119	16	13.4	C and A
PHYA (20 000)	$RE3_m$ (10 000)	132	0		C and A
PHYA (20 000)	$RE3_m$ (100 000)	133	0		C and A
CaM (10 000) + cGMP (50 μM)		129	0		C and A
CaM (10 000) + cGMP (50 μM)	RE3 (1000)	121	1	0.8	C and A
CaM (10 000) + cGMP (50 μM)	RE3 (10 000)	124	10	8.1	C and A
CaM (10 000) + cGMP (50 μM)	RE3 (100 000)	131	15	11.5	C and A
CaM (10 000) + cGMP (50 μM)	$RE3_m$ (10 000)	132	0		C and A
CaM (10 000) + cGMP (50 μM)	$RE3_m$ (100 000)	131	0		C and A

PHYA and signalling intermediates were co-injected with *AS1–GUS* (5000 molecules) and competitor plasmids in white light into hypocotyl cells of etiolated *aurea* seedlings. Competitor plasmids contain tetramers of either the RE3 or $RE3_m$ sequences (see Materials and methods). GUS activity was examined after 48 h white light incubation. Concentrations are in number of molecules unless stated otherwise. Abbreviations are as used in previous tables. As in the experiments for Table II, a subset of injected cells were examined for chlorophyll (C) and anthocyanin (A) fluorescence, to confirm previous observations (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994a).

Table VI. Expression of *35S–RE3–GUS* and *35S–RE3$_m$–GUS* in wild-type and *aurea* hypocotyl cells

Genotype	Signaling compound	Construct	Light conditions	No. injections	No. activations	Efficiency (%)
wt		*35S–RE3–GUS*	D	85	9	10.6
wt		*35S–RE3–GUS*	L	151	0	
wt		*35S–RE3$_m$–GUS*	D	85	8	9.4
wt		*35S–RE3$_m$–GUS*	L	149	18	12.1
au		*35S–RE3–GUS*	D	80	7	8.8
au		*35S–RE3–GUS*	L	112	15	13.4
au		*35S–RE3$_m$–GUS*	D	85	8	9.4
au		*35S–RE3$_m$–GUS*	L	129	17	13.2
au	PHYA (20 000)	*35S–RE3–GUS*	D	86	7	8.1
au	PHYA (20 000)	*35S–RE3–GUS*	L	141	0	
au	PHYA (20 000)	*35S–RE3$_m$–GUS*	D	89	8	9.0
au	PHYA (20 000)	*35S–RE3$_m$–GUS*	L	139	16	11.5
au	Ca^{2+} (2 μM)	*35S–RE3–GUS*	L	125	20	16.0
au	CaM (10 000)	*35S–RE3–GUS*	L	121	19	15.7
au	cGMP (50 μM)	*35S–RE3–GUS*	L	131	19	14.5
au	CaM (10 000) + cGMP (50 μM)	*35S–RE3–GUS*	L	145	0	
au	CaM (10 000) + cGMP (50 μM)	*35S–RE3$_m$–GUS*	L	109	18	16.5

Etiolated seedlings maintained in the dark (D) after injection were initially injected under green safelight conditions, while those incubated in white light (L) subsequent to injection were injected under normal white light conditions. GUS activity was analyzed 48 h post-injection. *GUS* reporter gene constructs were injected at estimated final intracellular concentrations of 10 000 molecules. Abbreviations are as used in previous tables.

by both calcium and cGMP. It is possible, for example, that RE3 is a target for only one of the PfrA signalling intermediates and that repression of *AS1–GUS* by calcium and cGMP is mediated by interactions between the RE3 binding factor and other DNA binding proteins recognizing different *cis*-elements within the *AS1* promoter. To test the role of the RE3 element more precisely, we inserted the RE3 tetramer between the *35S* B domain (−343 to −90) and the minimal −46 *35S* TATA box, which is normally constitutively expressed in both light and dark (Lam and Chua, 1990). The artificial promoter was placed upstream of *GUS* (*35S–RE3–GUS*). When injected into wild-type cells, we found that expression of this reporter gene was now repressed in light, in the same way as was *AS1–GUS* (Table VI). Furthermore, consistent with the behaviour of *AS1–GUS*, *35S–RE3–GUS* was not repressed in injected *aurea* cells by light (Table VI). The $RE3_m$ tetramer, however, could not confer light repression on the reporter gene in either wild-type or *aurea* cells. Clearly then, the RE3 element is both necessary and sufficient to mediate light repression and can function in a heterologous context.

We tested whether RE3 itself was a target for PfrA, and for calcium and cGMP, by injecting *35S–RE3–GUS* into *aurea* cells together with these signalling intermediates. Consistent with the above data, co-injection with PfrA in light resulted in repression of *GUS* expression from *35S–RE3–GUS* but not from *35S–RE3$_m$–GUS*, whereas injection of PrA (performed under a green safelight) and subsequent incubation of seedlings in darkness did not result in *35S–RE3–GUS* or *35S–RE3$_m$–GUS* repression (Table VI). Furthermore, although injection of neither calcium, activated CaM nor cGMP alone had any repressive effect, as with *AS1–GUS*, a combination of activated CaM with cGMP resulted in repression of *35S–RE3–GUS*. This was not observed with *35S–RE3$_m$–GUS* (Table VI). These experiments therefore demonstrate that RE3 is a

target for both calcium and cGMP and that, at least in these experiments, it can mediate light repression in an identical manner to the intact *AS1* promoter.

Discussion

The work presented in this manuscript is a continuation of our use of the tomato *aurea* mutant for dissection of PHYA signal transduction. We have previously used this mutant to identify positively acting signalling intermediates controlling PfrA-activated chloroplast development and anthocyanin biosynthesis (Neuhaus *et al*., 1993; Bowler *et al*., 1994a). Unfortunately, several inadvertant mistakes in data presentation were made in these articles [see Erratum, *Cell*, 1994, **79**(4)]. Although these mistakes did not affect the conclusions of our experiments, we have nonetheless repeated key experiments relevant to the essential features of the scheme. Our new experiments confirming the identity of the three signalling pathways controlling *CAB*, *CHS* and *FNR* gene expression are shown in Figure 1 and Table II. Additionally, a combination of microinjection experiments in *aurea* and physiological analyses in SB-P cultures has allowed us to begin to understand cross-talk phenomena acting between different PfrA signal transduction pathways (e.g. reciprocal control) (Bowler *et al*., 1994b) and other experiments have revealed that distinct phytochrome-responsive *cis*-elements are controlled by calcium and cGMP (Wu *et al*., 1996).

The phenotype of the *aurea* mutant is rather complex. Although likely to be a mutation affecting chromophore biosynthesis (Terry and Kendrick, 1996), biochemical and physiological experiments have clearly indicated that the mutation affects primarily PHYA, i.e. functional PHYA is absent in dark-grown seedlings and the mutant displays type II phytochrome-regulated end of day far-red responses (van Tuinen *et al*., 1996). However, the *aurea* phenotype is not wholly consistent with that of *Arabidopsis phyA* null mutants (Whitelam and Harberd, 1994) nor with recently isolated tomato *phyA* mutants (van Tuinen *et al*., 1995). We nonetheless believe that the signalling pathways elucidated in this and previous articles are controlled by PHYA, because injection of PHYA should rescue only PHYA-mediated events. This is supported by recent observations that injection into *aurea* of recombinant reconstituted PHYA produces identical responses, whereas equivalent concentrations of PHYB do not (Kunkel *et al*., 1996).

In the present report, we have performed a series of microinjection experiments to elucidate how PHYA down-regulates expression of certain nuclear genes in light. In particular, we have used the promoter of the pea *AS1* gene as a target. Tsai and Coruzzi (1990) have previously shown that this gene is highly expressed in the dark but rapidly down-regulated in light. Moreover, down-regulation is mediated by phytochrome primarily at the transcriptional level (Tsai and Coruzzi, 1991). In agreement with this data, our current results show that an *AS1–GUS* chimeric gene is down-regulated by phytochrome in wild-type tomato cells, whereas in the *aurea* mutant it is expressed constitutively, regardless of the irradiation conditions (Figure 1 and Table I). Furthermore, by restoring negative light regulation of the *AS1–GUS* gene in *aurea* by co-injection with PfrA, we have been able to show that PHYA can specifically mediate this expression pattern (Figure 1 and Table II).

The down-regulation of *AS1–GUS* by PfrA requires G proteins, calcium and cGMP (Figure 1 and Table II), previously characterized as signalling intermediates for PfrA-mediated activation of anthocyanin biosynthesis and chloroplast development (Bowler *et al*., 1994a). By all known criteria (Bowler *et al*., 1994b), this down-regulation appears to be controlled by the same signal transduction pathway that is used to activate the *FNR* promoter: down-regulation is blocked by high concentrations of cGMP but not CaM, it can be inhibited by trifluoperazine and staurosporine but not by genistein and it requires only low concentrations of cGMP (Tables II and III). Hence, PfrA appears to use the same signal transduction pathway to both activate (e.g. *FNR*) and down-regulate (e.g. *AS1*) different genes. This implies that there are different oppositely acting targets for the same PfrA signal transduction pathway, an efficient and mechanistically simple means for concurrently activating and repressing different responses. The identification of other phytochrome responses that are oppositely regulated by a single PfrA signalling pathway will allow a better assessment of the physiological importance of this novel regulatory mechanism.

It has been proposed that expression of genes that are down-regulated by light, such as *AS1*, may be modulated by phytochrome either by Pfr repression in light or by Pr activation in the dark (Bruce *et al*., 1991). Although current knowledge would tend to favour the former mechanism, the lack of experimental tools has made it impossible to distinguish definitively between these two possibilities. Concerning the regulation of *AS1* by phytochrome, our current experiments have demonstrated: (i) that in wild-type cells *AS1–GUS* can be down-regulated by red light whereas in *aurea* it cannot (Table I); (ii) that co-injection of PfrA in *aurea* can prevent *AS1–GUS* expression in light (Table II). This information would suggest that PfrA is the mediator of *AS1* down-regulation in light and that in its absence *AS1–GUS* is expressed regardless of whether PrA is present or not. However, as with previous data, these experiments do not prove that PfrA, and not PrA, is the active molecule. More definitive experiments, however, have shown: (i) that it is possible to prevent PfrA-mediated down-regulation of *AS1* by co-injection of a specific tetramer sequence corresponding to a putative *cis*-element within the *AS1* promoter (Table IV); (ii) that this sequence by itself is sufficient to confer PfrA-mediated down-regulation on a heterologous constitutively active *35S* promoter (Table VI). These observations therefore provide compelling evidence that PfrA is the mediator of *AS1* down-regulation in light and that it functions by activating a putative repressor that binds to this *cis*-element.

The 17 bp *cis*-element, denoted RE3, that we have identified as the binding site of the putative repressor, is centered at $^{-}43$ and contains the TGGG core motif that is present within the promoters of all other genes so far characterized as being down-regulated by light (Figure 2). Another similar sequence is centered at $^{-}160$ bp (although its activity has not currently been tested). The importance of *cis*-elements containing the TGGG core motif was initially inferred from studies with the oat *PHYA* promoter.

In this latter case, linker scanning mutagenesis indicated that an 11 bp sequence containing a TGGG motif, denoted RE1, was a target for Pfr-mediated negative regulation (Bruce *et al*., 1991). In loss-of-function experiments, we have corroborated this data by showing that a co-injected RE3 tetramer can prevent down-regulation of *AS1–GUS* mediated by light in wild-type cells (Table IV) and by PfrA and CaM and cGMP in *aurea* cells (Table V), although a tetramer containing a mutated core sequence ($RE3_m$) is ineffective. The simplest interpretation of these results is that the RE3 tetramer is able to compete away a repressor that binds this sequence within the *AS1* promoter. If this is the case, a constitutively active activator/enhancer must also interact with the *AS1* promoter. Consequently, the *AS1* promoter would be constitutively active in the dark, due to the absence of active repressor, whereas activation of the repressor by PfrA in light would block activity of this positive element and hence inhibit expression. Such a mechanism has also been proposed for autoregulation of oat *PHYA* (Bruce *et al*., 1991).

The most convincing evidence that this sequence binds a PfrA-generated repressor was derived from gain-of-function experiments: when placed within the constitutive *35S* promoter (between the B domain and the $^{-}46$ TATA box) the RE3 tetramer was sufficient to confer light repression in wild-type cells and PfrA-mediated repression in *aurea* cells (Table VI). Again, the $RE3_m$ tetramer was ineffective. Furthermore, both calcium (or CaM) and cGMP were required to reproduce the repression mediated by PfrA in *aurea* (Table VI), indicating that the putative repressor that binds to RE3 requires both signalling molecules for activation.

The homology between RE1 and RE3, at both the structural and functional levels, would strongly suggest that they are binding sites for the same (or at least a highly related) repressor, even though the RE1 and RE3 *cis*-elements are present on opposite DNA strands within their respective promoters. It has been proposed that RE1 binds a critical repressor that acts as the molecular switch controlling expression of oat *PHYA* (Bruce *et al*., 1991). Our current results support this view and indicate, in addition, that the activity of this repressor is not limited to *PHYA* regulation but is also utilized in inactivating other genes that are down-regulated by light. Indeed, we have found that the RE3 sequence is both necessary and sufficient to mediate this expression pattern. This information, together with the fact that RE sequences are present within the promoters of all light down-regulated genes, infers that the repressor is well conserved and that it may be critical for inactivating expression of such genes in light. The isolation and characterization of this factor or complex will clearly be important for elucidation of the light-mediated repression mechanism.

Although we have found that the RE3 repressor requires both calcium and cGMP for activation (Table VI), it is not known whether RE elements within the promoters of other light down-regulated genes are regulated in the same manner. The presence of a family of repressor proteins each with a particular requirement for calcium and/or cGMP and with different binding affinities for the RE sequence, controlled by sequences around the TGGG core, would allow fine tuning of individual responses in spite of the utilization of common signalling molecules. How these signalling molecules may activate the RE repressor is currently open to speculation, although they may not modify DNA binding directly, because no differences in binding of nuclear factors in response to changing Pfr levels have been detected in footprint analyses of the oat *PHYA* promoter (Bruce *et al*., 1991).

In summary, the results presented here provide a good view of a plant signal transduction pathway. We have identified both the most upstream component (PfrA) and the most downstream component (a 17 bp *cis*-element) and have information about some of the signal transduction intermediates and their effective concentrations. Most significantly, it is becoming clear that different responses can be controlled via the same signalling network. In the future, as other specific gene targets are linked to specific pathways, as their activation/repression thresholds in response to calcium and/or cGMP become defined and as the influence of reciprocal control on their expression is investigated, it may become possible to interpret complex physiological responses to light in terms of the functioning of this rather simple signal transduction circuitry.

Materials and methods

DNA constructs

The *AS1* promoter was cloned using the polymerase chain reaction (PCR) from pea genomic DNA prepared as described (Pruitt and Meyerowitz, 1986). Based on the original published sequence, primers were designed for PCR in order to generate an *Xba*I–*Sca*I fragment of 559 bp, which has been shown to be sufficient to mediate light down-regulation in transgenic tobacco (Tsai, 1991). This fragment contains 559 bp of promoter sequence upstream of the transcription start site. The fragment was cloned as a transcriptional fusion to a *GUS* reporter gene (Jefferson *et al*., 1987) containing a downstream poly(A) addition sequence from the pea *RBCS3C* gene (Fluhr *et al*., 1986) in plasmid pBluescript IISK. Other reporter gene constructs, *CAB–GUS*, *FNR–GUS* and *CHS–GUS*, have been previously described (Bowler *et al*., 1994a).

RE3 was made by annealing the following two sets of oligonucleotides: 5′-GATCTGGTGGGAGCTAG-3′ and 5′-GATCCTAGCTCCCACCA-3′. $RE3_m$ was made with 5′-GATCTGGACCGAGCTAG-3′ and 5′-GATCCTAGCTCGGTCCA-3′. Each of the two sets of oligonucleotides were ligated and the 68 bp fragments (tetramers) were cloned into the *Bam*HI site of pBluescript IISK. To construct *35S–RE3–GUS* and *35S–*$RE3_m$*–GUS*, the *RE3* and $RE3_m$ tetramer fragments respectively were inserted between the CaMV *35S* B domain ($^{-}343$ to $^{-}90$) and the $^{-}46$ minimal promoter. The synthetic promoters were then fused upstream of a *GUS* reporter gene and the pea *RBCS3C* poly(A) sequence (Benfey and Chua, 1990).

Microinjection

Seven- to 10-day-old etiolated seedlings of wild-type tomato (*Lycopersicon esculentum* cv. Moneymaker) and the long hypocotyl mutant *aurea* (W616 genotype *au/au*) were used in all experiments. Techniques for the microinjection and subsequent analysis of *aurea* subepidermal hypocotyl cells have been described (Neuhaus *et al*., 1993; Bowler *et al*., 1994a) and essentially the same protocols were followed for injection and analysis of wild-type seedlings. Preparation and handling of injection solutions were performed as described previously (Neuhaus *et al*., 1993; Bowler *et al*., 1994a), as was the treatment of injected seedlings with pharmacological inhibitors (Bowler *et al*., 1994b). Purified oat PHYA was stored in the dark as the PrA form. Hence, PHYA injections in green safelight conditions introduced PrA into the cells, whereas injections under normal (i.e. white light) conditions introduced PfrA.

Plasmids for microinjection were prepared using Qiagen and were stored in injection buffer at concentrations between 0.2 and 1 μg/μl (Neuhaus *et al*., 1993, 1994). Both reporter gene and competitor plasmids were injected in the circular form. For competition experiments, the target and competitor DNA were mixed immediately prior to injection.

Due to a technical refinement we now routinely use micropipettes with an aperture diameter of 0.3–0.5 μm (calculated as described; Schnorf *et al*., 1994). The estimated volume delivered during each

injection is 1 pl and the estimated cell volume is 160 pl (calculated as described previously; Neuhaus *et al*., 1993). Hence, pipette concentrations of reagents are 160 times higher than those shown in the tables. This differs from our initial microinjection protocol (Neuhaus *et al*., 1993), in which we estimated an injection volume of 5 pl.

Phytochrome photo-reversibility experiments

For red/far-red experiments, all procedures for the preparation and subsequent injection of etiolated seedlings were performed under green safelight conditions (0.25 $\mu E/m^2/s$) [type TL40W/17 (Phillips) with plexiglass filters PG303/3 mm and PG627/3 mm]. Injected seedlings were then either irradiated with red light (1.18 $\mu E/m^2/s$) [type TL40W/15 (Phillips) with a red plexiglass filter (PG501/3 mm)] for 1 min or were irradiated for 1 min with red light (1.18 $\mu E/m^2/s$) followed by 10 min of far-red light (0.2 $\mu E/m^2/s$) [120W Linestra lamp (Osram) with a plexiglass combination of one layer red (PG501/3 mm) and two layers blue (PG627/3 mm)]. Subsequently, the seedlings were returned to darkness for 48 h before analysis.

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