Phytochrome-induced SIG2 expression contributes to photoregulation of phytochrome signalling and photomorphogenesis in Arabidopsis thaliana

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Abstract

Chloroplast-localized sigma factor (SIG) proteins promote specificity of the plastid-encoded RNA polymerase. SIG2 function appears to be necessary for light-grown Arabidopsis thaliana plants. Specific photoreceptors or light-dependent factors that impact the light-induced accumulation of SIG2 have not been reported. A molecular link between phytochromes and nuclear-encoded SIG2, which impacts photomorphogenesis specifically under red (R) and far-red (FR) light, is described here. Both phyA and phyB promote SIG2 transcript accumulation. Disruption of SIG2 results in R- and FR-specific defects in the inhibition of hypocotyl elongation and cotyledon expansion, although no impairments in these responses are detected for sig2 mutants under blue (B) or white (W) light. SIG2 also impacts root elongation under W and R, and the R-dependent expression of PIF4, encoding a phytochrome-interacting factor, and HY2, which encodes a phytochrome chromophore biosynthetic enzyme. Whereas SIG2 apparently impacts the accumulation of the phytochromobilin (PΦB) phytochrome chromophore, sig2 mutants differ significantly from PΦB mutants, primarily due to wavelength-specific defects in photomorphogenesis and disruption of a distinct subset of phytochrome-dependent responses. The molecular link between phytochromes and SIG2 is likely to be an important part of the co-ordination of gene expression to maintain stoichiometry between the nuclear-encoded phytochrome apoprotein and plastid-derived PΦB, which combine to form photoactive phytochromes, and/or light-dependent SIG2 accumulation is involved in an inductive light signalling pathway co-ordinating components between nucleus and plastids.

Key words: Light signalling, nuclear gene expression, plastid gene expression, photomorphogenesis, phytochrome, sigma factor.

Introduction

The establishment of photosynthesis during plant development requires the assembly of functional chloroplasts, which is a light-regulated process requiring fine co-ordination of the expression of nuclear- and plastid-encoded genes. The major photosynthetic enzyme Rubisco is composed of a small subunit RbcS, which is encoded in the nuclear genome, and the chloroplast genome-encoded large subunit RbcL. The assembly of Rubisco has often been used as a model for understanding the mechanism(s) by which plants maintain stoichiometry between nuclear- and plastid-encoded components (Rodermel, 1999; Wostrikoff and Stern, 2007; Suzuki and Makino, 2012). The involvement of photoreceptors in regulating the expression of a number of nuclear-encoded genes required for chloroplast development has been reported (Tyagi and Gaur, 2003; Larkin and Ruckle, 2008). In recent studies investigating the function of mesophyll-localized phytochromes, it was noted that both RbcL and RbcS protein levels were reduced in lines depleted of phytochromes, which...
suggested a distinct role for phytochromes in co-ordinating the stoichiometry of nuclear- and plastid-derived components of Rubisco (Oh and Montgomery, 2011).

Plant photoreceptors mediate a number of light-dependent growth and developmental responses, including seed germination, inhibition of hypocotyl elongation, chloroplast development, cotyledon and leaf expansion, promotion of root elongation, and the photoperiodic induction of flowering, among others (Franklin and Quail, 2010; Kami et al., 2010). Phytochromes are photoreceptors that consist of two distinct components, i.e. a nuclear-encoded apoprotein and a plastid-synthesized linear tetrapyrrrole chromophore. There are five nuclear-encoded genes for phytochrome apoproteins in Arabidopsis, i.e. PHYA to PHYE (Sharrock and Quail, 1989; Quail, 1994). A single chromophore exists, i.e. phytochromobilin (PΦB), which is synthesized fully in the plastid and exported to the cytosol, where it becomes covalently attached to all of the apoproteins to produce photoactive holophytochromes (Terry et al., 1993).

Among the phytochrome family members, phyA is primarily responsible for perceiving far-red (FR) light (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993), although it also functions in sensing blue (B) light (Neff and Chory, 1998; Poppe et al., 1998; Whitelam et al., 1998; Chun et al., 2001; Weller et al., 2001; Duek and Fankhauser, 2003; Yadav et al., 2005; Stephenson and Terry, 2008; Warnaoooriya et al., 2011) and high irradiance red (R) light (Franklin et al., 2007). The remaining four phytochromes, i.e. phyB to phyE, primarily contribute to the regulation of photomorphogenesis in response to perceiving R light (Nagatani et al., 1993; Reed et al., 1993; Aukerman et al., 1997; Devlin et al., 1998, 1999; Franklin et al., 2003; Monte et al., 2003; Warnaoooriya et al., 2011). Phytochromes can function as homodimers (Jones and Quail, 1986; Wagner et al., 1996; Sharrock and Clack, 2004; Clack et al., 2009) or, in some cases have been reported to function as obligate heterodimers (Sharrock and Clack, 2004; Clack et al., 2009).

A number of plastid-encoded genes involved in photosynthesis are transcribed by the plastid-encoded RNA polymerase (PEP), whose activity can be regulated by nuclear-encoded sigma (SIG) factor proteins (Kanamaru et al., 1999). SIG proteins are promoter-specificity factors that function with PEP in Arabidopsis (Hanaoka et al., 2003). There are six such proteins (SIG1 to SIG6) in Arabidopsis (Allison, 2000). SIG2 (Shirano et al., 2000) and SIG6 (Ishizaki et al., 2005) have been implicated in the regulation of chlorophyll synthesis and/or accumulation in Arabidopsis.

Expression of SIG2 was previously shown to be green-tissue- or leaf-specific (Isono et al., 1997), as well as light-induced in Arabidopsis (Isono et al., 1997; Privat et al., 2003). Prior studies also showed that SIG2 is a chloroplast-localized protein (Kanamaru et al., 1999; Woodson et al., 2013). Light-grown sig2 mutants have a pale-green phenotype and abnormal plastids, whereas normal etioplasts form in the mutants grown in the dark (Shirano et al., 2000; Kanamaru et al., 2001). In this regard, the SIG2 function appears to be light dependent. Tetrapyrrrole synthesis and accumulation are impaired in the sig2-2 mutant with reduced levels of several tetrapyroles, including δ-aminolevulinic acid (ALA), protochlorophyllide, chlorophyll, and haem (Woodson et al., 2013). Mutation of SIG2 is associated with reduced glutamyl-tRNA (tRNA glut ) levels (Kanamaru et al., 2001; Hanaoka et al., 2003; Woodson et al., 2013), which results in the reported reduced levels of tetrapyroles in sig2 mutants.

A SIG2-dependent link between the plastid and nuclear genome has been previously reported (Shirano et al., 2000). Recent work indicated that a sig2 mutant is impacted in plastid transcription and retrograde signalling from the plastid to the nucleus that co-ordinates gene expression between these two organelles in response to the functional status of plastids (Woodson et al., 2013). Relatedly, SIG2 is expressed early in development (Ishizaki et al., 2005), suggesting important roles during the light-dependent establishment of seedlings. One such role could be in co-ordinating gene expression to maintain stoichiometry of nuclear- and plastid-encoded components in light-grown plants, e.g. nuclear-encoded apoproteins and chloroplast-synthesized chromophores of tetrapyrole-containing proteins. A previous study indicated a link between photoreceptors cry1 and cry2 and B light-dependent regulation of SIG5 (Onda et al., 2008). SIG5 expression had been shown previously to be particularly responsive to B light, and SIG5 shown to function in induction of photosystem II reaction center protein D (psbD) (Tsunoyama et al., 2002). The specific photoreceptors or light-dependent factors that impact light-induced accumulation of other SIG proteins have not been reported.

Here, we describe a molecular link between phytochromes and SIG2, which impacts distinct aspects of photomorphogenesis in Arabidopsis. The role of phytochromes in regulating the induction of SIG2 may be an important part of maintaining the balance between the nuclear-encoded phytochrome apoprotein and the plastid-derived PΦB chromophore, which combine to form photoactive phytochrome photoreceptors, and/or a component of anterograde signalling, i.e. forward communication from the nucleus that controls plastid development and signalling. In the latter regard, SIG2 may function both in retrograde signalling from the plastid to the nucleus (Woodson et al., 2013), as well as in an inductive light signalling pathway between the nucleus and plastids.

Materials and methods

Plant materials

*Arabidopsis thaliana* WT Col-0 ecotype, T-DNA insertional mutant lines sig2-2 (SALK_045706; Kanamaru et al., 2001; Woodson et al., 2013), sig2-3 (SALK_022546), sig1-1 (SAIL_983_C09; Woodson et al., 2013), hy5 (SALK_056805), and pif4 (SALK_140393) were obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.biocsi.ohio-state.edu/pcmb/Facilities/abbr/abbrhome.htm, last accessed 3 September 2013; Alonso et al., 2003). No-0 WT and transgenic *BVR* (i.e. 35S::pBVR3 and C35:3pBVR2) lines have been described previously (Montgomery et al., 1999; Warnaoooriya and Montgomery, 2009). All mutant genotypes for SALK lines were confirmed by PCR using primers designed with the web-based SALK T-DNA Primer Design tool (http://signal.salk.edu/tdnaprimers2.html, last accessed 3 September 2013) and primer sequences are listed in Table 1. Confirmed homozygous mutant plants were used in this study.
Table 1. Primers used in this study

| AGI number | LP or forward primer sequence (5′–3′) | RP or reverse primer sequence (5′–3′) | Purpose |
|------------|-------------------------------------|-------------------------------------|---------|
| At1g08540  | CCTTCTGCTTCTCATCATCCG               | CATAGAAAGTATTGGGAACCCG              | sig2-2  |
|            | GTACAGTGTTCCTGGCTGCCT               | GAGACAAGCATGAGCTGGCG                | T-DNA screening |
|            | TGGCGGAGGAATACAGGACCTT              | TCCCATTTCTGGTCTAGGGACCTC            | RT-PCR analysis |
|            | AGAGGAACAGAAAGCAACTGTGA             | AGGAGAGATGAAGACCGCAT                | qRT-PCR analysis |
| At1g08550  | CACCTCGAGATGGCGTCTGAG               | CACCTCGAGATGGCGTCTGAG               | T-DNA mutant screening |
|            | TGCTTGGTGGTCTCATGGTGTCGA            | TTGCGAGATGGAGATGTTGAGA              | qRT-PCR analysis |
|            | ATCGGTGACTGACCAGACGG                 | CTCCGCATGTGGACTGACCAGG              | qRT-PCR analysis |
|            | CGGCGAGGCGTGTATACATTTG              | CTGAAAACACCGGCTTCTCCG               | qRT-PCR analysis |
| At2g43010  | CTTCTGCAAACTTGGCGG                 | CTTCTGCAAACTTGGCGG                 | T-DNA mutant screening |
|            | CATCTGCAAACTTGGCGG                 | CATCTGCAAACTTGGCGG                 | T-DNA mutant screening |
|            | CACCTGTATTGTGACCTGGA                | CACCTGTATTGTGACCTGGA                | T-DNA mutant screening |
|            | CTGAAAACACCGGCTTCTCCG               | CTGAAAACACCGGCTTCTCCG               | T-DNA mutant screening |

T-DNA insertion mutants

phyA (SALK_014575; Mayfield et al., 2007; Ruckel et al., 2007) and phyB (SALK_022035; Mayfield et al., 2007; Ruckel et al., 2007) used in these studies have been previously described; whereas the double mutant phyAphyB was obtained from a genetic cross between the two single mutants.

Additional mutant allele for SIG2

Arabidopsis transgenic line sig2-4 was used as an additional mutant allele for SIG2. sig2-4 was a line derived from NPQ1 complementation of the sig2-4npq1-2 mutant (SALK_124757). pVC1, which contained a WT copy of NPQ1 in vector pBIN19 (a gift from Dr Niyogi) was used to complement SALK_124757. A T1 single copy, homozygous complementation line was used in this study.

Light sources

Light sources for far-red (FR; λmax~735 nm), red (R; λmax~670 nm), blue (B; λmax~470 nm), and white (W) light were described previously by Warnasooriya and Montgomery (2009). Fluence rates of R, B, and W were measured using a LI-250A Light Meter (LI-COR) connected to a Li-Cor quantum sensor. Fluence rates for FR were measured using a StellarNet EPP2000 spectroradiometer (Apogee Instruments).

Reverse transcriptase PCR (RT-PCR) analyses

Total RNA was extracted from 7-d-old whole seedlings grown at 22 °C in a growth chamber with continuous FR (FRc) illumination (2.5 or 5 μmol m⁻² s⁻¹) or continuous R (Rc) illumination (50 μmol m⁻² s⁻¹) using the RNeasy Plant Minikit (Qiagen, CA). cDNA was synthesized using a Reverse Transcription System (Promega, WI) following the instructions of the manufacturer. The first-strand cDNA was used as the template in PCR reactions performed with GoTaqGreen (Promega, WI). Ubiquitin-conjugating enzyme 21 (UBC21) was used as an internal control gene, and oligonucleotides were designed using AtRTPrimer (Han and Kim, 2006). The following standard thermal profile was used for PCR: (i) 1 cycle of denaturation at 94 °C for 3 min; (ii) 27–30 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s; and (iii) final extension at 72 °C for 5 min. PCR products were visualized using 2% (w/v) agarose gel electrophoresis with ethidium bromide staining. The primers used for RT-PCR are listed in Table 1 and also in Supplementary Table S1 at JXB online.

For quantitative RT-PCR (qRT-PCR), cDNA was synthesized using 100 ng of total RNA and random primers as described above and the cDNA was mixed with Fast SYBR Green Master Mix (Applied Biosystems). qPCR was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems) in three technical and three biological replicates. UBC21 was used as a standard for normalization for SIG2 in the experiments. The primers used for qRT-PCR are indicated in Table 1 and also in Supplementary Table S1 at JXB online.

Hypocotyl length and cotyledon area measurements

Seeds were surface-sterilized using 35% (v/v) bleach solution and germinated on Murashige and Skoog (MS) media containing 1% (w/v) sucrose adjusted to pH 5.7 with KOH and 0.7% (w/v) Phytoblender agar (Caisson Labs, UT). After cold-stratification at 4 °C for 4 d in darkness, plates were transferred to a growth chamber with appropriate light conditions at 22 °C for 7 d. To quantify hypocotyl lengths, seedlings were scanned and measured using Image J software (NIH). Cotyledons were detached from each seedling, scanned, and cotyledon area measured using Image J software (NIH).

Root length measurements

Seeds were grown vertically on MS medium containing 1% (w/v) Suc, 0.05% (w/v) MES, 1% (w/v) Phytoblender agar (Caisson Labs, UT) for 7 d under appropriate light conditions. To quantify root lengths, seedlings were scanned and measured using Image J software (NIH).

Results

Expression of SIG2 in phytochrome-inactivation lines

It was demonstrated previously that the hypocotyls of the CAB3:pBVR2 line, which exhibits mesophyll-specific inactivation of phytochromes due to CAB-driven expression of the chromophore-degrading enzyme biliverdin reductase (BVR), are longer than the hypocotyls of No-0 wild-type (WT) and a 35S::pBVR3 line exhibiting constitutive inactivation of phytochromes under continuous FR (FRc) (Warnasooriya and Montgomery, 2009). These results support a role for mesophyll-specific phytochromes in the inhibition of hypocotyl elongation. The more severe phenotype of the CAB3::pBVR2 line under FRe relative to a constitutive BVR expression line,
i.e. 35S::pBVR3, has previously been attributed to differences in the pattern of BVR accumulation and the associated degradation of the phytochrome chromophore precursor biliverdin in cotyledons of these distinct lines (Oh et al., 2013). It is well established that 35S-driven gene expression is vascular enriched in transgenic plants (Sunilkumar et al., 2002; Hraška et al., 2008); however, CAB3 promoter-driven expression is widespread specifically in mesophyll cells of light-grown plants (Mitka et al., 1989).

From a microarray-based comparison of CAB3::pBVR2, 35S::pBVR3, and No-0 WT lines grown in FRc light (Rosa et al., 2010; Oh et al., 2013), it was found that, among other genes, some of which encode chloroplast-localized and photosynthesis-related proteins and those impacting light-dependent hypocotyl elongation (Oh et al., 2013), SIG2 was misregulated. SIG2 (At1g08540), which encodes nucleus-encoded sigma factor 2 for the plastid-encoded RNA polymerase (PEP), was down-regulated by 2.2-fold in CAB3::pBVR2 compared with WT (Fig. 1A). Using qRT-PCR analyses, the down-regulation of SIG2 was validated in lines exhibiting BVR-dependent inactivation of phytochromes grown under FRc (Fig. 1B). Notably, this misregulation of SIG2 expression was light-dependent, as SIG2 mRNA levels were not appreciably down-regulated in dark-grown BVR lines (see Supplementary Fig. S1A at JXB online).

Regulation of SIG2 by phytochromes is light-dependent
As holophytochrome levels are correlated with SIG2 expression, it was tested whether the expression of SIG2 is regulated by phytochrome A- and/or B-mediated light signalling pathways. The expression of SIG2 in phyA, phyB, or phyA-phyB mutants was examined in dark- and light-grown seedlings. The levels of SIG2 mRNA accumulation in dark-grown phytochrome-deficient lines was ~90% of WT levels for single phy mutants, and nearly 80% of WT for the phyAphyB double mutant (see Supplementary Fig. S1B at JXB online), suggesting a minimal impact on SIG mRNA accumulation in darkness. Using qRT-PCR analyses, it was shown that SIG2 mRNA accumulation was reduced in light-grown phyA, phyB, and phyAphyB mutants (Fig. 1C). Notably, down-regulation of SIG2 was most prominent in the phyAphyB double mutant, i.e. down by ~3-fold, compared with single mutants (Fig. 1C). These data suggested that both phyA and phyB contribute to positive, light-dependent transcriptional regulation of SIG2.

Expression of SIG2 in different tissues and light conditions
The expression of SIG2 was analyzed in different tissues and light conditions using public microarray data for Col-0 WT from...
AtGenExpress (http://www.weigelworld.org/resources/microarray/AtGenExpress, last accessed 3 September 2013) (Fig. 2). SIG2 is highly expressed in cotyledon and young leaf tissues from seedlings, as well as in the rosette leaves of adult plants (Fig. 2A). This observation corresponds with prior studies in which SIG2 expression was shown to be green-tissue- or leaf-specific (Isono et al., 1997) and chloroplast-specific (Kanamaru et al., 1999; Woodson et al., 2013). The public microarray data analysis from AtGenExpress showed the up-regulation of SIG2 after exposure to 4 h of light treatment, including B (10 µmol m⁻² s⁻¹), R (10 µmol m⁻² s⁻¹), FR (10 µmol m⁻² s⁻¹), and W (10 µmol m⁻² s⁻¹) (Fig. 2B), which corresponds to prior observations that SIG2 expression is light-induced (Isono et al., 1997; Privat et al., 2003). Our qRT-PCR analyses confirmed that SIG2 expression is light responsive (Fig. 2C). Expression of SIG2 was up-regulated to a greater degree in response to continuous R (Rc; 50 µmol m⁻² s⁻¹) or white (W; 100 µmol m⁻² s⁻¹) compared with FRc (5 µmol m⁻² s⁻¹) in 7-d-old seedlings (Fig. 2C).

Identification of sig2 mutants

Two homozygous SALK T-DNA mutant lines for SIG2 (sig2-2 and sig2-3) were identified and the T-DNA insertion site of each mutant line was verified by PCR using a T-DNA left border primer coupled with a SIG2-specific primer and a pair of SIG2-specific primers downstream and upstream of the insertion (Fig. 3A; for primer sequences see Table 1). sig2-2 (Woodson et al., 2013) and sig2-3 have T-DNA insertions in the first and third introns, respectively (Fig. 3A). A reduced level of expression of SIG2 in the isolated T-DNA mutants was confirmed (Fig. 3B). Notably, SIG2 was heavily down-regulated in sig2-2 compared with sig2-3, suggesting that the sig2-2 allele is stronger than the sig2-3 allele (Fig. 3B).

An additional mutant allele was identified for SIG2 (Fig. 3C). In an assessment of the SALK mutant line SALK_124757, which contains a T-DNA insertion in gene At1g08550 (NON-PHOTOCHEMICAL QUENCHING 1, NPQ1), it was noted that, unlike previously identified npq1 mutant alleles (e.g. npq1-2) that exhibited no obvious change in the cotyledon colour (Niyogi et al., 1998), SALK_124757 showed a pale-green phenotype (data not shown). Noticeably, expression of SIG2, in addition to NPQ1, was down-regulated in SALK_124757 (Fig. 3C), suggesting a polar effect of the T-DNA in this mutant. Given this observation, NPQ1 expression was tested in sig2-2 and sig2-3, which was not changed in these mutant alleles (Fig. 3B). SALK_124757 was
Oh and Montgomery

complemented with a WT copy of the \textit{NPQ1} gene, using a construct previously shown to complement \textit{npq1} mutants fully (Niyogi \textit{et al.}, 1998), and complementation was validated by checking the expression of \textit{NPQ1} and \textit{SIG2} via RT-PCR analysis (Fig. 3C). The confirmed T\textsubscript{3} transgenic line still exhibited a pale-green phenotype and complete down-regulation of \textit{SIG2} (Fig. 3C), suggesting that the resultant T\textsubscript{3} transgenic complementation line could be used as an additional mutant allele for \textit{SIG2}. This line was designated as \textit{sig2-4-3} and it was used in further studies.

\textit{SIG2} contributes to inhibition of hypocotyl elongation and expansion of cotyledons under FRc and Rc illumination

As previously reported, a lesion in \textit{SIG2} resulted in pale-green seedlings under light conditions (Fig. 4A; Shirano \textit{et al.}, 2000). Among the tested mutant alleles, \textit{sig2-2} and \textit{sig2-4} exhibited obvious pale-green phenotypes in Rc, continuous B (Bc), and W, whereas \textit{sig2-3} did not (Fig. 4A), supporting the idea that \textit{sig2-2} and \textit{sig2-4} were strong alleles. It was observed that the hypocotyls of strong \textit{sig2} mutant alleles were longer than those of Col-0 WT when plants were grown in FRc or Rc, but not under Bc, W, or in D (Fig. 4). The longer hypocotyl phenotype was most obvious in Rc-grown \textit{sig2-2} and \textit{sig2-4} (Fig. 4; greater than 2-fold), which may correspond to a greater expression of \textit{SIG2} under Rc, when compared with FRc (Fig. 4A). However, \textit{sig2-3}, which was considered a weak mutant allele, did not exhibit any significant changes in hypocotyl length under any light condition (Fig. 4B). These data suggest that \textit{SIG2} is required for the accurate inhibition of hypocotyl elongation in FRc and Rc, specifically.

In addition to the noted hypocotyl phenotypes, \textit{sig2-2} and \textit{sig2-4} exhibited smaller cotyledons compared with Col-0 WT in FRc or Rc (Fig. 5). However, these mutant alleles did not show changes in cotyledon size when they were grown in Bc or W. The smaller cotyledon phenotype was most obvious in Rc-grown \textit{sig2-2} and \textit{sig2-4} (about 2-fold, Fig. 5B), similar to the more severe hypocotyl elongation phenotype of strong \textit{sig2} mutant alleles under Rc (Fig. 4). \textit{sig2-3} did not exhibit any significant changes in cotyledon size under any light condition tested (Fig. 5). Collectively, our data suggested that \textit{SIG2} contributes to the inhibition of hypocotyl elongation and expansion of cotyledons in specific light conditions, namely FRc and Rc.
SIG2 impacts root elongation under Rc and W illumination

To determine whether other light-dependent phenotypes were observed in sig2 mutants, root phenotypes were assessed under monochromatic light. In previous studies, it had been demonstrated that both shoot- and root-localized phytochrome-dependent signals can impact the photoregulation of root elongation (Costigan et al., 2011). Although there was no apparent impact on root elongation of sig2 mutant seedlings in complete darkness compared with WT, strong sig2 mutants exhibited strongly impaired light-induced elongation of roots relative to WT under Rc and W light conditions, with less severe impacts under Bc (Fig. 6). There was no significant defect observed in the photoregulation of root elongation under FRC for sig2 mutants when compared with WT (Fig. 6).

Light-dependent expression of phytochrome genes and phytochrome-signalling component genes in sig2 mutants

The down-regulation of SIG2 was observed in phytochrome inactivation lines or phytochrome T-DNA mutants (Fig. 1). Therefore, it was investigated whether SIG2 is required...
Oh and Montgomery

LONG HYPOCOTYLS 5 (HY5), encoding a bZIP transcription factor, contributes to the promotion of photomorphogenesis and is downstream of phytochromes (Oyama et al., 1997; Chattopadhyay et al., 1998). PHYTOCHROME-INTERACTING FACTOR 4 (PIF4) encodes a bHLH factor, which functions as a negative regulator of phytochrome signalling (Huq and Quail, 2002). LONG HYPOCOTYLS 1 (HY1) and LONG HYPOCOTYLS 2 (HY2) encode a haem oxygenase and a ferredoxin-dependent biliverdin reductase, respectively, which are required for the synthesis of the phytochrome chromophore (i.e. phytochromobilin) (Davis et al., 1999; Kohchi et al., 2001; Emborg et al., 2006). Using qRT-PCR analyses, it was determined that the accumulation of PHYA and PHYB mRNA was reduced by only ~20–30% in strong sig2 mutants (see Supplementary Fig. S2 at JXB online), although the expression of SIG2 was significantly down (i.e. ~2-fold or greater) in phytochrome-deficient mutants (Fig. 1).

For phytochrome signalling components, it was determined that the expression of HY5, PIF4, HY1, or HY2 genes was not significantly changed in sig2 mutants grown in FRc for 7 d using RT-PCR analyses (see Supplementary Fig. S3A at JXB online). In reciprocal analyses, it was noted that the expression of SIG2 was not changed in hy5 or pif4 mutants grown in FRc for 7 d (see Supplementary Fig. S3B at JXB online). These data suggested that, in FRc conditions, these genes are not involved in the SIG2-mediated phytochrome-signalling pathway.

To test the possibility that the role of SIG2 in impacting components of the phytochrome-signalling pathway might be wavelength-specific, qRT-PCR of PIF4 was performed in sig2 mutants grown in Rc (50 µmol m⁻² s⁻¹), FRc (2.5 µmol m⁻² s⁻¹), Bc (25 µmol m⁻² s⁻¹), and W (100 µmol m⁻² s⁻¹) for 7 d. In Rc, PIF4 was up-regulated (more than 2-fold) in strong sig2 mutant alleles (i.e. sig2-2 and sig2-4), whereas no significant change was apparent.

Fig. 5. Cotyledon expansion assay for Col-0 WT and sig2 mutant seedlings. Seedlings were grown on MS medium containing 1% Suc and 0.7% Phytoblend agar at 22 °C for 7 d under FRc (2.5 µmol m⁻² s⁻¹), Rc (50 µmol m⁻² s⁻¹), Bc (25 µmol m⁻² s⁻¹), or W (100 µmol m⁻² s⁻¹). (A) Representative images of cotyledons are shown. Scale bars indicate 0.5 cm. (B) Data points in bar graphs represent mean cotyledon area of seedlings (±SD, n=34). Unpaired, two-tailed Student’s t-test comparing mutants to WT, **P <0.01, ***P <0.005.
in a weak sig2 mutant allele (sig2-3) (Fig. 7). However, in FRc or W, the expression of PIF4 in most sig2 mutants was comparable with Col-0 WT (Fig. 7). In FRc, a moderate down-regulation of PIF4 was observed in sig2-4. Line sig2-4 exhibited a great down-regulation of SIG2 and moderate up-regulation of NPQ1, which is a neighbouring gene of SIG2, relative to the WT (Fig. 3C). Thus, the observed moderate down-regulation of PIF4 in FRc-grown sig2-4 may be caused by ectopic expression of NPQ1 in FRc-grown sig2-4 or could be caused by another difference in the sig2-4 background independent of SIG2 or NPQ1 expression. Under Rc, it was also determined that the phytochrome chromophore biosynthesis gene HY2 was down-regulated more than 2-fold in the strong sig2 mutant, whereas expression of HY1 was not significantly impacted (Fig. 8). To

**Fig. 6.** Root assay for Col-0 WT and sig2 mutant seedlings. Seedlings were grown vertically on MS medium containing 1% Suc, 0.05% MES, and 1% Phytoblend agar at 22 °C for 7 d under FRc (2.5 µmol m⁻² s⁻¹), Rc (50 µmol m⁻² s⁻¹), Bc (25 µmol m⁻² s⁻¹), W (100 µmol m⁻² s⁻¹) or in darkness (Dc). (A) Representative image of seedlings is shown. Scale bar indicates 1 cm. (B) Data points in bar graphs represent mean root lengths of seedlings (±SD, n ≥34). Unpaired, two-tailed Student’s t-test comparing mutants to WT, *P <0.05, **P <0.01, ***P <0.005.
address whether the differences in gene expression in FR versus R are due to wavelength-dependent differences in SIG2 expression in sig2 mutants, the down-regulation of the SIG2 gene in Rc-grown sig2 mutants was confirmed (Fig. 7). Again, no differences were observed in the expression of SIG2 in hy5 or pif4 mutants grown in Rc for 7 d (Fig. S4B).

**Fig. 7.** Expression of PIF4 in Col-0 WT and sig2 mutant seedlings. Quantitative RT-PCR (qRT-PCR) analyses were performed using 7-d-old Col-0 wild-type (WT) and mutant seedlings grown on MS medium containing 1% Suc and 0.7% Phytoblend agar at 22 °C for 7 d under FRc (2.5 µmol m⁻² s⁻¹), Rc (50 µmol m⁻² s⁻¹), or W (100 µmol m⁻² s⁻¹). Relative gene expression level compared to UBC21 is shown (±SD, n=3). Unpaired, two-tailed Student's t-test comparing mutants to WT, *P < 0.05, ***P < 0.005.

SIG6 does not impact the light-dependent inhibition of hypocotyl elongation

It was assessed whether the impacts observed for sig2 mutants were specific to SIG2 function when compared with another SIG protein also shown to impact chloroplast development and plastid transcription, i.e. SIG6 (Ishizaki et al., 2005; Woodson et al., 2013). Both SIG2 (Shirano et al., 2000) and SIG6 (Ishizaki et al., 2005) have been implicated in the regulation of chlorophyll synthesis and/or accumulation in Arabidopsis. Recent work also indicated that sig2 and sig6 mutants are impacted in plastid transcription and retrograde signalling from the plastid to nucleus that co-ordinates gene expression between these two organelles (Woodson et al., 2013). Given these similarities in SIG2 and SIG6 function, a sig6-1 mutant was grown under D, FRc, and Rc conditions in order to determine whether it exhibited light-dependent defects in hypocotyl elongation similar to strong sig2 mutants. Although the sig6-1 mutant was severely impaired in SIG6 function as evidenced by the severe chlorophyll deficiency, hypocotyl lengths were not significantly different from WT under any of the conditions that were used to grow the seedlings (Fig. 9). Thus, SIG6 did not have an impact on the regulation of light-dependent hypocotyl elongation, which
SIG2 impacts photomorphogenesis in Arabidopsis

was in contrast to the FRc- and Rc-dependent hypocotyl elongation observed for strong sig2 alleles (Fig. 4). These data suggest a distinct role for SIG2 in phytochrome-dependent photomorphogenesis.

Discussion

Although the role of SIG2 in the regulation of plastid gene expression and retrograde regulation of nuclear-encoded photosynthesis-associated genes is not light dependent, i.e. the expression of Photosynthetic Associated Nuclear Genes (PhANGs) and plastid-encoded transcripts was also down-regulated in dark-grown sig2 mutant seedlings (Woodson et al., 2013), expression of SIG2 is impacted by phytochromes (Fig. 1) and light (Fig. 2). Furthermore, SIG2 impacts hypocotyl elongation (Fig. 4) and root elongation (Fig. 6) in a light-dependent manner, i.e. the lengths of hypocotyls or roots of sig2 mutant seedlings did not differ from WT in complete
darkness. Consistent with our observation of light-specific roles for SIG2 in the regulation of growth responses, no phenotype was noted in prior studies of etioplasts in dark-grown sig2 mutants although plastids were aberrant in light-grown sig2 seedlings (Shirano et al., 2000; Kanamaru et al., 2001).

Of note, not all phytochrome-dependent phenotypes are disturbed in sig2 mutants. For example, none of the sig2 alleles tested differed in the regulation of the expression of a number of known phytochrome biosynthesis or target genes, with the exception of PIF4 (Fig. 7; see Supplementary Fig. S4 at JXB online) and HY2 (Fig. 8; see Supplementary Fig. S4 at JXB online) expression under Rc. Thus, the reduced accumulation of tetrapyrroles in sig2 mutants (Woodson et al., 2013), which would result in a reduced synthesis of the haem-derived phytochrome chromophore, could be associated specifically with the disruption of the phytochrome-dependent R- and FR-induced inhibition of hypocotyl elongation and cotyledon expansion (Figs 4, 5), R- and W-induced root elongation (Fig. 6), and PIF4 (Fig. 7) and HY2 (Fig. 8) expression under Rc. It has been debated previously whether plastid-derived signals may be associated with the phytochrome control of PhANGs (Vinti et al., 2005) or plant development (Moller et al., 2001). In this regard, our results support a role for SIG2 in the promotion of the expression of a plastid-derived gene(s) or protein(s) that impacts expression of PIF4 and HY2 in the nucleus.

SIG2 is expressed early in seedling development (Kanamaru et al., 1999), which may be correlated with a link to phytochrome apoprotein and chromophore co-ordination that is critical for holophytochrome production that functions in early light-dependent seedling development. Thus, phytochrome-dependent regulation of SIG2 expression could represent a mechanism for co-ordinating plastid-localized tetrapyrrole synthesis with nuclear-encoded apophytochrome gene expression, which is still not well understood. However, the observed results are distinct from hy1 and hy2 mutants, which exhibit elongated hypocotyls and reduced cotyledon expansion under R, FR, B, and W illumination (Neff and Van Volkenburgh, 1994; Parks and Quail, 1993), suggesting that the absence of SIG2 does not lead to a simple phytochrome chromophore deficiency. Indeed, the expression of phytochrome chromophore biosynthesis genes HY1 and HY2 is not apparently impacted in sig2 mutants under FRc (see Supplementary Fig. S3A at JXB online); whereas HY2 expression is reduced by more than 2-fold under Rc in strong sig2 mutants (Fig. 8). Thus, the sig2 mutant phenotype is probably due to a lack of substrate, i.e. haem-derived biliverdin (BV) due to a haem deficiency (Woodson et al., 2013) and, in the case of Rc, an additional enzymatic limitation of sig2 mutants to synthesize the Pd PhB chromophore. Thus, there is likely to be a chromophore deficiency in sig2 lines that results in a phenotype similar in some regards, but not identical, to hy1 and hy2 phytochrome chromophore-deficient mutants. These latter mutants may have distinct aspects of their phenotypes associated with the accumulation of tetrapyrrole intermediates in the PdPhB synthesis pathway.

In addition to being different from phytochrome chromophore-deficient mutants, the phenotypes of sig2 mutants are different from CAB UNDEREXPRESSED (cue) mutants, the latter of which exhibit defects in chlorophyll synthesis and/or accumulation similar to sig2 mutants, but very limited to no impacts on light-dependent control of hypocotyl elongation and cotyledon opening under R, FR, B or W light (Vinti et al., 2005). Furthermore, cue mutants exhibit light-dependent and light-independent defects in plastid development (Vinti et al., 2005), unlike sig2 mutants that exhibit defects that appear to be specific to photomorphogenesis.

Among other mutants impacting light-dependent growth and plastid metabolism, only laf6 has a somewhat similar phenotype to the strong sig2 mutants described here. A laf6 mutant has elevated protoporphyrin IX levels, reduced chlorophyll levels, and elongated hypocotyls specifically under FR light (Moller et al., 2001). LAF6 encodes an ABC transporter (i.e. AtABC1), which was purported to function as a chloroplast transporter, perhaps transporting tetrapyrroles out of the plastid (Moller et al., 2001). The elevated protoporphyrin IX levels have been postulated to be associated with a reduced phytochrome chromophore pool in laf6 mutants (Cornah et al., 2003). However, why this would result in a FR-specific defect in hypocotyl elongation, and not also impact Rc-dependent development, is unclear.

SIG2 expression is regulated by both phyA and phyB in a light-dependent manner (Fig. 1C; see Supplementary Fig. S1 at JXB online), whereas SIG2 has a minor impact on PHYA and PHYB mRNA accumulation (see Supplementary Fig. S2 at JXB online). Prior experiments demonstrated a decrease of SIG2 mRNA accumulation in a phyA mutant using microarray analyses (Ma et al., 2001) and in a phyB mutant by Northern analysis (Nagashima et al., 2004). Notably, SIG2 appears to accumulate to a greater level under Rc and W light than under FRc in 7-old seedlings (Fig. 2C). Similar up-regulation of SIG2 in response to FR light was reported using microarray analysis (Ma et al., 2001). Although SIG2 is up-regulated under Bc in addition to other light conditions, including UV, W, R, and FR (Fig. 2B), hypocotyl length is increased under R and FR, but essentially the same as WT under Bc for strong sig2 mutants (Fig. 4). However, strong sig2 mutants are still severely chlorophyll-deficient under Bc (Fig. 5A), which suggests that the B-dependent role of SIG2 includes the regulation of chloroplast development and chlorophyll synthesis.

The extended hypocotyl phenotype and up-regulation of expression of the PIF4 gene in Rc-grown sig2 mutants (Figs 4, 7) are reminiscent of phenotypes observed in PIF4-overexpressing lines (Huq and Quail, 2002). In Rc, PIF4-overexpressing lines show shorter hypocotyls whereas PIF4 knock-down lines exhibit shorter hypocotyls, suggesting that PIF4 is a negative regulator of phyB signalling (Huq and Quail, 2002). Up-regulation of PIF4 in sig2 mutants may explain, in part, the longer hypocotyl phenotypes observed in sig2 mutants in Rc. The elongated hypocotyl and down-regulation of expression of HY2 under Rc are also consistent with phenotypes of the hy2 mutants (Koornneef et al., 1980). Thus, it is proposed that SIG2 contributes to phyB signalling by negative transcriptional regulation of PIF4 and positive regulation of HY2 under R, which could be a direct
regulation or, alternatively, could be the result of reduced levels of holophytochromes due to reduced chromophore levels (Woodson et al., 2013) in the sig2 mutants (Fig. 10). Notably, expression of CIPK20 was also identified as down-regulated in a sig2 mutant under W light (Woodson et al., 2013) and in phytochrome-deficient lines under R and FR light (Oh et al., 2013). Recently, it was demonstrated that CIPK20 contributes to phytochrome-dependent inhibition of hypocotyl elongation under R and FR (Oh et al., 2013). Taken together, these results suggest a potential for CIPK20 to be regulated by phytochromes in a SIG2-dependent manner to impact photomorphogenesis in Arabidopsis.

In chloroplasts, two types of RNA polymerase complexes, nuclear-encoded RNA polymerase (NEP) and plastid-encoded RNA polymerase (PEP) perform transcriptional events. Interaction of PEP with sigma factors (SIGs) at the promoters of target genes in chloroplasts is required for the initiation of transcription of many genes (Beardslee et al., 2002; Suzuki et al., 2004). In Arabidopsis, PEP also physically interacts with Transcriptionally Active Chromosome protein 12 (pTAC12), also named HEMERA (HMR; Pfalz et al., 2006; Chen et al., 2010), which functions in phytochrome signalling in the nucleus (Chen et al., 2010). HMR has recently been shown to interact physically with phytochromes (Galvão et al., 2012). HMR is essential for the proteolysis of phytochrome, PIF1, and PIF3 in the nucleus, yet is also localized to plastids and has a function in these organelles as nuclear-only HMR expression does not fully rescue the hmr mutant phenotype (Chen et al., 2010). HMR has been considered an indirect transcriptional regulator for PIF1/PIF3-controlled genes encoding chloroplast proteins (Chen et al., 2010). Since PIF4 shares significant homology with other PIF family members, including PIF1 and PIF3 (Leivar and Quail, 2011), it could be interesting to test whether chloroplast-localized HMR is associated with the SIG2-dependent phenotypes that were observed. Notably, expression of HMR does not appear to be impacted by light, although HMR protein accumulates preferentially in light (Chen et al., 2010), which has recently been shown to be due to light-dependent promotion of HMR protein accumulation due to interactions with light-activated phytochromes (Galvão et al., 2012). SIG2 expression and protein accumulation are both light-dependent (Iso no et al., 1997; Privat et al., 2003).

It has been suggested previously that levels of SIG proteins, which are enriched in leaf tissues, could be involved in the regulation of plastid-localized photosynthesis genes in a tissue-specific manner (Iso no et al., 1997). Part of the tissue-specific phytochrome responses involve the regulation of genes encoding tissue-enriched factors such as SIG2, CIPK20 and other genes (Oh et al., 2013). Although the expression of SIG2 is leaf-specific (Iso no et al., 1997), a clear impact on hypocotyl and root elongation is seen in sig2 mutants. These observations correspond to prior findings that light perception in cotyledon or leaf tissue results in a signal(s) that inhibits growth in hypocotyls (Tanaka et al., 2002; Endo et al., 2005; Montgomery, 2009; Warnasooriya and Montgomery, 2009; Oh et al., 2013) and impacts root elongation (Costigan et al., 2011). Although phytochrome-dependent impacts of light on root elongation have been reported previously (Costigan et al., 2011; Dyachok et al., 2011), an indirect impact of aberrant chloroplast development on the inhibition of root elongation that is observed in strong sig2 mutants cannot summarily be ruled out.

As a result of reciprocal effects of light during de-etiolation, elongation of hypocotyls is inhibited, but cotyledon expansion is enhanced. It has been suggested that

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**Fig. 10.** Model depicting the roles of phyA or phyB under FR or R light, respectively, in the regulation of distinct genes and phytochrome-dependent responses. Sigma factor 2 (SIG2), phytochrome-interacting factor 4 (PIF4), phytochrome chromophore phytochromobilin (PDB), phytochrome chromophore biosynthesis enzymes haem oxygenase (HY1), and phytochromobilin synthase (HY2). Solid lines indicated confirmed relationships, whereas dotted lines represent pathways for which factors are not definitively confirmed or are still unknown. FR, far-red light; R, red light.
these opposite, organ-specific responses can be used as an excellent screening method to distinguish between mutants related to general cell expansion and those specifically related to light-regulated cell expansion (Quail, 2002). In this regard, sig2 mutants exhibited FR- or Rc-dependent defects in cotyledon expansion, inhibition of hypocotyl elongation, and promotion of root elongation, which differ from mutants showing general defects in seedling growth and development (e.g. dwarf mutants). Therefore, SIG2 most likely contributes to light-signalling events that are vitally important for apposite regulation of distinct aspects of photomorphogenesis.

Supplementary data
Supplementary data can be found at JXB online.

Supplementary Table S1. Additional primer sequences used in this study

Supplementary Fig. S1. Expression of SIG2 in Dc-grown phytochrome-deficient lines.

Supplementary Fig. S2. Expression of PHYA and PHYB in sig2 mutants.

Supplementary Fig. S3. Expression of phytochrome-related genes in sig2 mutants (A) and expression of SIG2 in hy5 mutants (SALK_056405 or pif4 mutants (SALK_140393) (B) under FRC.

Supplementary Fig. S4. Expression of phytochrome-related genes in sig2 mutants (A) and expression of SIG2 in hy5 (SALK_056405) or pif4 (SALK_140393) mutants (B) under Rc.

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