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Blue-Light-Independent Activity of *Arabidopsis* Cryptochromes in the Regulation of Steady-State Levels of Protein and mRNA Expression

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ABSTRACT Cryptochromes are blue-light receptors that mediate blue-light inhibition of hypocotyl elongation and blue-light stimulation of floral initiation in *Arabidopsis*. In addition to their blue-light-dependent functions, cryptochromes are also involved in blue-light-independent regulation of the circadian clock, cotyledon unfolding, and hypocotyl inhibition. However, the molecular mechanism associated with the blue-light-independent function of cryptochromes remains unclear. We reported here a comparative proteomics study of the light regulation of protein expression. We showed that, as expected, the protein expression of many metabolic enzymes changed in response to both blue light and red light. Surprisingly, some light-regulated protein expression changes are impaired in the *cry1cry2* mutant in both blue light and red light. This result suggests that, in addition to mediating blue-light-dependent regulation of protein expression, cryptochromes are also involved in the blue-light-independent regulation of gene expression. Consistent with this hypothesis, the *cry1cry2* mutant exhibited reduced changes of mRNA expression in response to not only blue light, but also red light, although the cryptochrome effects on the red-light-dependent gene expression changes are generally less pronounced. These results support a hypothesis that, in addition to their blue-light-specific functions, cryptochromes also play roles in the control of gene expression mediated by the red/far-red-light receptor phytochromes.

INTRODUCTION

Cryptochromes are photolyase-like proteins that regulate development in plants and the circadian clock in plants and animals. Cryptochromes often exert their function by regulating gene expression (Cashmore, 2003; Lin and Shalitin, 2003; Sancar, 2003). The *Arabidopsis* genome encodes at least three cryptochrome sequences, of which CRY1 and CRY2 mediate primarily blue-light inhibition of hypocotyl elongation and photoperiodic promotion of floral initiation, respectively (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Guo et al., 1998; El-Assal et al., 2001). CRY3 is a mitochondria/chloroplast protein that acts as a single-strand DNA-repairing enzyme (Kleine et al., 2003; Selby and Sancar, 2006). In addition to regulating growth inhibition and flowering time, cryptochromes have also been found to regulate other physiological or cellular activities in response to blue light. For example, *Arabidopsis* cryptochromes are known to mediate light regulation of the circadian clock (Somers et al., 1998), cotyledon expansion (Lin et al., 1998), anion channel activity (Folta and Spalding, 2001), fruit traits (El-Assal et al., 2004), stomata opening (Mao et al., 2005), root elongation (Canamero et al., 2006), programmed cell death (Danon et al., 2006), and chromatin modulation (Tessadori et al., 2007). The molecular mechanisms underlying those cryptochrome responses remain largely unknown.

Cryptochrome is defined historically as the pigment that absorbs and responds to blue light (approximately 400–500 nm) and UV-A light (approximately 320–400 nm) (Gressel, 1979; Lin and Shalitin, 2003). Cryptochromes possess two chromophores—flavin (Flavin adenine dinucleotide, FAD) and folate (methenyltetrahydrofolate, MTHF), which, depending on the redox status and pH, absorb primarily blue and UV light (Lin et al., 1995; Malhotra et al., 1995; Sancar, 2003). *Arabidopsis* CRY1 purified from insect cells contains oxidized FAD, which can be photoreduced in vitro to a neutral flavin radical (FADH•) intermediate that absorbs green light

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(approximately 500–600 nm), and the UV-absorbing reduced-flavin final products (Lin et al., 1995; Banerjee et al., 2007; Bouly et al., 2007). It has been proposed that the neutral flavin radical may represent the active redox status of *Arabidopsis* cryptochromes (Banerjee et al., 2007; Bouly et al., 2007). No anion flavin radical ($FAD^{-\bullet}$) that absorbs a broader wavelength of light (550–700 nm) has been detected for plant cryptochromes, although such a red-light-absorbing anion flavin radical was found for *Drosophila* dCRY (Berndt et al., 2007). The present consensus is that plant cryptochromes probably do not absorb red light at all, although the exact absorption spectrum of a cryptochrome remains unclear until the holoprotein that contains both MTHF and flavin can be purified and analyzed.

Most cryptochrome-mediated responses in *Arabidopsis*, such as growth inhibition and floral promotion, are known to have the wavelength specificity in primarily the blue-light region of the electromagnetic spectrum (Guo et al., 1998; Mockler et al., 1999; Lin, 2000; Ahmad et al., 2002; Valverde et al., 2004). However, not every cryptochrome function is dependent on blue light. It is well known that mammalian cryptochromes, being components of the central oscillator, function in light-independent modes (Griffin et al., 1999; van der Horst et al., 1999), whereas the mammalian cryptochromes in the retinal ganglion cells mediate light responses (Thresher et al., 1998; Selby et al., 2000; Van Gelder et al., 2003). Although plant cryptochromes have been well established to act as blue-light receptors, blue-light-independent functions have also been reported for the *Arabidopsis* cryptochromes. For example, *Arabidopsis* loss-of-function cryptochrome mutants exhibited longer periods of the circadian rhythm of the *CAB2* (*chlorophyll a/d-binding protein 2*) promoter activity not only in blue light, but also in red light (Somers et al., 1998; Devlin and Kay, 2000). The naturally occurring gain-of-function Cape Verde Islands QTL allele of the *CRY2* gene (CRY2-Cvi), which encodes a $M^{367}V$ alteration, was found to enhance cotyledon unfolding in the absence of blue light (El-Assal et al., 2001; Botto et al., 2003). Although cryptochrome mutants show no obvious phenotypic alteration in the hypocotyl inhibition response when tested under continuous red light or far-red light (Koornneef et al., 1980; Ahmad and Cashmore, 1993; Lin et al., 1998; Mockler et al., 1999), the loss-of-function *cry2* mutant exhibited reduced hypocotyl inhibition when grown under white-plus-far-red light (Mas et al., 2000), indicating a blue-light-independent role of CRY2 in de-etiolation. It has been shown that *Arabidopsis* CRY1 and CRY2 physically interact with phyA and phyB, respectively (Ahmad et al., 1998; Mas et al., 2000), which may explain blue-light-independent physiological activities of cryptochromes.

Arabidopsis cryptochromes regulate gene expression to affect developmental responses (Jackson and Jenkins, 1995; Lin et al., 1996; Yanovsky and Kay, 2002; Valverde et al., 2004; Zhao et al., 2007). DNA microarray analyses showed that, depending on the experimental conditions, approximately 5–40% of the genes encoded by the *Arabidopsis* genome change the levels of mRNA expression in response to light, and that most of the red-light- and blue-light-dependent mRNA changes are regulated by phytochromes and cryptochromes, respectively (Ma et al., 2001; Tepperman et al., 2001; Folta et al., 2003; Ohgishi et al., 2004). Up to one-third of the genome-wide mRNA expression changes are commonly induced by red light or blue light (Ma et al., 2001), which is believed to result from co-action of phytochromes and cryptochromes. Recently, the 2D/MS (two-dimensional electrophoresis and mass spectrometry) method has been used to investigate light and photoreceptor regulation of changes in protein expression in *Arabidopsis*. As expected, phytochromes were found to affect changes of protein expression in response to red/far-red light, whereas CRY1 was shown to mediate blue-light regulation of changes in protein abundance (Kim et al., 2006; Phee et al., 2007). Given that cryptochromes are blue-light receptors and that most phenotypic alterations resulting from the cryptochrome mutations are apparent in blue light but not in the dark, red light, or far-red light, it is not surprising that none of the previous genome-wide expression studies specifically addressed the question of whether cryptochromes affect gene expression changes in response to non-blue light.

We report here a comparative proteomic study of protein expressions in the wild-type *Arabidopsis* and the *cry1cry2* mutant seedlings grown under continuous blue light or red light. As expected, the *cry1cry2* mutation altered protein expression profile in response to blue light. Surprisingly, we also found differences of the protein expression profiles between wild-type and the *cry1cry2* mutant seedlings grown in red light. RNA expression analyses showed that the *cry1cry2* mutation affects not only blue-light-dependent but also blue-light-independent mRNA expression changes. These results support a hypothesis that cryptochromes may act in a blue-light-independent manner to affect phytochrome regulation of gene expression and development.

RESULTS

The Steady-State Protein Expression Changes in Both Blue Light and Red Light

We compared protein abundance changes, using the conventional 2D/MS method similar to that described previously (Kim et al., 2006; Phee et al., 2007). To access the steady-state level of changes in the absence of both *Arabidopsis* cryptochromes (CRY1 and CRY2) that have partially redundant functions, we examined the samples prepared from wild-type and the *cry1cry2* double mutant grown in blue light or red light of the same fluence rate. Proteins extracted from 7 d old seedlings grown in continuous blue light (80 μmol m^{-2} s^{-1}) or red light (80 μmol m^{-2} s^{-1}) were separated by two-dimensional gel electrophoresis; the silver-stained gels containing different samples were scanned and digitized; protein spots in the samples of comparisons (wild-type vs *cry1cry2* grown in dark vs light) were aligned; and each protein spots in the respective gels were normalized against the total signals

(see Materials and Methods). To minimize variations resulting from sample preparations, three independent sets of gels per sample were prepared for statistic analyses. The protein spots that showed statistically significant changes ($p < 0.05$) between the samples of comparison were excised, trypsin-digested, and the resulting peptide mass and sequence were analyzed using MALDI-TOF-TOF mass spectrometry (Yergey et al., 2002).

We initially isolated 110 protein spots from the 2D gel that showed different abundance between the samples of the comparison. The peptide sequences were successfully identified by MALDI-TOF-TOF analyses for 75 of the 110 protein spots. These 75 sequences represent 61 genes (Supplemental Table 1), as some differentially expressed protein spots isolated from the 2D gels appear to be the same gene products. Among those 61 genes, 39 (64%) showed expression changes in response to both blue light and red light (Supplemental Table 1), which is somewhat higher than the 34% estimated by a DNA microarray analysis of mRNA expression (Ma et al., 2001). Nevertheless, this result demonstrates that a considerable number of proteins changed their steady-state levels in response to both blue light and red light.

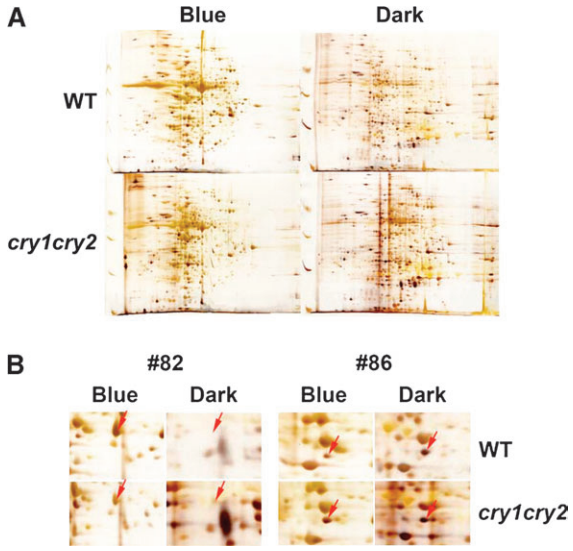

Figure 1. Representative 2D Gel Images Showing Protein Expression Changes in Response to Blue Light.

(A) Representative gel image showing proteins of 7 d old *Arabidopsis*Col-4 (WT) and the *cry1cry2*mutant seedlings grown in the dark or blue light (80 μmol m^{-2} s^{-1}).

(B) Representative gel images of spot #82 (carbonic anhydrase) and spot #86 (dehydroascorbate reductase) that exhibited blue-light-induced and suppressed expression, respectively.

The *cry1cry2* Mutant Affects Changes of Protein Expression in Response to Not Only Blue Light but Also Red Light

Regulation of protein expression by different wavelengths of light may result from actions of different types photoreceptors or one type of photoreceptors. To test these possibilities, we examined whether changes of protein abundance of the same gene in red light and blue light may be both affected in the *cry1cry2* mutant. Among the 39 proteins identified that showed light-induced protein expression changes, 16 (41%) were apparently regulated by cryptochromes for their blue-light-regulated expression changes. The expression profile of these 16 proteins showed more than two-fold changes between etiolated and blue-light-grown wild-type seedlings, whereas the blue-light-dependent protein expression change was reduced by at least two-fold in the *cry1cry2* mutant (Figure 1A and Table 1). Figure 1 shows two examples of cryptochrome-regulated protein expression—one (#82, carbonic anhydrase) was induced in blue light and the other (#86, dehydroascorbate reductase) was suppressed in blue light (Figure 1B). Table 1 summarizes the 16 cryptochrome- and blue-light-regulated proteins identified.

As shown in Table 1, the 16 blue-light- and cryptochrome-regulated proteins represent 18 differentially expressed protein spots identified. For example, the spots #51 and #52 were identified as the same gene product (putative glycine dehydrogenase, GDH), whereas spots #7 and #65 were both identified as serine hydromethyltransferase, which apparently result from different post-translational modifications of the same gene products. Many blue-light-regulated proteins are metabolic enzymes (52.8%, including those with functions in metabolism and energy production), and more than two-thirds of those proteins are expected chloroplast (29.4%) or mitochondrial proteins (41.2%) (Figure 3A and 3B). These results are consistent with a previous proteomics study showing that most light-induced proteins were metabolic enzymes (Kim et al., 2006). Given the relatively low sensitivity of our 2D-gel/silver-stain system that resolves up to approximately 1000 protein spots, it may not be surprising that the expression changes of metabolic enzymes, which are generally more abundant than other types of proteins, were more readily detected. Over 75% of the cryptochrome- and blue-light-regulated proteins identified (12 of 16) showed increased protein expression in blue-light-grown wild-type seedlings than that in etiolated ones (Table 1). This observation seems consistent with the known light stimulation of plant metabolic activities in general and photosynthesis-related activities in chloroplasts in particular.

Unexpectedly, a similar percentage of proteins (36% or 14/39) showed cryptochrome-dependent regulation of protein expression in response to red light (Figure 2 and Table 2). Those 14 proteins exhibited more than two-fold expression changes between etiolated and red-light-grown wild-type seedlings, but the red-light-induced expression changes were reduced by at least two-fold in the *cry1cry2* mutant (Figure 2 and Table 2). Table 2 summarizes the 14 cryptochrome- and red-light-regulated proteins, which represent 17 protein spots identified. Interestingly, the putative glycine dehydrogenase and serine hydromethyltransferase were also each identified twice as red-light-regulated proteins (Table 2). In addition, a LEA (late embryogenesis abundant) protein was identified twice as

Table 1. Proteins Showing Cryptochrome-Dependent Expression in Response to Blue Light

Group	Spot No.	Locus	Protein	WT B/D	*cry1cry2* B/D	RNA (WT) B/D	Expected function	Expected location
I	82	AT3G01500*	Isoform 3 of Carbonic anhydrase	84.63	7	3.272	Metabolism	Chloroplast
	73	AT3G63140*	mRNA-binding protein	23.57	2.59	2.434	Cellular structural organization	Plastoglobule
	51	AT4G33010*	Putative glycine dehydrogenase	22.76	2.21	4.07	Metabolism	Mitochondrion
	65	AT4G37930*	Serine Hydroxymethyltransferase	22.16	0.313	3.763	Metabolism	Mitochondrion
	52	AT4G33010*	Putative glycine dehydrogenase	15.41	1	4.07	Metabolism	Mitochondrion
	26	AT4G26530	Pructose bisphosphate aldolase	11.24	1.11	4.348	Energy	Unknown
	7	AT4G37930*	Serine Hydroxymethyltransferase	9.9	0.595	3.763	Metabolism	Mitochondrion
	75	ATIG20020*	Ferredoxin-NADP(+) reductase	8.72	1.1	1.905	Unclassified protein	Chloroplast
	53	AT2G26080	Glycine dehydrogenase	4.24	1.13	1.392	Metabolism	Mitochondrion
	19	AT5G61410	Ribulose-phosphate 3-epimerase	3.84	1.13	1.431	Energy	Chloroplast
	24	AT1G42970	Glyceraldehyde-3-phosphate dehydrogenase B	6.71	2.88	3.128	Energy	Chloroplast
II	34	AT5G17710*	Co-chaperone grpE family protein	3.57	1.47	1.039	Cellular transport and transport mechanisms	Chloroplast
	29	AT1G16880	Uridylytransferase-related	7.24	2.07	0.98	Unclassified protein	Chloroplast
	118	AT2G22780*	Probable malate dehydrogenase	0.12	0.53	1.004	Energy	Peroxisome
III	11	AT3G08030	Expressed protein	0.15	0.45	3.763	Unclassified protein	Endomembrane system
	58	AT5G67360	Subtilisin-like protease precursor	0.22	1.74	1.23	Protein fate	Extracellular matrix
	d	AT5G44120	12S seed storage protein (CRAI)	0	1.34	1.53	Unclassified protein	Endomembrane system
	86	AT1G19570*	Dehydroascorbate reductase	0.52	1.13	1.415	Metabolism	Mitichondrion

Protein spots that show blue-light-induced expression changes in the wild-type seedlings but the change was reduced in the *cry1cry2* mutant selected (from Supplemental Table 1) and shown. The protein expression changes (B/D) of the wild-type (WT) and the *cry1cry2* mutant (*cry1cry2*) in response to blue light were calculated by dividing the amount of protein signal derived from seedlings grown in blue light by that of seedlings grown in the dark. The mRNA expression changes (WT B/D RNA) in response to blue light obtained from Genevestigator are shown for comparison. Proteins are divided into three groups, which contain loci that showed similar protein and mRNA expression changes (I), changes in protein expression but not in mRNA expression (II), or opposite protein and mRNA expression changes (III). Asterisks indicate proteins that are included in both Table 1 and Table 2.

a red-light-induced spot. As with blue-light treatment, red-light illumination also results in protein abundance change for many metabolic enzymes and chloroplast proteins (Figure 3B and 3E). A comparison of Table 2 to Table 1 shows that 57% (eight of 14) of the cryptochrome- and red-light-regulated proteins (Table 2, marked by asterisks) are identical to those regulated by cryptochromes and blue light (Table 1, marked by asterisks). Among them, six showed cryptochrome-dependent light induction of protein expression, and two showed cryptochrome-dependent light suppression of protein expression. Importantly, the blue-light- or red-light-induced changes of protein expression of all those eight proteins were diminished in the *cry1cry2* mutant (Tables 1 and 2). Therefore, cryptochromes appear to regulate protein expression of at least some genes in a light-dependent but blue-light-independent manner.

The *cry1cry2* Mutant Also Affects Light-Regulated mRNA Expression in Response to Both Blue Light and Red Light

To examine whether the cryptochrome- and light-regulated changes of protein expression are due to changes of mRNA expression of the respective genes, we compared mRNA expression changes of the light-regulated genes identified in this study in the wild-type seedlings, using Genevestigator and its *Arabidopsis* microarray database (Zimmermann et al., 2004). According to this analysis, 10 of the 16 (62.5%) cryptochrome- and blue-light-regulated genes showed similar blue-light regulation of mRNA and protein expression (Table 1), three genes (17.5%) showed change in protein abundance but little change in mRNA expression (Table 1, group II), whereas three genes (17.5%) showed changes in protein abundance change in the opposite direction to that of the mRNA changes in response to blue light (Table 1, group III). The cryptochrome- and red-light-regulated genes showed comparable results. Among the 14 cryptochrome- and red-light-regulated genes, seven (50%) showed similar red-light regulation of mRNA and protein expression changes in response to red light (Table 2, group I), five (36.7%) genes showed no mRNA expression changes (Table 2, group II), and two genes (14%) showed opposite mRNA expression changes in response to red light (Table 2, group III). Therefore, approximately half of the cryptochrome- and light-regulated genes showed correlated changes in the

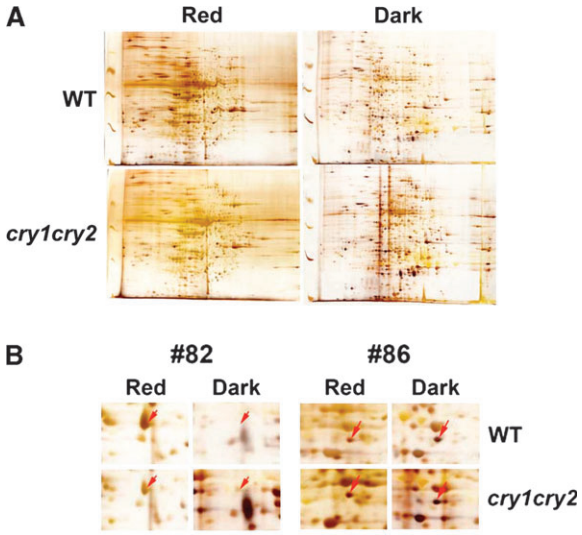

Figure 2. Representative 2D Gel Images Showing Protein Expression Changes in Response to Red Light.

(A) Representative gel image showing proteins of 7 d old *Arabidopsis* Col-4 (WT) and the *cry1cry2* mutant seedlings grown in the dark or red light (80 μmol m^{-2} s^{-1}).

(B) Representative gel images of spot #82 and spot #86 that exhibited red-light-induced and suppressed expression, respectively.

mRNA and protein expression, and more than half of the 'group I' genes (eight of 16 total) are positively regulated by cryptochromes in both red light and blue light (Tables 1 and 2, comparing that indicated by asterisks). On the other hand, the reason for the lack of correlation of mRNA and protein expression patterns for the group II and II genes, at least one of which has been confirmed by Q-PCR analyses (data not shown), remain to be investigated.

To test further whether cryptochromes may regulate mRNA expression in both blue-light-dependent and blue-light-independent manners to cause changes of protein abundances, we examined mRNA expression of the wild-type and the *cry1cry2* mutant seedlings grown in dark, red light, or blue light, using both conventional RT-PCR and real-time Q-PCR methods (Figure 4A and 4B). As the controls, we first examined mRNA expression of the two blue-light-regulated genes that have been extensively studied—chalcone synthase (*CHS*) and chlorophyll a/b-binding protein 2 (*CAB2*). Figure 4 shows that, as previously reported (Batschauer et al., 1991; Kubasek et al., 1992; Jackson and Jenkins, 1995), *CHS* mRNA expression is strongly or modestly induced by blue light or red light, respectively (Figure 4A and 4B). Importantly, the blue-light-induced increase in *CHS* mRNA is markedly reduced in the *cry1cry2* mutant, but the red-light-induced increase in the *CHS* mRNA level was apparently reduced in the *cry1cry2* mutant as well (Figure 4A and 4B). This result is consistent with hypothesis that cryptochromes are the major photoreceptors mediating blue-light induction of *CHS* transcription, whereas phytochromes are the major photoreceptors mediating the relatively weaker red-light induction of *CHS* transcription (Batschauer et al., 1991; Kubasek et al., 1992; Jackson and Jenkins, 1995). But it also suggests that cryptochromes may be involved in the phytochrome regulation of *CHS* expression in response to red light. Similarly, the *CAB2* mRNA expression showed stronger and modest induction by blue light and red light, respectively. The blue-light-induced *CAB2* expression was markedly reduced in the *cry1cry2* mutant, whereas the red-light-induced *CAB2* mRNA expression was slightly reduced in the *cry1cry2* mutant (Figure 4A and 4B). We then tested mRNA expression of two genes identified in this study (Tables 1 and 2), the putative glycine dehydrogenase (*GDH*) and ferridoxin-NADP-reductase (*FNR*) genes. Figure 4 shows that, compared with the dark controls, the mRNA expression of *GDH* and *FNR* increased in the wild-type seedlings grown in blue light or red light. The blue-light-induced mRNA expression of both genes was dramatically reduced in the *cry1cry2* mutant (Figure 4A and 4B), whereas the red-light-induced *GDH* and *FNR* mRNA expression was also reduced, albeit modestly, in the *cry1cry2* mutant (Figure 4A and 4B). These results are consistent with the change of abundance of these two proteins (Figure 4C), although the effects of cryptochromes on red-light-induced mRNA expression changes seem not as pronounced as that on the changes of protein abundance. We conclude that, in addition to the major roles that cryptochromes play in the regulation of blue-light-dependent gene expression changes, cryptochromes also affect gene expression changes in response to non-blue light.

DISCUSSION

We showed in this report that *Arabidopsis* cryptochromes affect gene expression in response to not only blue light, but also red light. Several lines of evidence indicate that the observed effects of cryptochromes on the red-light-induced gene expression changes are unlikely to be experimental artifacts. First, the *cry1cry2* mutant grown in the LED red light used in this study showed hypocotyl elongation indistinguishable from that of the wild-type seedlings (not shown), indicating that the LED red-light source was not contaminated by blue light. Second, the conventional proteomics method employed in our study has been used previously to study light regulation of protein expressions regulated by phytochromes and CRY1 (Kim et al., 2006; Phee et al., 2007), indicating general feasibility of this methodology. Third, cryptochrome regulation of gene expression in response to red light was found at both the protein level (Table 1) and the mRNA level (Figure 4C), using 2D/MS and Q-PCR methods, respectively, providing independent lines of evidence supporting the same conclusion.

The cryptochrome- and light-regulated changes of protein and mRNA expression shown in this report are based on comparisons between etiolated seedlings and seedlings grown in continuous light. Therefore, our results present only the steady-state level of gene expression changes. This would be consistent with the fact that most proteins identified in this study are metabolic enzymes that are most likely indirect

Table 2. Proteins Showing Cryptochrome-Dependent Expression in Response to Red Light

Group	Spot No.	Locus	Protein	WT R/D	*cry1cry2* R/D	RNA (WT) R/D	Expected function	Expected location
I	82	AT3G01500*	Isoform 3 of Carbonic anhydrase	74.58	12.8	2.715	Metabolism	Chloroplast
	51	AT4G33010*	Putative glycine dehydrogenase	11.51	1	3.37	Metabolism	Mitochondrion
	92	AT4G33010*	Putative glycine dehydrogenase	10.3	1	3.37	Metabolism	Mitochondrion
	73	AT3G63140*	mRNA-binding protein	10.09	3.02	1.999	Cellular structural organization	Plastoglobule
	75	AT1G20020*	Ferredoxin–NADP(+) reductase	7.73	2.6	1.712	Unclassified protein	Chloroplast
	7	AT4G37930*	Serine Hydroxymethyltransferase	4.15	2.5	3.123	Metabolism	Mitochondrion
	65	AT4G37930*	Serine Hydroxymethyltransferase	3.5	0.87	3.123	Metabolism	Mitochondrion
	15	AT3G26650	Glyceraldehyde-3-phosphate dehydrogenase A	3.1	0.78	1.265	Energy	Chloroplast
II	34	AT5G17710*	Co-chaperone grpE family protein	4.13	1	1.025	Cellular transport and transport mechanisms	Chloroplast
	92	AT2G44060	Late embryogenesis abundant family protein	2.12	1	1.063	Unclassified protein	Unknown
	118	AT2G22780*	Probable malate dehydrogenase	0.06	0.12	1.029	Energy	Peroxisome
	57	AT5G67360	Subtilisin-like protease precursor	3.32	0.85	1.148	Protein fate	Extracellular matrix
	93	AT2G44060	Late embryogenesis abundant family protein	6.08	2.92	1.063	Unclassified protein	Unknown
	102	AT1G54870	Similar to short-chain dehydrogenase/reductase	0.16	0.27	0.947	Metabolism	Chloroplast
III	6	AT4G35090	Similar to catalase 3 (SEN2)	0.49	0.79	1.254	Defence stress and detoxification	Mitochondrion
	86	AT1G19570*	Dehydroascorbate reductase	0.26	1.3	1.193	Metabolism	Mitochondrion
	5	AT5G47380	Hypothetical protein	0.13	1.89	2.137	Unclassified protein	Unknown

Protein spots that show red-light-induced expression changes in the wild-type seedlings but the change was reduced in the *cry1cry2* mutant selected (from Supplemental Table 1) and shown. The protein expression changes (R/D) of the wild-type (WT) and the *cry1cry2* mutant (*cry1cry2*) in response to red light were calculated by dividing the amount of protein signal from seedlings grown in blue light by that of seedlings grown in the dark. The mRNA expression changes (WT R/D RNA) in response to red light obtained from Genevestigator are shown for comparison. Proteins are divided into three groups, which contain loci that showed similar protein and mRNA expression changes (I), changes in protein expression but not mRNA expression (II), or opposite protein and mRNA expression changes (III). Asterisks indicate proteins that are included in both Table 1 and Table 2.

targets of the cryptochrome action. Whether cryptochromes also affect rapid and/or transient gene expression changes in response to red light remains to be further investigated. Curiously, only one cryptochrome-regulated protein (At3g01500, carbonic anhydrase) identified in our experiment was also similarly identified in a previous report as a cryptochrome-regulated protein (Phee et al., 2007). A possible interpretation of this apparent lack of consistency would be due to different experimental conditions (such as light intensity, growth conditions, etc.) used in different studies. Alternatively, it may also be due to the relatively low sensitivity of the 2D/MS methodology. For example, the *CAB2* mRNA was strongly induced by blue light in the cryptochrome-dependent manner and modestly induced by red light (Figure 4A and 4B), and the CAB2 protein was also clearly shown to be strongly induced by blue light and modestly induced by red light (Kim et al., 2006). Therefore, the failure to identify this protein in our 2D/MS analysis (Supplemental Table 1) is most likely because our 2D gel somehow failed to resolve the CAB2 spots or our MS analysis failed to identify the CAB2 protein spots. It seems apparent that a more sensitive technology with higher resolution and reproducibility will be needed to investigate light regulation of protein expression further.

Arabidopsis cryptochromes are not known to absorb red light (Lin et al., 1995; Banerjee et al., 2007; Bouly et al., 2007), which raises the question as to how cryptochromes affect gene expression in response to red light. It is conceivable that the observed activity of cryptochromes in non-blue light may result from physical interaction between cryptochromes and phytochromes. It has been well established that phytochromes regulate plant development by direct interaction with transcription regulators such as PIF1 and PIF3 (Ni et al., 1998; Huq et al., 2004), and that phytochromes physically interact with cryptochromes (Ahmad et al., 1998; Mas et al., 2000). Therefore, loss of cryptochromes might disrupt the phytochrome-regulated transcription in response to red light. Alternatively, cryptochromes may interact with a protein that also interacts with phytochromes, so that loss of cryptochromes may alter such common partners to affect phytochrome-mediated red-light regulation of gene expression

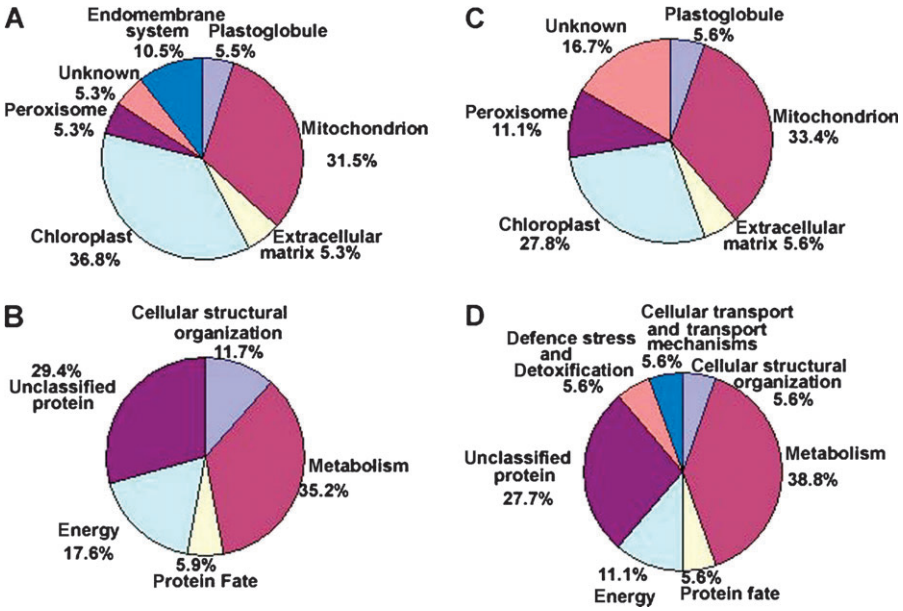

Figure 3. Functional and Cellular Localization Categories of Proteins Identified.

Proteins included in Table 1 (A, B) and Table 2 (C, D) are categorized for their expected cellular localization (A, C) and predicted function (B, D) (http://www.*Arabidopsis*.org/).

indirectly. It has been shown that the E3 ubiquitin ligase COP1 is involved in the function of both cryptochromes and phytochromes (Wang et al., 2001; Yang et al., 2001). Therefore, altered COP1 activity may also explain the observed effects of the *cry1cry2* mutation on gene expression changes in response to blue light and non-blue light. These possibilities remain to be further investigated directly.

MATERIALS AND METHODS

Plant Growth Conditions and Light Sources

The *Arabidopsis cry1cry2* mutant and the wild-type Col-4 accession have been described previously (Mockler et al., 1999). Seeds of col-4 and *cry1cry2* were sown on the Murashige and Skoog (MS) growth medium, stratified at 4°C in the dark for 4 d, and transferred to a temperature-controlled (23–25°C) growth room in the dark, or under continuous red light or blue light for the fluence rate and time indicated. Blue and red lights used are LED-B (peak: 470 nm, half-bandwidth: 30 nm) and LED-R (peak: 660 nm, half-bandwidth: 20 nm), respectively. Fluence rates of red and blue light were measured using a Li-250 quantum photometer (Li-Cor, Inc, Lincoln, NE).

Protein Sample Preparation and Analyses

The 7 d old seedlings were harvested, grinded in liquid nitrogen, and suspended in 10% TCA in acetone containing 0.3% DTT. The homogenates were kept for 24 h at –20°C, and centrifuged at 34 900 *g* at 4°C for 1 h. The precipitates were washed with acetone containing 0.07% β-mercaptoethanol, lyophilized, and kept at –80°C. Frozen powders were suspended in lysis buffer (8 M urea, 4% CHAPS (3-(3-cholamidopropyl)-dimethylammonio-1-propanesulfonate), 40 mM Tris-HCl and 2 mM PMSF), mixed for 30 min, and centrifuged at 18 900 *g* for 10 min to remove the insoluble debris. Protein concentration was estimated using the Bio-Rad Bradford reagent. The supernatant was then mixed with two volumes of the IEF (isoelectric-focusing) sample buffer (8 M urea, 4% CHAPS, 2% Pharmalyte 3–10, and 1% DTT). Samples (450 μg protein per sample) were analyzed by 2D gel electrophoresis with a IPGphor Isoelectric Focusing Unit (Amersham Biosciences). The IPG (Focused immobilized pH gradient) strip gel was rehydrated for 13 h, running at 30 V, and the proteins were separated using the following stepwise increases in voltage and running times: 500 V for 1 h, 1000 V for 1 h, 8000 V for 8.5 h (to a total of, 69 890 Vh). Focused strip gels were incubated for 15 min at room temperature with equilibration buffer I (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 1% DTT) and transferred to equilibration buffer II (50 mM Tris-HCl, pH 8.8, 6 M urea, 30% glycerol, 2% SDS, 2.5% iodoacetamide). After equilibration, the strip gels were placed on 10% SDS-PAGE gels and sealed with a 3% agarose solution. SDS-PAGE was performed at 2.5 W for 30 min and 15 W/gel constant current, respectively. Gels were stained with silver nitrate according the published protocol (Blum et al., 1987).

Image Acquisition and Analysis

The silver-stained gels were scanned using an Amersham imageScanner at an optical resolution of 300 dpi in transmission model. Approximately 950–1000 protein spots were detected in one 2D gel. Image analyses were carried out with PDQUEST software Version 7.1 (Bio-Rad Laboratories, Hercules,

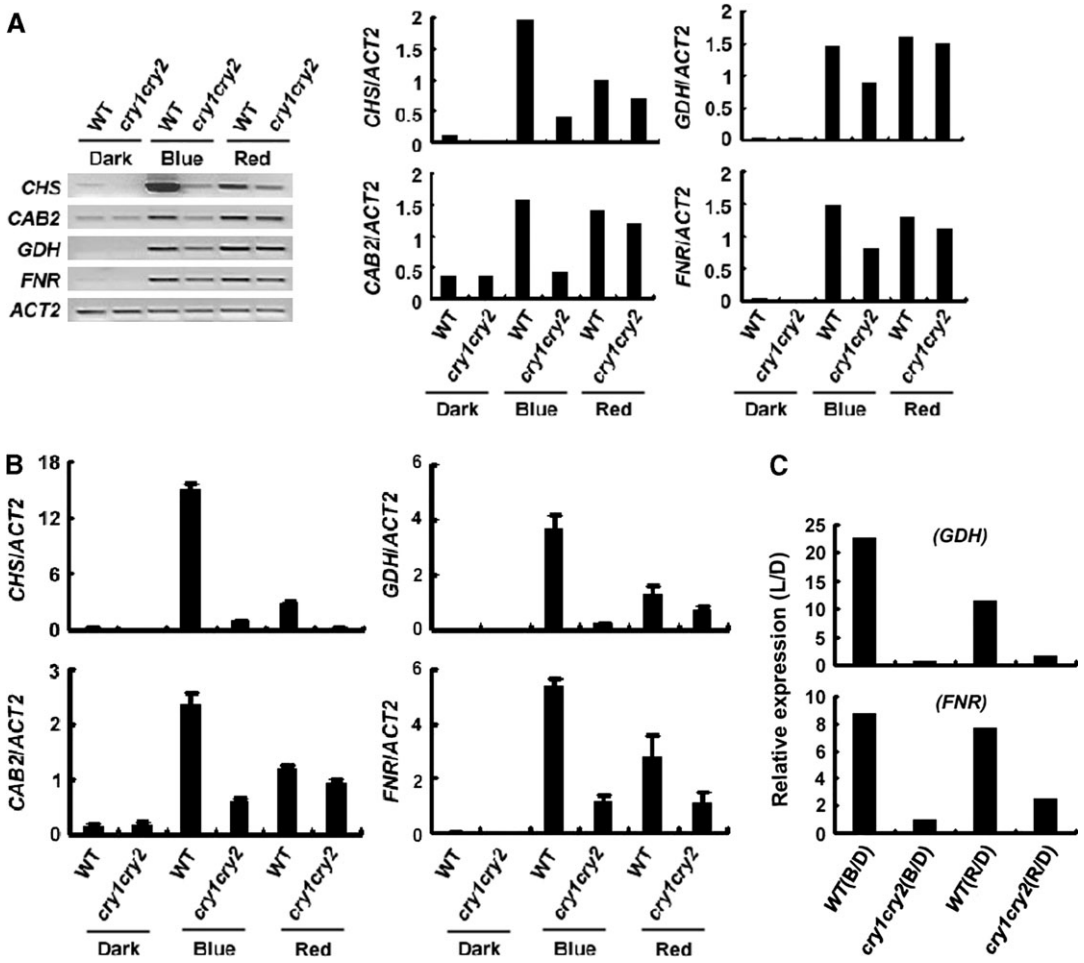

Figure 4. RT-PCR and Q-PCR Analyses of mRNA Expression.

(A) Semi-quantitative RT-PCR analyses of mRNA expression changes of *CHS* (Chalcone synthase), *CAB2* (chlorophyll a/b-binding protein 2), *GDH* (putative glycine dehydrogenase), and *FNR* (ferridoxin-NADP reductase) genes in different light conditions in the genotypes indicated. The representative RNA gel images (left) and relative expression (right) are shown. RNAs were prepared from 7 d old seedlings of the indicated genotypes grown in the dark, continuous blue light (100 μmol m^{-2} s^{-1}) or red light (100 μmol m^{-2} s^{-1}).

(B) The real-time Q-PCR analyses of mRNA expression changes of the *CHS*, *CAB2*, *GDH*, and *FNR* genes in seedlings (of the indicated genotypes) grown in different light conditions. The Q-PCR results shown are representative of the three independent sets of RNA samples analyzed. The means of three Q-PCR reactions of the same set of RNA samples and standard deviations are shown. RNAs were prepared from 7 d old seedlings of the indicated genotypes grown in the dark, continuous blue light (80 μmol m^{-2} s^{-1}) or red light (80 μmol m^{-2} s^{-1}).

(C) Relative changes of the GDH and FNR protein abundances in WT and *cry1cry2* seedlings in response to blue light (B/D) or red light (R/D) are derived from Table 1and Table 2.

CA, USA). After spot detection and background subtraction (mode: average on boundary), 2D gels were aligned, matched, and the quantitative determination of the spot volumes was performed (mode: total spot volume normalization). Digitized signals were normalized against the total signal. Triplicates from two independent protein extractions were analyzed for each comparison. Quantification and statistical analyses were carried out using the PDQUEST software.

In-Gel Trypsin Digestion

Protein spots that showed significant difference between the samples of comparison were selected and excised from preparative gels using a puncher and placed in Eppendorf microtubes. The proteins were digested in gel with trypsin as described previously (Gharahdaghi et al., 1999; Zhang et al., 2006). Briefly, the spots were washed three times with the double-distilled water. The fresh solution containing 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ was used to decolor. The cysteine reduction and alkylation steps consisted of incubation in 10 mM DTT/100 mM NH_4HCO_3 for 1 h at 57°C, and then in freshly prepared 55 mM iodoacetamide/100 mM NH_4HCO_3 solution for 30 min at room temperature in the dark. The gel pieces were dried and incubated with trypsin (0.02 μg μl^{-1}) in 25 mM NH_4HCO_3 and 10% ACN at 37°C overnight. The

digests were desalted with ZipTip™ column (Millipore, Bedford, MA, USA) according to the manufacturer's instructions and subjected to analysis using MALDI-TOF-TOF mass spectrometry.

MALDI TOF-TOF Mass Spectrometry

The tryptic digested samples were loaded onto AnchorChip™ target plate as described previously (Gobom et al., 2001). The peptides mass was obtained by using a MALDI-TOF-TOF mass spectrometer (UltraFlex I, Bruker Daltonics) equipped with nitrogen laser (337 nm) and operated in reflector/delay extraction mode for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF-TOF with a fully automated mode using the flexControl™ software. An accelerating voltage of 25 kV was used for PMF. The instrument was calibrated externally with $[M+H]^+$ ions of angiotensin I, angiotensin II, substance P, bombesin, and adrenocorticotropic hormones (clip 1–17 and clip 18–39). Each spectrum was produced by accumulating data from 100 consecutive laser shots and the spectra were interpreted with the aid of the Mascot Software (Matrix Science Ltd, London, UK). The peaks with S/N $\geqslant$ 5, Resolution $\geqslant$ 2500 were selected and used for LIFT from the same target. A maximum of five precursor ions per sample were chosen for MS/MS analysis. In the TOF1 stage, all ions were accelerated to 8 kV under conditions promoting metastable fragmentation. After selection of a jointly migrating parent and fragment ions in a timed ion gate, the ions were lifted by 19 kV to a high potential energy in the LIFT cell. After further acceleration of the fragment ions in the second ion source, their masses could simultaneously be analyzed in the reflector with high sensitivity. LIFT spectra were interpreted by the Mascot software. PMF and LIFT datasets were combined via BioTools 2.2 software (Bruker) and used for protein identification. The parameters are the following: mass tolerance in PMF of 50 ppm, MS/MS tolerance of 1.0 Da and one missing cleavage sites and cysteines modified by carbamidomethylation. The protein identifications were considered to be confident when the protein score of the hit exceeded the threshold significance score of 58 ($p < 0.05$). When there were several hits, the first hit was selected. The peptide masses were input in software PeptIdent (http://www.expasy.ch), using the database SWISS-PROT and TrEMBL. The database searches were performed using the following values: *Arabidopsis* species, protein molecular weight range, and p*I* range, trypsin digest (two missed cleavages allowed), cysteines modified by carbamidomethylation, mass tolerance $\pm$50 ppm using internal calibration. The identification was based on four matching peptides and 15% coverage. Tryptic autolytic fragments and contamination were removed from the set of data used for the database search.

RNA Analysis

Total RNA was isolated using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd). DNA-free RNA was obtained by RQ1 DNase I treatment according to the manufacturer's instructions (Promega). RT-PCR and Real-time Q-PCR was used to quantitatively measure CHS (At5g13930), CAB2 (At1g29920), spot #75 (FNR, AT1G20020) and spot #51 (GDH, AT4G33010). ACT2 was used as the internal control. SYBR green sequence detection reagents (Invitrogen, USA), Taq polymerase, sense and antisense primers were assayed on an iCycler iQ Detection System (stratagene). The reaction was performed in Mx3000p (Stratagene), and the reaction conditions were: one cycle of 95°C for 10.0 min, and then 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s.

The primers used for Q-PCR are:

*Act2*F(5 CACTGTGCCAATCTACGAGGGT-3),
*Act2*R(5CACAAACGAGGGCTGGAACAAG-3);
CAB2F (5TAAGGCTACCGACCCAGAGG-3)
CAB2R (5CAGTAGCGATGGCTTGAACG-3)
CHSF (5TGGTGCCATAGACGGACATT-3)
CHSR (5GGTGGGCTATCCAGAAGAGG-3′)
Spot #75(AT1G20020)F (5′GATAAGCAGAGAACAAGCGAAC-3)
Spot #75(AT1G20020)R (5′AATCAAACCAGTCAATACCGTC-3)
Spot #51AT4G33010F (5′GGTTTTATTGGAGCGGATGTGTG-3)
Spot #51AT4G33010R (5′GTGCTGTCTTCTCGGGTTGTG-3)

RT-PCR was as described previously. cDNA was prepared from 2 μg total RNA using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Promega). The cDNA was diluted 10-fold, and 1 μl of diluted cDNA was used in a 20-μl PCR reaction.

PCR was generally performed with a 5-min denaturation at 95°C followed by 24 cycles for *ACT2*, 31 cycles for *CHS* with each cycle composed of 95°C for 30 s, 55–60°C for 30 s, and 72°C for 30 s. PCR products were analyzed using 1.5% agarose gel. RT-PCR reactions for each experiment were repeated at least three times, and the representative gel images were shown. The expression level of the *ACT2* was used as the internal control to normalize and calculate relative expression levels of genes tested using ImageJ (http://rsb.info.nih.gov/ij/).

Supplemental Figure 1. Representative 2D Gel Images Showing Protein Expression Changes in Response to Blue (A) and Red (B) Light.

Protein spots were identified that showed significant changes of protein expression in the wild-type seedlings grown in dark or light. Representative images of differentially expressed protein spots are shown.

Supplemental Table 1. A List of Proteins Identified that Showed Expression Changes in Response to Blue Light or Red Light.

Protein spots that showed changes of protein expression in the wild-type (WT) or the *cry1cry2* mutant (*cry12*) seedlings grown in the dark (D) or light (B or R) are listed. The relative protein abundances were calculated by dividing the amount of signal derived from samples prepared from seedlings grown in light by that from seedlings grown in the dark (B/D or R/D). Data of mRNA expression changes

(RNA) in response to light of the wild-type seedlings were obtained from Genevestigator (https://www.genevestigator.ethz.ch/). Genes for which the protein expression changes are similar to that of the mRNA expression changes (I), little mRNA expression changes (II), opposite to mRNA expression changes (III), or that the mRNA expression data are not available in Genevestigator.

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