Rapid, Organ-Specific Transcriptional Responses to Light Regulate Photomorphogenic Development in Dicot Seedlings

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ABSTRACT

The dicotyledon seedling undergoes organ-specific photomorphogenic development when exposed to light. The cotyledons open and expand, the apical hook opens and the hypocotyl ceases to elongate. Using the large and easily dissected seedlings of soybean (Glycine max cv. Williams 82), we show that genes involved in photosynthesis and its regulation dominate transcripts specific to the cotyledon, even in etiolated seedlings. Genes for cell wall biosynthesis and metabolism are expressed at higher levels in the hypocotyl, while examination of genes expressed at higher levels in the hook region (including the shoot apical meristem) reveals genes involved in cell division and protein turnover. The early transcriptional events in these three organs in response to a one-hour treatment of far-red light are highly distinctive. Not only are different regulatory genes rapidly regulated by light in each organ, but the early-responsive genes in each organ contain a distinctive subset of known light-responsive cis-regulatory elements. We detected specific light induced gene expression for the root phototropism gene RPT2 in the apical hook, and also phenotypes in Arabidopsis rpt2 mutants demonstrating that the gene is necessary for normal photomorphogenesis in the seedling apex. Significantly, expression of the RPT2 promoter fused to a GUS reporter gene shows differential expression across the hook region. We conclude that organ-specific, light-responsive transcriptional networks are active early in photomorphogenesis in the aerial parts of dicotyledon seedlings.
INTRODUCTION

Photomorphogenic responses control a wide range of important developmental events throughout the lifetime of plants, including seed germination, de-etiolation, shade avoidance and flowering (Josse et al., 2008; Monte et al., 2007). Seedling photomorphogenesis (or de-etiolation) is the phenomenon whereby a dark-grown seedling, which features an elongated hypocotyl, closed cotyledons, an apical hook and undifferentiated chloroplasts, displays an inhibition of hypocotyl elongation, opening of cotyledons and apical hook, and chloroplast maturation after it is exposed to light. Photomorphogenesis is thus both a developmental process and a response to environmental stimuli. The timing of de-etiolation is of key importance to the survival of plants. Early opening of the hook and cotyledons while still in the soil will lead to the damage of young embryos. A delayed de-etiolation response may result in late initiation of photosynthesis and depletion of nutrients, ultimately limiting the ability of the seedling to survive.

The phytochrome family mediates photomorphogenesis in response to red and far-red (FR) light. In Arabidopsis, the five phytochromes (phyA-phyE) perform overlapping yet distinct physiological functions. In particular, phyA mediates the response of etiolated seedlings to FR, while phyB-E are largely responsible for the response to larger doses of continuous red light in etiolated seedlings (Sharrock and Quail, 1989; Clack et al., 1994; Devlin et al., 1998; Quail, 2002). The photosensory activity of the phytochromes results from their capacity to undergo light-induced, reversible switching between the biologically inactive Pr (for red-light-absorbing) form and the biologically active Pfr form (for far-red-light-absorbing) (Borthwick et al., 1952; Smith, 2000; Quail, 2002). The active Pfr form is translocated from the cytoplasm to the nucleus (Fankhauser and Chen, 2008) where it interacts with transcription factors which likely trigger changes in downstream gene expression and subsequently morphological changes (Ni et al., 1998; Quail, 2002).

Many regulatory factors in phytochrome signaling have been reported in recent years (Josse et al., 2008; Sharrock, 2008; Franklin et al., 2005; Monte et al., 2007), greatly increasing our understanding of molecular and cellular mechanism of photomorphogenesis. However, another important feature of seedling photomorphogenesis, the cellular specificity of photoreceptors and photomorphogenic responses at the molecular level, is poorly understood. In seedling photomorphogenesis, for example, different organs display distinct responses to the light stimulus. During de-etiolation, cell expansion occurs in the
cotyledon and the concave side of apical hook, while inhibition of cell growth is observed in the hypocotyl and the convex side of apical hook. Questions such as how the same light signal triggers distinct, even opposite, responses in different tissues and organs, and whether there is co-ordination or communication between organs, have been of interest in the field (Bou-Torrent et al., 2008). As early as in 1995, some light-responsive genes, e.g. TUB1 (β-Tubulin 1) and SPA (SUPPRESSOR OF PHYA), were shown to be regulated organ-specifically (Leu et al., 1995; Zhu et al., 2008). As the expression microarray became a standard tool for global expression analysis, the organ-specific light response began to be examined by profiling light responsive gene expression in individual organs such as cotyledon, hypocotyl, root and shoot apex (Ma et al., 2005; Lopez-Juez et al., 2008). However, our knowledge of the tissue-specific regulation of light signaling and thus regulation of photomorphogenesis at the cellular level is far from complete. For example, the apical hook plays a key role in early seedling establishment by protecting cotyledons and the meristematic primordia in the etiolated seedling during soil penetration. The timing of hook opening is therefore critical to the survival of the young seedling. Despite its distinct photomorphogenic behavior and importance to seedling survival, the light response of the apical hook has only recently begun to be explored (Li et al., 2004; Khanna et al., 2007). A global study of transcriptional responses to light in the apical hook has not yet been performed, to our knowledge, perhaps due to the significant difficulty of extracting sufficient RNA from this small organ in Arabidopsis seedlings.

We argue that our knowledge of the photomorphogenic control of plant development can be refined by examining the response of the transcriptome to light thoroughly in seedlings at the organ level. We performed an organ-specific expression profiling study with soybean (Glycine max cv. Williams 82), which permits accurate expression profiling of multiple tissues thanks to its large etiolated seedlings, without resorting to RNA amplification. The response to a short FR treatment was studied in order to identify the early regulatory events as well as to eliminate the effects of photosynthesis. In this work, gene expression in the cotyledon, apical hook (including the apical meristem) and hypocotyl was compared between seedlings treated with continuous far-red light (FRc) for one hour, and dark-grown seedlings using microarrays. FRc responsive genes were identified and their regulation by FRc was shown to be organ-specific. To identify organ-specific FRc responses and to investigate the relationship between organ-specific expression and light-induced expression, a single-channel analysis was performed with the microarray data to identify genes showing expression that was significantly stronger in individual organs. Both analyses were selectively verified by quantitative real time reverse transcription
PCR (QRT-PCR). The de-etiolation responses in FRc of Arabidopsis mutants carrying defects in an ortholog of one of the identified genes, the root phototropism gene \textit{RPT2}, were examined to study the function of the gene in photomorphogenesis.

RESULTS

Global expression analysis identified 27 genes as early far-red light responsive genes in soybean seedling photomorphogenesis

We conducted an expression profiling experiment to identify the organ-specific gene regulation triggered in the early stage of seedling de-etiolation. In order to remove the potential complications of 1) signaling via multiple photoreceptor pathways and 2) gene expression responses to the initiation of photosynthesis, non-photosynthetic FRc was given for one hour to induce the changes in gene expression associated with de-etiolation via the phyA signaling pathway (Tepperman et al., 2001). The expression profiles of the cotyledon, apical hook and hypocotyl of soybean seedlings were compared between FRc treated seedlings and dark controls using the soybean cDNA microarray described by Vodkin et al., 2004 (Fig. 1). Four independent biological replicates, each containing the tissue of a single seedling, were used in separate hybridizations. To control for labeling differences the dyes used for labeling the co-hybridized samples (Cy3 and Cy5) were swapped in the biological replicates. Microarray data was preprocessed and normalized by Global Locally Weighted Scatterplot Smoothing (GLOWESS) and then statistically analyzed using the rank product method (Breitling et al., 2004). Genes with mRNAs differentially expressed in FR vs. dark as identified by an absolute fold change (FC) of greater than two-fold, and which were statistically significant after applying a false discovery rate (FDR) correction of 5%, were defined as “FR-responsive genes”. In total, microarray analysis identified 27 FR responsive genes according to these criteria, including four genes identified as differentially expressed between the cotyledon plus/minus FR samples, fifteen genes identified as responding in apical hook, and ten genes identified in hypocotyl (Table I). Intriguingly, only two of the genes fulfilled these criteria in multiple tissues (Glyma02g04170 is responsive to FRc in both hook and hypocotyl, and Glyma06g14170 is FRc responsive in cotyledon and hook).

An organ-specific gene expression pattern in response to FRc

With the previously described criteria (FDR<0.05 and fold change greater than 2), we found that only
two genes out of the 27 FRc responsive genes meet these criteria in more than one organ. The early FR regulation of most genes detected thus seems to be organ-specific. However, it is possible that some of those 27 FRc responsive genes are also induced or repressed by FRc in a second organ, but fail to pass the arbitrary cutoff due to array noise and/or differences in statistical power. Therefore the fold changes of the 27 FRc regulated genes were compared across all three organs using replicated microarray data to get a clear picture of the organ specificity of FR gene response (Fig. 2). In addition to the organ where the gene was identified to be FR-responsive, if the gene is also responsive in another organ with a fold change greater than 1.5 (equal to a log 2 fold change 0.6), the gene is considered to be regulated by FRc in more than one organ. Such stringent parameters for calling organ-specific gene regulation allow us to identify organ specific gene regulation with confidence. According to these criteria, 81.5% (22/27) of the FR responsive genes are regulated by FRc specifically in only one organ (Fig. 2A, 2B & 2C). Ten genes are specifically regulated only in the apical hook (Fig. 2B), nine genes were regulated specifically in hypocotyl (Fig. 2C), while only three genes were shown to be specifically regulated in cotyledon (Fig. 2A). Some genes that are regulated in more than one organ were also identified (Fig. 2D). Overall, the result supports the hypothesis that many light-triggered mRNA-level changes are likely to be tissue-specific during the early stage of the transcriptional signaling cascade. The organ specificity of transcriptional light responses is a possible reason for discrepancies between the FRc responsive genes identified in this experiment and those identified in whole-seedling Arabidopsis experiments (Tepperman et al., 2001). For example, the Arabidopsis ortholog of Glyma09g05180 (AT4G02570), which was up-regulated specifically in the apical hook by 2.41 fold in our experiment, was not found to be significantly regulated by FRc in either of the two previously published whole-seedling Arabidopsis microarray experiments (Tepperman et al., 2001; Ghassemian et al., 2006).

Because false positive results from arrays of the type used for this study have been reported (Woo et al., 2004), QRT-PCR was performed to confirm the gene regulation patterns. Gene expression was monitored in one-hour FRc-treated samples versus dark controls by means of an independently conducted, replicated and controlled set of QRT-PCR experiments. Five reference gene candidates, ubiquitin (Glyma20g27950), α-tubulin (Glyma04g09350), β-tubulin (Glyma04g02610), Histone H3.2 Minor (Glyma14g40590) and Phosphoenolpyruvate carboxylase (PEPC; Glyma13g36670) were chosen based on microarray data and literature (Tuteja et al., 2004). These five candidates were tested for their stability in expression level across three organs in both FRc and dark conditions using QRT-PCR. Histone H3.2 Minor was chosen to be the reference gene because it was most consistently expressed in different organs.
in response to either treatment among the five candidates (Supplemental Fig. S1). Eight genes that display distinct organ-specific regulation patterns were chosen from the 27 FR responsive genes identified by the microarray for QRT-PCR analysis. The mRNA levels of genes were measured with three biological replicates where each biological replicate was a pool of eight seedlings. Three technical replicates were performed for each biological replicate sample. The results of these highly replicated QRT-PCR experiments thus allow greater statistical power. The data was first converted to replicated expression values normalized with the internal reference gene and then a fold change (FR/dark) was calculated, using the \( \Delta \Delta CT \) method with experiment-determined amplification efficiency incorporated (Livak and Schmittgen, 2001). QRT-PCR results are in agreement with the organ-specific regulation of mRNA levels revealed by microarray analysis for all but one of the genes tested (Fig. 3) although the magnitude of the change observed is generally greater from the QRT-PCR. We interpret this as being the result of background hybridization to the microarray limiting the observable fold change. In the case of Glyma11g03850, a discrepancy between microarray data and QRT-PCR results was observed (Fig. 3). Although the direction of change in expression level in response to light is consistent between the QRT-PCR and microarray results for Glyma11g03850, the organ specificity of mRNA regulation is switched from hook-specific to hypocotyl-specific. Since the QRT-PCR was performed as an entirely separate experiment with an independently collected plant sample set, the discrepancy may be due to variation in dissection of hook and hypocotyl between the two batches of samples, which, in the case of a gene whose expression is confined to a small group of cells (e.g. the region where hypocotyl starts and apical hook ends), could create a difference in the observed regulation pattern. Overall the QRT-PCR results supports the organ-specific regulation pattern revealed by the microarray data, and hence suggest that light responsive organ-specific transcriptional regulation early in the transcriptional cascade is part of the mechanism underlying the tissue-specific photomorphogenesis.

**Functional annotation of the soybean cDNA microarray sheds light on the roles of organ-specific FRc responsive genes**

To allow further investigation of the biological meaning of the microarray data, functional annotation was generated for the array probes. Functional assignments of the 27 genes of interest are listed in Table II. We identified Arabidopsis orthologs for 21 out of 27 FR responsive genes, among which 16 genes have well-annotated functions while the other 5 have poorly-known functions. Among the well-annotated genes, some were previously reported to be involved in light signal transduction, such as Root Phototropism 2 (RPT2) (Glyma18g05720, Arabidopsis ortholog AT2G30520) (Sakai et al., 2000; Inada et
al., 2004) and ATHB-2 (Glyma11g03850, Arabidopsis ortholog AT4G16780) (Ohgishi et al., 2001). Some other genes are known as downstream effectors for light response, e.g. chalcone synthase (Glyma11g01350, Arabidopsis ortholog AT5G13930), which is involved in generation of protective anthocyanin pigments in response to light (Batschauer et al., 1991; Kubasek et al., 1992&1998), and early light-inducible protein (ELIP1) (Glyma20g28890, with Arabidopsis ortholog AT3G22840). Some other genes are involved in protein regulation and modification, e.g. the ubiquitin-dependent protein catabolic process (Glyma09g05180, Arabidopsis AT4G02570; Glyma20g38030, Arabidopsis AT1G09100) and protein phosphorylation/de-phosphorylation (Glyma12g13290, Arabidopsis AT4G28400), which are two known mechanisms of controlling protein activity in the light signaling pathway (Wei and Deng, 2003; Monte et al., 2007). Two genes (Glyma02g04170 and Glyma02g42500) that encode proteins with the domain with unknown function DUF231, including the freezing tolerance regulator Eskimo 1 (ESK1) (Xin et al., 2007), were both down-regulated by FRc. Six genes of the identified 27 FR responsive genes (22%) do not have a clear Arabidopsis homolog identifiable by TBLASTX with an e-value cutoff of 1E-6. The annotation of identified FR-responsive genes agrees well with our current knowledge of photomorphogenesis, confirming that our microarray experiment led to the identification of photomorphogenic regulators, and suggesting that not all photomorphogenesis-related genes in soybean have orthologs in Arabidopsis.

**Single channel analysis of the microarray data reveals organ specific gene expression pattern**

Spotted microarray data can be analyzed in multiple dimensions in order to compare samples across multiple microarrays, in addition to comparing samples physically hybridized into the same array (Dhaubhadel et al., 2007). To address whether the FR responsive genes were also genes that are expressed in an organ specific pattern regardless of light response, a single channel approach was taken to re-analyze the microarray data. The gene expression profile of one organ (either the Cy3 or Cy5 channel in one hybridization array) was compared to that of other organs (profiled in the Cy3 or Cy5 channel from a second array) in either FRc treated or dark control samples (Fig. 1). The microarray data was normalized and processed as described in methods. Statistical analysis was performed again using the rank product method with FDR cutoff 5% and a greater fold change, four fold cutoff, to minimize the influence of between-array noise and to identify organ-specific expression with confidence. Transcripts that are four or more fold more abundant in one organ relative to the other two organs and show statistical significance were considered to be organ-specific transcripts.
The analysis revealed that in etiolated seedlings, 459 genes are expressed at statistically significant, fourfold higher levels in the cotyledon with respect to apical hook and hypocotyl. These 459 represent the cotyledon-specific transcripts in seven-day-old etiolated seedlings. In seedlings treated with FRc for one hour, 559 transcripts are more abundant in the cotyledon compared to apical hook and hypocotyl, which represent the cotyledon specific transcripts in the seedlings exposed to 1h FRc. Comparing the two gene lists led to identification of the overlapping set of 389 transcripts, henceforth referred to as “cotyledon-specific genes” in this study, which represent genes with four-fold higher mRNA levels in cotyledon irrespective of light conditions (Fig. 4A & 4B; Supplemental Fig. S2A; Supplemental Table S1). With the same process 40 “hook-specific genes” and 262 “hypocotyl-specific genes” were identified (Fig. 4A & 4B; Supplemental Fig. S2B & S2C; Supplemental Table S2 & S3). We expected to see more cotyledon-specific genes than hook-specific genes and hypocotyl specific genes, because the cotyledon is distinct in morphology, function and organogenesis from the other two organs in question. The result agrees well with our expectation. Some of the organ-specific transcripts were selected and confirmed by replicated QRT-PCR experiments (The gene Glyma15g09750 was just short of the fourfold criterion for "hook specific" (FC of hook vs. hypocotyl as 3.64 fold) but was included because of its potential biological significance as an ARF6 like gene). The QRT-PCR results agreed well with those from the microarray (Fig. 4C) confirming the reproducibility of the single-channel analysis method.

We investigated whether Gene Ontology (GO) terms were over-represented in the genes that were significantly more abundant in each organ. For this analysis, we included all genes whose mRNA levels are significantly higher with respect to the other two organs with FDR < 5% (without the four-fold cutoff criterion) allowing greater statistical power for detection of over-represented GO terms. GO term annotations were assigned to soybean genes by determining the GO annotations of their Arabidopsis orthologs using the Arabidopsis Information Resource (TAIR) (Huala et al., 2001). The percentage of genes annotated with a given GO term in the organ-specific genes was compared to the percentage of genes annotated with the same GO term in the complete probe set of the soybean cDNA microarray, and the statistical significance of any difference assessed by means of the hypergeometric distribution. Raw P-values calculated using the hypergeometric method were then submitted to false discovery rate control for multiple-testing error with FDR <5%, to find the statistically significant GO terms. The over-represented GO terms in cotyledon specific genes include chloroplast thylakoid membrane (GO:0009535), chloroplast ribulose bisphosphate carboxylase complex (GO:0009573), oxygen evolving complex (GO:0009654), cysteine protease inhibitor activity (GO:0004869), and cytochrome b6f complex.
Taken together these terms indicate an expected predominance of photosynthesis-associated genes that are specific to the cotyledon (Table III). The higher expression levels of photosynthesis and chloroplast-related genes in the cotyledons are observed even before the seedlings are exposed to a light signal. This suggests that most components of the photosynthetic machinery are already expressed in a cotyledon-specific manner in the darkness. Hook specific genes are enriched with GO terms related to cell division and protein turnover (Table III). This could indicate that active cell division has a role in hook opening or subsequent development, especially considering the meristemic tissue is closely adjacent to the apical hook. The most significant GO term in hypocotyl specific genes was “cell-wall related genes”, which agrees well with an expected importance of cell wall synthesis activity in hypocotyls in either elongating etiolated seedlings or during seedling de-etiolation, where the elongation of the hypocotyl is inhibited (Table III). Overall, the significantly over-represented GO terms identified in the organ-specific genes agree well with our current knowledge of seedling photomorphogenesis.

We next compared the set of genes regulated by FR in an organ-specific manner and the set of genes expressed in an organ-specific pattern, to investigate whether the FR-responsive genes are also expressed in an organ-specific pattern. The result shows that there is little overlap between the two gene sets, with three exceptions. Glyma15g16190 and Glyma07g21150 are expressed in high abundance in the apical hook of etiolated seedlings (Supplemental Table S2), meanwhile they are down-regulated by FRc specifically in the apical hook. Glyma01g38590 is highly expressed in cotyledon and induced by FRc only in the cotyledon. However, the remainder of the FR responsive genes identified (24 out of 27) were not shown to be expressed at significantly higher levels in the organ where they show organ-specific regulation, suggesting there is no strong correlation between organ-specific gene expression and organ-specific transcriptional responses to light.

cis-regulatory elements identified in the promoters of organ-specific genes
The regulatory nucleotide sequence in gene promoters plays a key role in transcriptional response of plants to light stimuli (Hudson and Quail, 2003). A number of cis-regulatory elements have been characterized, and for light-responsive elements their specific binding to trans-regulatory factors in the phytochrome-mediated light signaling pathway is known in many cases, e.g. GATA, G-box, I-box and the CCA1 binding motif (Donald and Cashmore, 1990; Wang et al., 1997; Teakle et al., 2002). To search for potential organ-specific light-responsive cis-regulatory elements, two enumerative-approach-based motif-
finding tools, Sift and Elefinder, were used to identify over-represented promoter motifs in the microarray-identified co-regulated gene sets (a co-regulated gene set is a group of genes regulated by FRc in the same manner e.g. the same organ). Sift was developed for identifying over-represented promoter motifs in co-regulated Arabidopsis genes sampled from the Arabidopsis Affymetrix array (Hudson and Quail, 2003). In this study, we used an updated version of Sift, which allows the detection of motifs including degenerate nucleotides and with more rigorous statistics, with promoter sequences from the now-completed genome of soybean (Schmutz et al., 2010). Elefinder is a new program similar to Sift but rather than detecting new motifs, it is designed to detect previously characterized motifs that are over-represented. Both tools are available at http://stan.cropsci.uiuc.edu/tools.php. The abundance of a motif in promoters of co-regulated genes was compared to the abundance of the same motif in promoters of all the genes presented on the microarray, using both Elefinder and Sift. Motifs that are significantly more abundant in co-regulated gene sets with respect to the rest of the microarray were determined by first calculating a P-value using the hypergeometric method, and then correcting for multiple tests by using FDR < 5%.

We investigated the over-represented motifs in the co-regulated genes identified by the microarray experiment at multiple levels, by testing sets of promoters from FR responsive genes, FR up-regulated genes, FR down-regulated genes, organ-specific FR responsive genes and organ specific genes. Fig. 5 shows the significantly over-represented known regulatory motifs identified in those groups by Elefinder. Significantly over-represented motifs in the 27 FR responsive genes include the formerly characterized GATA binding site and CCA1 binding site, which are known light-responsive motifs (Fig. 5A) (Wang et al., 1997; Teakle et al., 2002). FR up-regulated genes and FR down-regulated genes have distinct over-represented motifs in their promoters (Fig. 5B & 5C), suggesting the same light signal is transduced through two separate pathways leading to positive and negative regulation of downstream effectors. In the promoters of FR up-regulated genes, the GATA binding motif and CCA1 binding motif are again over-represented, as well as another formerly described light responsive motif, I-box (Donald and Cashmore, 1990). In the promoters of FR down-regulated genes, SORLREP1, first reported as a light-repressed motif by Hudson and Quail in 2003, is the most over-represented motif (Fig. 5C). Motif analysis also revealed the most over-represented motif in FRc-induced genes is distinct in different organs. The I-box was most over-represented in the promoters of cotyledon FR responsive genes, the CCA1 binding motif in the hook and the GATA binding site in hypocotyl (Fig. 5D, 5E & 5F). This suggests that distinct interactions between transcription factors and cis-regulatory elements occur in
different organs and tissues in response to light stimulus.

The significant motifs identified in organ-specific FR-responsive promoters could either be involved in FR-induced photomorphogenesis, or simply over-represented in promoters of genes expressed in the organ in question. To address this issue, we prepared the list of over-represented cis-regulatory motifs in genes from the “organ specific” gene lists, and compared it with the motifs associated with organ-specific FR regulation. In cotyledons and hook, organ-specific gene expression and organ-specific FR gene regulation are associated with different motifs (Fig. 5), therefore, the light responsive motifs identified in cotyledon and hook are likely to be truly light responsive. Note that SORLIP1, a motif known to be over-represented in light-induced genes (Hudson and Quail, 2003), appears in this experiment to be specific to cotyledon-expressed genes and not to light-induced genes. The most over-represented light responsive motif in hypocotyl, the GATA motif, is also observed to be present at significantly higher levels in hypocotyl specific gene promoters (Fig. 5I), while the mean number of GATA motifs per promoter in hypocotyl FR responsive genes is higher than that in hypocotyl specific genes. The hitherto undescribed differentiation of known light-regulatory motifs into cotyledon, hook and hypocotyl has several implications for the mechanism of photomorphogenesis in different tissues.

In addition to searching for known motifs in co-regulated gene groups using Elefinder, we also analyzed all 6, 7, 8 and 9mers in those promoters using Sift (Hudson and Quail, 2003; Walley et al., 2007), to search for novel cis-regulatory motifs. A new motif, TGNGCNANT, was identified as significantly over-represented in FR up-regulated gene promoters. Another motif, CNACGTGG, which shares strong similarity with the known light responsive element G-box, was identified as significantly over-represented in the cotyledon specific gene expression (Table IV). No other motifs were identified as significant using the FDR cutoff at 5%. The fact that most of the motifs identified as significant by Elefinder were not detected by Sift is likely a result of the relatively low statistical power of Sift when corrected for false discovery, since Sift examines millions of putative motifs while Elefinder examines only hundreds of known motifs.

**Identification of new signaling factors in FRc regulated photomorphogenesis**

The microarray experiment allowed the identification of genes that are regulated by light in an organ-specific manner. We then investigated whether any genes identified can be shown to play an organ-specific role in FRc-induced photomorphogenesis. Because the apical hook has a critical role in seedling
de-etiolation but the mechanism underlying hook opening is poorly understood, we investigated genes involved in the hook-specific transcriptional regulation.

Glyma18g05720 is up-regulated in response to FRc with a larger fold change in apical hook than in cotyledon or hypocotyl, as indicated by the microarray and confirmed by QRT-PCR (Table I; Fig. 2B and Fig. 3). Glyma18g05720 encodes a likely ortholog of the Arabidopsis Root Phototropism 2 (RPT2) (Sakai et al., 2000; Inada et al., 2004), hence the protein encoded by this transcript is referred to as GmRPT2L (for *Glycine max* RPT2 like) hereafter. GmRPT2L shares 66.4% amino acid identity and 89.5% similarity with Arabidopsis RPT2. RPT2 was first reported as a positive regulator downstream of PHOT1 in the blue light signaling pathway of root phototropism (Motchoulski and Liscum, 1999; Sakai et al., 2000) and later shown to mediate light-induced stomatal opening by associating with PHOT1 (Inada et al., 2004).

To better understand the role of RPT2 in FRc-induced photomorphogenesis, we studied the spatial expression pattern of RPT2 during de-etiolation. The GUS-reporter system was used to test the expression pattern of pRPT2:GUS (Inada et al., 2004) in 1h FRc treated Arabidopsis seedlings as well as dark-grown seedlings (Fig. 6A-F). In darkness, the promoter of RPT2 drives GUS expression in the concave side of the hook and the root tip, as well as in cotyledons (Fig. 6A-C). The spatial expression pattern of RPT2:GUS in 1h FRc treated seedlings is very similar to that in the etiolated seedlings (Fig. 6D-F). Because one hour is unlikely to be long enough for distribution of the GUS protein to change significantly, we further examined the GUS expression pattern in seedlings treated with 4h FRc. The GUS activity in the hook expanded from the concave side of the hook to the convex side in 4h FRc treated seedlings (Figure 6G-I), which agrees well with the observed induction of RPT2 in the apical hook overall in response to FRc.

To investigate any role of RPT2 in FRc-induced photomorphogenesis, we exploited the available Arabidopsis mutant resources to obtain two EMS-induced alleles of *rpt2*, *rpt2-1* (null mutant, Sakai et al., 2000) and the previously undescribed *rpt2-101* (where a G-A transition at position 115 in the open reading frame causes a G39R substitution). Seed of *rpt2-101* was obtained as a TILLING line from the Arabidopsis Biological Resource Center at the Ohio State University with accession number CS91521 (Till et al., 2003). Seeds of these lines were first grown in darkness for 3 days and then given 10μM·s⁻¹·m⁻² FR light for 24 or 27 hours. After transfer to FRc the angle of cotyledons and apical hook were
measured every three hours for \textit{rpt2-1} and once per hour for the first 12 hours followed by once every three hours for \textit{rpt2-101} (since \textit{rpt2-101} displays a more rapid opening curve compared to \textit{rpt2-1}). The background accessions of the two mutant lines (\textit{Landsberg erecta} for \textit{rpt2-1} and \textit{Col er105} (Big Mama, Torii et al., 1996) for \textit{rpt2-101}) were also included in the assay as controls. The result suggests that although the mRNA for \textit{RPT2} is regulated by FR more strongly in apical hook than the other two organs, the light-induced hook opening rate of \textit{rpt2} mutants is similar to that of the wild type. However, \textit{rpt2-101} showed an altered hook angle in darkness (Fig. 7B & 7D). For both \textit{rpt2} mutants, cotyledon opening is significantly faster in the mutants compared to their corresponding wild type (Fig. 7A & 7C). \textit{RPT2} is thus necessary for normal photomorphogenesis in FRc. The more rapid opening of the cotyledon in the mutants may indicate a negative regulatory role of \textit{RPT2} in seedling photomorphogenesis.

**DISCUSSION**

**Spatial specificity of gene regulation in photomorphogenesis**

Using microarrays, it is now possible to study the role of spatial specificity of gene regulation in photomorphogenesis, as others have done (Ma et al., 2005; Lopez-Juez et al., 2008). In our study, we were able to use the large seedling size and genomic resources available for soybean to show that several genes are expressed at significantly higher levels in cotyledon, hook or hypocotyl. When the genes responsive to FRc within one hour are considered, some genes show stronger responses in one organ than in other organs. We identified more organ-specific genes than FRc-regulated genes in this experiment, which is not surprising considering the large differences in the biological roles the three organs play. The cotyledons showed a larger number of organ-specific genes, consistent with the special role played by the cotyledon in energy supply. Hook and hypocotyl cells have similar fates in seedling development and thus fewer genes are expected to be specific to one of these two tissues. Such organ-specific expression and regulation of gene expression provides a reasonable explanation for the mechanism of organ-specific photomorphogenic responses.

In our study we identified 27 genes that were regulated in cotyledons, hook and hypocotyl by 1h FRc. Seven genes were repressed and 20 genes were induced. A previous study (Tepperman et al., 2001) performed on Arabidopsis whole seedlings using an Affymetrix assay identified 56 genes induced and 6 genes repressed by 1h FRc via phyA. Among the 56 genes, transcription factors (TFs) were significantly enriched. We identified a similar trend of repression vs. induction, but a smaller number of regulated
genes and fewer transcription factors. A comparison of our regulated gene list with the Arabidopsis gene list based on orthologous genes showed that: (1) 2 out of the 27 genes, RPT2 and ATHB2, were also reported to be regulated in the same direction by FRc in the Arabidopsis affymetrix data; and (2) the majority of the 27 genes were not identified as being regulated in the Arabidopsis Affymetrix data. A few differences in experimental approaches may have contributed to this. Firstly, a biological replicate in our microarray experiment contained tissues from a single seedling to enhance confidence in true positives by intentionally allowing between-individual variance to be measured. However, this could lead to false negatives due to higher noise, especially for low-expression genes such as transcription factors. In contrast, Tepperman et al. used a pool of many Arabidopsis seedlings as a single biological replicate. Secondly, Tepperman et al. used an Affymetrix array while in this study a mechanically spotted array was used. Overall the level of noise and the resolution provided by the array differ substantially between the two experiments. Moreover, Tepperman et al. reported 62 early responsive genes of which 21 are TFs. Of these 21 TF genes only 11 are represented by soybean orthologs in the soybean cDNA microarray. Six of these 11 genes showed consistent repression/induction by FRc greater than 1.6 fold in our array data (thus we consider them to be confirmed in both species) while the others showed smaller fold changes, possibly due to array noise as described earlier. Two of these six transcription factors were reported in our study as significantly responsive genes (RPT2 like and HAT4/ATHB2 like). Therefore, the correlation of the two gene lists may be greater than apparent at first sight. The discrepancies were most likely caused by the fold-change/FDR cutoff due to the sensitivity of the microarray and the stringency of the statistical methods applied. That our study identified some FR-regulated genes that were not reported to be responsive in the Tepperman et al. study might be due to the difference in spatial resolution (organ level versus the whole seedling). Although the fundamental mechanisms of photomorphogenesis are likely conserved among dicots, timing and magnitude differences in light responsive gene regulation between Arabidopsis and soybean might exist and might also account for differences in the significant gene lists.

The difference in over-represented cis-regulatory motifs in different organs provides more insight into the mechanism of organ specification, suggesting that organ-specific interaction of transcription factors and cis-regulatory motifs occurs, even for motifs already known to mediate light responses. This finding suggests that photomorphogenic regulatory networks vary in different organs and tissues. Such distinctive, tissue-specific regulatory networks provide a mechanism for the organ-specificity of seedling photomorphogenesis at the tissue level. SORLIP1, which was reported to be a motif over-represented in light-induced genes, appears in this experiment to be specific to cotyledon-expressed genes and not to
light-induced genes. It is hard to distinguish between a cotyledon-specific motif and a light-inducible, photosynthesis-related motif in this context. The FR-regulated genes described here are regulated two-fold within one hour at organ-specific level, but the dataset in which SORLIP1 was discovered consists of genes induced by FR within 24 hours in the whole seedlings (Hudson and Quail, 2003). Thus, this motif could be correlated with genes that are light responsive beyond the one-hour time point.

The apical hook and the role of RPT2

The apical hook displays distinct morphological behavior in de-etiolation. As a result of the interplay of light stimulus and hormonal regulation, the apical hook shows localized cell expansion, leading to unfolding of the hook. However, apical hooks were not included in the previous organ-specific light response studies (Ma et al., 2005; Lopez-Juez et al., 2008), presumably due to the extremely small size of the hook in Arabidopsis seedlings. In our study, we took advantage of the relatively large soybean seedlings to show a list of genes regulated by FRc in the apical hook in dicot seedlings for the first time. However, the apical hook region we dissected was still necessarily heterogeneous, limited by how visually distinguishable the organs in question are, the need for a sample of large enough size for effective RNA isolation, the need to rapidly process the samples to avoid RNA degradation and touch responses, and the maintenance of consistent light conditions. First, the concave and convex sides of the hooks play very different, almost opposing roles during hook opening. For the apical hook to open, the concave side of the hook has to show active growth, primarily by cell elongation, while the convex side of the hook has to show slower or no cell wall expansion. Cambial cell division may also be asymmetric. Second, the hook section of the seedling as used in this study contains the shoot apical meristem and also likely some hypocotyl tissue. Since the shoot apical meristem and leaf primordia, with many cells undergoing cell fate determination and rapid expansion, were included in apical hook tissue, the “hook” sample contains the most rapid regions of cell division. This provides an explanation of why the largest number of FRc responsive genes were detected in the apical hook region, and that the over-represented GO terms for the hook specific genes are related to cell division and protein turnover. Also, the junction of the apical hook and hypocotyl is not a clearly defined line that could be seen visually. Thus, the hook sample may contain a variable number of cells where gene regulation responsible for the hypocotyl elongation is active. This may explain the discrepancy between QRT-PCR and microarray result for gene Glyma11g03850 (ATHB2-like), whose organ specificity is flipped between the hook and hypocotyl in these experiments.
To further investigate whether organ-specific transcriptional regulation can influence photomorphogenesis, we investigated Arabidopsis mutants in a gene with a known role in light-regulated cell elongation (RPT2/Glyma18g05720) to determine whether hook-specific developmental defects were present. This mutant has a known role in tropic responses, but no previously known effects on phytochrome-mediated, non-directional photomorphogenic responses. A missense mutant allele of *rpt2* showed an altered hook angle in darkness (Fig. 7D). The missense mutation in this gene may be affecting other systems as a result of gain of function, or alternatively, this mutation may have a dominant negative effect over redundant similar genes. Both the missense and stop codon alleles displayed faster cotyledon opening (Fig. 7A&C). This result indicates that this gene does play a role in the morphogenesis of the apical zone but that it is likely more important for cotyledon angle than for hook angle. Therefore, while a gene expression profile in the apical hook does not always predict a mutant phenotype in that structure, a previously undescribed phenotype for *rpt2* was observed, which is consistent with a role of this gene in photomorphogenesis of the apical zone during de-etiolation. Since cotyledon opening and hook opening are both rapid responses in the apical area mediated largely by cell expansion, it is likely that these responses are related and possible that the cotyledon-opening response is the more sensitive of the two to perturbation of regulatory factors. Alternately, it is also possible that a signal causing cotyledon opening originates in the hook and is transported to the base of the cotyledons, thus affecting cotyledon opening. Such intercellular signaling, induced by phytochrome, between different tissues has been reported in tobacco (Bischoff et al., 1997). A careful examination of the spatial gene expression pattern of RPT2 in the seedlings at the developmental stages concerned in this study was thus necessary.

We therefore examined the spatial expression pattern of *RPT2*, with Arabidopsis transgenic plants carrying the pRPT2:GUS reporter system. In dark-grown seedlings, the GUS signal was observed most strongly in the hook, cotyledons and roots (Fig. 6A-C). The signal in the hook was asymmetric with a strong bias towards the concave side of the hook. After giving etiolated seedlings 1h FRc, no strong change was observed (Fig. 6D-F), although the microarray and QRT-PCR suggested a major induction of RPT2 in the hook. This is not unexpected, because the microarray and QRT-PCR assay examined the mRNA level of RPT2, while the GUS assay examined a combination of transcriptional and translational regulation of RPT2. Therefore, one hour is likely too short for the GUS protein levels to change significantly, even though the transcriptional regulation likely occurs as early as 1h. We thus also examined the GUS expression pattern of RPT2 after 4h of FRc treatment. The region strongly stained by GUS expanded from the hook concave region to the hook convex region, as well as in the direction of
hypocotyl, in 4h FRc treated seedlings (Fig. 6G-I). This agrees well with the induction of RPT2 in response to FRc in the apical hook observed by microarray and QRT-PCR.

Given the evidence described above, we postulate that RPT2 is expressed preferentially in the concave side of the hook, cotyledons and root in dark grown seedlings. When stimulated by FRc, RPT2 is significantly induced by light in the apical zone of the seedlings, with an expansion of the region of expression from the concave side of the hook to the whole hook (Fig. 2B, Fig. 3 and Fig. 6G-I). The spatial expression and light induction of RPT2 in the apical zone agrees well with its proposed role as a signaling factor in cotyledon and hook opening. Since mutants in RPT2 open their cotyledons more quickly, RPT2 may function to fine-tune the speed of cotyledon opening to prevent damage to seedlings caused by premature opening of the cotyledons. The signal of pRPT2:GUS is strongest in the concave side of the hook in dark-grown seedlings and the gradient disappears in FRc, consistent with a role in the suppression of cell expansion in the concave region of the hook in darkness. However, the hook opening of one allele of the rpt2 mutant is comparable to that of wild type. A potential explanation is that the function of RPT2 in hook opening is redundant, therefore more robust to perturbation. It is also possible that RPT2 does not control hook opening but rather functions as a sensor of the hook opening process to signal the subsequent cotyledon opening. RPT2 has already been shown to be involved in the blue light responses of root phototropism and stomatal opening in the leaf (Sakai et al., 2000; Inada et al., 2004). This multiplicity of roles suggests that RPT2 acts downstream of multiple photoreceptors to control differential cell expansion responses, but plays little role in determining organ development. Instead, spatio-temporal changes in RPT2 expression are likely interpreted within the organellar and/or developmental context.

**Novel regulatory factors in photomorphogenesis**

Several more genes of interest were identified from the organ-specific regulated gene list and the organ-specific expressed gene list, further study of which may lead to a better understanding of seedling de-etiolation. Glyma02g42500 encodes a protein similar to ESK1/AT3G55990 (67% identity and 83% similarity), which is a negative regulator of cold tolerance in Arabidopsis (Xin and Browse, 1998; Xin et al., 2007) (Table II). ESK1 contains a conserved domain of unknown function (DUF) 231. In our study, Glyma02g42500 was repressed significantly by 1h FRc (Table I), indicating that cold-tolerance was induced by FRc. The possible adaptive value of crosstalk between the cold tolerance and light regulation responses has been reported (Franklin and Whitelam, 2007). An increased FR: R ratio (as a result of FR
being preferentially transmitted from low-angle sunlight) may indicate a shorter day length and longer twilight period as winter approaches (Franklin and Whitelam, 2007). Therefore, induction of cold tolerance genes by FRc may help plants prepare for winter. In the case of germination and seedling establishment, as our study imitates, a higher FR:R ratio may be an indicator that germination is in a period of low-angle sunlight where cold tolerance is required for the plants to survive. Interestingly, another gene containing a DUF231 domain, Glyma02g04170, was also down regulated by 1h FRc in our study (Table I&II). The significant selection advantage to be gained by early germination and establishment (e.g. canopy penetration) could have lead to the development of interaction between cold acclimation and light adaptation responses.

Another interesting finding is that Glyma09g05180, regulated by FRc specifically in the apical hook (Table I; Fig. 2B and Fig. 3), encodes a cullin1-like protein (CUL1-like). The soybean CUL1-like protein shares 82.7% identity and 96.1% similarity with AtCUL1 (AT4G02570) by protein-protein Smith-Waterman alignment. CUL1 is a key subunit of the ubiquitin protein-ligase (E3) complex SCF (SKP1-CUL1-F-box), which specifies the substrate proteins for 26S proteasome in the ubiquitin/26S proteasome pathway (Pintard et al., 2004). SCF complex-associated protein degradation controls the turnover of important regulatory proteins in light signaling, including the light receptors (Dieterle et al., 2001; Harmon and Kay, 2003; Wei and Deng, 2003; Franklin et al., 2005). An Arabidopsis mutation in CUL1 was reported to display hypersensitivity to far-red light and delay in phyA degradation in response to far-red light (Quint et al., 2005). In addition, the SCF complex was also shown to degrade AUX/IAA proteins under auxin stimuli (Gray et al., 2001). Auxin is known to play a key role in apical hook maintenance (Liscum and Reed, 2002; Zádníková et al., 2010; Vandenbusshe et al., 2010). Therefore, CUL1 may be involved in the apical hook opening processes, either by targeting photomorphogenic regulators for degradation, or recruiting auxin-responsive factors for degradation. It may also act as a cross-talk point for hormone pathway and light signaling pathway in the apical hook opening process.

CONCLUSION

Taken together, our data demonstrate that even the transcriptional responses to FRc that occur within 1h of illumination are organ specific and developmentally regulated. The expressions of several genes show a light response that is specific to one or more organs. In addition, many genes show tissue-specific expression during photomorphogenesis. Thus, even studies of early signal-transduction events in
phytochrome signaling should be informed by the knowledge that the transcriptional networks and cascades mediating photomorphogenesis are likely to be distinct in different plant tissues. We have demonstrated that organ-specific profiling can be helpful in predicting the morphogenic roles of genes involved in spatially-controlled developmental processes, and that organ-specific genes regulated by a light stimulus can be correlated with a distinct subset of known cis-regulatory elements.

METHODS

Plant material and light treatment
For the microarray experiments, etiolated soybean seedlings (Glycine max cv. Williams 82) were first surface sterilized in a solution of 5% Clorox and 0.1% Tween 20 for 10 minutes, and then grown hydroponically in water, with sterile glass beads as the solid matrix, in transparent Magenta boxes at 20°C in darkness for 8 days. Seedlings were irradiated with FRc (peak 733nm) generated by Snap-Lite Light Emitting Diode arrays (Quantum Devices, Inc, Barneveld, WI). The FRc irradiation was given at 24.7 μmol m-2 s-2 for one hour. FRc treated samples were harvested and immediately frozen in liquid nitrogen at one hour after the start of irradiation. Dark control samples were harvested at 0 h time point, before the beginning of the irradiation. Plant material for the QRT-PCR experiment was prepared under the same conditions except that seedlings were grown in compost (Sunshine Mix LC1). For the phenotypic study of rpt2, two lines, rpt2-1 (Sakai et al., 2000) and rpt2-101 (CS91521 from the Arabidopsis TILLING project; Till et al., 2003) were used. All genotypes were verified with PCR amplification with gene-specific primers and sequencing through the putative SNP region. rpt2-1 was compared to its background accession Landsberg erecta (CS20 from ABRC), rpt2-101 was compared to its background accession Col er105 Big Mama (CS89540). The seeds of mutants and their controls were surface sterilized by means of chlorine gas sterilization, then planted on MS agar plates and stratified for 5 days in darkness at 4°C. Then plates were treated with 120μmol•m-2•s-1 white light for 3 hour to synchronize germination. Seeds were kept in dark for 72 hours to germinate, then treated with 10 μmol m-2 s-1 FR light for 24 hours (for rpt2-1 the treatment continued to 27 hours). Pictures of seedlings were taken repeatedly of the same plants either once per hour or once per three hours under green safelight (Cool fluorescent light through color effect filters 119, 116 and 101 (Lee filters); supplemental Fig. S3) for measurement of cotyledon opening and hook opening. At least 30 seedlings of each genotype were included in each replicate of the experiment to increase the statistical power of the measurement. The mutant and its corresponding control lines were planted in the same plate for reliable comparison and at
least two plates are included in each comparison for better replication. Experiments were repeated at least twice to verify the results.

**RNA extraction**

For the microarray experiments, each seedling was frozen immediately in liquid nitrogen and transferred to RNAlater®-ICE (Ambion, Austin, TX) which was pre-cooled to -80°C. The purpose of this step was to fix samples while at the same time softening the samples to facilitate dissection. Frozen, fixed seedlings were then dissected into cotyledon, hypocotyl and apical hook regions (roots were discarded). RNA was isolated from the three different parts of an individual seedling in quadruplicates following the pine tree method (Chang et al., 1993) except for some minor modifications: The homogenization was performed using Ultra-Turrax T8 Homogenizers (IKA, Wilmington, NC) at top speed for one minute; phase lock gel (Eppendorf, Westbury, NY) was used to facilitate the chloroform:isoamyl alcohol extraction. For QRT-PCR, pools of 8 seedlings were harvested in triplicates from both dark control and one hour FR treated samples and immediately cryofrozen in liquid nitrogen. Seedlings were dissected into cotyledon, hypocotyl and apical hook regions on dry ice and then the same modified pine tree method was used for RNA isolation.

**Microarray**

Pairwise comparison of FRc vs. dark was performed with the 18K soybean cDNA arrays (Vodkin et al., 2004). Each set of the 18K soybean cDNA array contains two slides 18kA and 18kB; together they present a low redundancy set of approximately 36,000 sequenced cDNAs. Three two-color pairwise comparisons, each for one organ, were carried out between FRc treated samples and dark control samples in quadruplicates (Fig. 1). Each biological replicate is a total RNA sample extracted from one dissected organ from one single seedling. By examining the expression profile of a single seedling we wanted to detect gene regulation events consistent among all individuals. By contrast, significant changes due to a small number of responsive individuals in a pool of seedlings could cause false significant fold change of gene expression level. A dye-swap was included in the experimental design to control for the possible bias caused by labeling methods. The 3DNA Array Detection Array Kit (Genisphere Inc, Hatfield, PA) was used for cDNA synthesis, labeling and microarray hybridization. The 3DNA Array Detection Array 900™ Kit was used for labeling RNA samples from apical hook and hypocotyl, and the Array 50™ Kit was used for the cotyledon samples, because the Array 900™ kit affords better sensitivity for samples where mRNA is limiting (due to the limited amount of total RNA one can extract from the apical hook or
hypocotyl from one single seedling), at the expense of higher noise levels. Arrays were scanned with a Packard ScanArray Express scanner (PerkinElmer life sciences, Waltham, Massachusetts) to generate the false color array images. Images were then processed with Genepix Pro 4.0 (MDS Analytical Technologies, Concord, ON) to generate Genepix Results files (which contain general information on image acquisition and analysis, as well as the raw data of channel F635 (for Cy5) and channel F532 (for Cy3) extracted from each individual feature). F635 Median and F532 Median were used for the data analysis: A Perl script merge.pl was obtained courtesy of Min Li and Steven Clough (USDA-ARS and University of Illinois) and adapted to extract F635 median and F532 median values from the GPR files and perform data preprocessing including removing empty/blank spots and low expression features. Data was normalized using GLOWESS in the MAANOVA package, part of the Bioconductor package for the R computing language and environment, to generate normalized relative expression matrices (R Development Core Team, Vienna, Austria). Expression levels of each gene were then compared between dark control and FR treated samples in individual organ in order to determine which genes showed statistically significant changes, by using the rank product analysis method (Breitling et al., 2004) at a false discovery rate (FDR, Benjamini and Hochberg, 1995) cutoff of 5%. The above-described analysis methods were automated using an in-house Perl script, which carries out the entire data analysis pipeline automatically from GPR files to rank product statistical analysis. In order to refine our analysis to those transcripts showing strong changes in expression that are likely to be biologically significant, only genes showing expression changes greater than 2-fold that were also statistically significant were defined as “FR-responsive genes”.

Organ-specific gene expression was determined using an alternative approach with single channel data (F635 median or F532 median). Single channel information representing median gene expression level from cotyledon, apical hook and hypocotyl were compared across organs in both dark-grown seedlings and FRc treated seedlings (Fig. 1). Raw data were first preprocessed to remove empty/blank spots and flagged data. Normalization across all channels was then performed by first multiplying each channel by a specific constant to make the mean intensity the same for each individual channel, and then converting to base 2 log values. Another script was written and used to remove spots with expression value less than the negative control before submitting the data for differential expression analysis. Genes that are significantly more highly expressed in one organ compared to the other two organs were defined as “organ-specific genes” by the rank product method (FDR =5%, FC cutoff: 4-fold).
**Microarray Annotation**

The 18k cDNA soybean microarrays are supplied with annotation derived from a BLASTX search against the nonredundant protein database using 5’ and 3’ sequences of the cDNA clones (cutoff E value: 10E-6) (Vodkin et al., 2004). However, many of these annotations are outdated or absent. A combination of the annotation of soybean chromosome scale assembly ([ftp.jgi-psf.org/pub/JGI_data/phytozome/v5.0/Gmax/annotation/initialRelease/Glyma1.cDNA.fa.gz](ftp.jgi-psf.org/pub/JGI_data/phytozome/v5.0/Gmax/annotation/initialRelease/Glyma1.cDNA.fa.gz)) (Schmutz et al., 2010) and the soybean gene index (Quackenbush et al., 2001) followed by BLASTX of these sequences against the TAIR database ([www.arabidopsis.org](http://www.arabidopsis.org)) and the plant protein database ([http://www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/)) was used to generate additional annotation for the 18k cDNA microarray (Supplemental Table S4). The new method was able to provide information on protein function of 5327 out of 7950 previously “unknown genes” from the previous annotation spreadsheet supplied with the soybean microarray.

**Quantitative real-time reverse transcription PCR**

Primers for QRT-PCR were designed using Primer Express v2.0 (Applied Biosystems, Foster City, CA) based on the soybean EST sequences corresponding to cDNA microarray probes and were then used to search against the JGI soybean chromosome scale assembly (Soybean Genome Project, DoE Joint Genome Institute) to ensure the specificity of the primers. Four control genes derived from array probes, ubiquitin (Glyma20g27950), α-tubulin (Glyma04g09350), β-tubulin (Glyma04g02610) and Histone H3.2 Minor (Glyma14g40590) were chosen from the microarray as candidate reference genes because they showed relatively constant expression between different light conditions in the microarray data (data not shown). Phosphoenolpyruvate carboxylase (PEPC, Glyma13g36670) was chosen as an additional candidate reference gene, specifically for organ-specific gene verification, because it has been shown to be expressed constantly across different tissue types (Tuteja et al., 2004). These five candidates were tested for their stability in expression level across three organs in both FRc and dark conditions using QRT-PCR. Histone H3.2 Minor was chosen to be the reference gene because it was most consistently expressed in different organs in both experimental conditions among the five candidates (Supplemental Fig. S1). Residual genomic DNA was removed from RNA sample using TURBO DNA-free™ (Ambion, Austin, TX). First strand cDNA synthesis was accomplished using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, California). QRT-PCR was performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) and Mx3000P QPCR system (Stratagene, La Jolla, CA). Amplification efficiencies of all tested genes including reference candidates were determined by dilution
series. QRT-PCR products were analyzed using 3% agarose gel electrophoresis to ensure specific amplification of a single product. Data were analyzed using the $\varepsilon$-∆∆CT method with the experimentally determined amplification efficiency incorporated (Livak and Schmittgen, 2001). Additionally, analysis with multiple reference genes was performed using the geNorm method (Vandesompele et al., 2002). GeNorm analysis and results from the $\varepsilon$-∆∆CT method were directly comparable (data not shown).

**Promoter Motif Analysis**

Promoter motif analysis was performed using promoter motif analysis tools, Sift and Elefinder, to search for over-represented cis-regulatory elements in the promoters of co-regulated genes (a co-regulated gene set is a group of genes regulated by FRc in the same manner e.g. same organ or same direction) identified by the microarray experiments (Hudson and Quail, 2003). The abundance of motifs in the promoters of co-regulated genes was compared with the abundance of motifs in the promoters of all probes in the soybean cDNA microarray using the hypergeometric distribution combined with false-discovery rate control at 5% to identify over-represented motifs. The extraction of promoter sequences of soybean genes was facilitated by the Soybean Genome Project Glyma 1.0 gene set and the soybean chromosome-scale assembly (Soybean Genome Project, DoE Joint Genome Institute). The EST sequences of cDNA microarray probes were used to search against Glyma 1.0 predicted gene models by BLASTN (Supplemental Table S4, e-value cutoff: 1e-10, identity $\geq$95%). The resultant top hits are the corresponding predicted gene models of cDNA microarray probes (named the 31k set herein as it contains about 31,000 transcripts). An in-house Perl script was used to extract 2kb upstream genomic sequence from the starting site of each mRNA in the 31k set. The promoter sequences of co-regulated genes were compared with the promoter sequences of 31k set using Sift (Hudson and Quail, 2003) to identify any significantly over-represented motifs with the size of 6nt to 9nt, and Elefinder (http://stan.cropsci.uiuc.edu/tools.php) to search for significantly over-represented motifs that have been reported before.

**GUS staining assay**

The Arabidopsis pRPT2:GUS line was produced and kindly provided by Dr. Tatsuya Sakai (Inada et al., 2004). The seeds were first surface sterilized by chlorine gas sterilization, then planted on ½ MS agar plates without sucrose and stratified for 4 days in darkness at 4°C. Seeds were then treated with 120μmol•m⁻²•s⁻¹ white light for 2 hours to synchronize germination. Seeds were kept in darkness for 72
hours at 20˚C to germinate, and then treated with 20µmol m⁻² s⁻¹ FR light while the dark controls were maintained in darkness. After the FRc treatment, the GUS staining was performed with FRc treated seedlings and dark controls with a protocol previously described (Campisi et al., 1999). The staining was stopped after 3h. Photomicrographs were taken in whole-mount bright-field and phase contrast illumination.

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Figure 4. Identification and QRT-PCR confirmation of organ-specific transcripts. A. Organ-specific transcripts in dark-grown etiolated seedlings and seedlings treated with 1 h continuous far-red light (FRc) were identified by microarray using a single channel approach and rank product method. The intersection of these two groups represents the transcripts which are consistently organ-specific in this experiment, used hereafter as the “organ specific” gene lists. B. Heatmap showing normalized expression values of
organ-specific genes across three organs in two different light conditions with four biological replicates per organ per light condition. Yellow indicates high expression while orange indicates low expression. C. Confirmation of organ-specific expression by QRT-PCR. In each graph the relative expression levels (normalized to the mean expression level in all three organs) derived from the microarray (red lines) and QRT-PCR data (blue columns) were plotted for cotyledon, hook and hypocotyl. The error bars represent standard error of the mean of all biological replicates.

**Figure 5. Over-represented motifs involved in organ-specific and continuous far-red light responsive gene expression.** Known *cis*-regulatory motifs were detected in promoters of organ-specific and continuous far-red light (FRc) responsive genes using the Elefinder software. Motifs that were over-represented in genes identified in this study as FRc-responsive or organ-specific to a statistically significant degree were identified using a hypergeometric distribution-based algorithm. This analysis was conducted with the following gene lists: (A) genes that are responsive (in either direction) to 1h FRc; (B) genes that are up-regulated by 1h FRc; (C) genes that are down-regulated by 1h FRc; (D) genes that are responsive to 1h FRc in cotyledons; (E) genes that are responsive to 1h FRc in the apical hook; (F) genes that are responsive to 1h FRc in the hypocotyl; (G) genes that are expressed at higher levels in cotyledons compared to the apical hook and hypocotyl; (H) genes that are expressed at higher level in apical hook compared to the cotyledons and hypocotyl and (I) genes that are expressed at higher level in the hypocotyl compared to the cotyledons and apical hook. In each graph the mean number of motifs per promoter of the genes in the co-regulated gene set (solid grey) was compared to the mean number of motifs per promoter of the soybean cDNA microarray probe set (hatched).

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continuous FR, showing loss of the differential expression gradient across the apical hook.

**Figure 7. Angle of cotyledon and apical hook in *rpt2* mutants compared to their background accession (WT) during growth under continuous far-red light (FRc).** Three-day old dark-grown seedlings of *rpt2-1* (A&B) and *rpt2-101* (C&D) seedlings and their corresponding background accessions were transferred to FRc. The angle between the cotyledons (A&C) and the hook angle (measured as the angle between the cotyledon axis and the hypocotyl) (B&D) of both mutants and WT were measured at three-hour intervals, and at one-hour intervals in the most rapidly changing part of the curve. The same seedlings were measured repeatedly under dim green safelight, then returned to the continuous FR treatment. The error bars represent the standard error of the mean.
### TABLE I. FRc responsive genes identified by microarray analysis.

| Glycine max Identifier | Organ   | Log2 Fold Change\(^a\) | False Discovery Rate (%) |
|------------------------|---------|------------------------|--------------------------|
| Glyma02g42500          | Cotyledon | -1.86                  | 0                        |
| Glyma13g37320          | Cotyledon | 1.04                   | 0                        |
| Glyma01g38590          | Cotyledon | 1.22                   | 0                        |
| Glyma06g14170          | Cotyledon | 1.38                   | 0                        |
| Glyma11g03850          | Hook     | -1.31                  | 0                        |
| Glyma08g28730          | Hook     | -1.12                  | 0                        |
| Glyma15g16190          | Hook     | -1.1                   | 0                        |
| Glyma11g36210          | Hook     | -1.09                  | 0                        |
| Glyma07g21150          | Hook     | -1.02                  | 0                        |
| Glyma02g04170          | Hook     | -1.01                  | 0                        |
| Glyma18g05720          | Hook     | 1.02                   | 0                        |
| Glyma11g08850          | Hook     | 1.02                   | 0                        |
| Glyma08g45310          | Hook     | 1.02                   | 1.3                      |
| Glyma05g34870          | Hook     | 1.07                   | 2.1                      |
| Glyma02g13930          | Hook     | 1.1                    | 2                        |
| Glyma09g05180          | Hook     | 1.27                   | 0                        |
| Glyma08g45300          | Hook     | 1.4                    | 0                        |
| Glyma06g14170          | Hook     | 1.73                   | 0                        |
| Glyma20g28890          | Hook     | 1.89                   | 0                        |
| Glyma02g04170          | Hypocotyl| -1.16                  | 0                        |
| Glyma12g13290          | Hypocotyl| 1.07                   | 5                        |
| Glyma17g03350          | Hypocotyl| 1.09                   | 3.3                      |

\(^a\) Log2 Fold Change
| Gene ID          | Tissue       | Log2FC  | FR/dark |
|-----------------|--------------|---------|---------|
| Glyma20g38030   | Hypocotyl    | 1.12    | 3.8     |
| Glyma15g09150   | Hypocotyl    | 1.18    | 0       |
| Glyma10g33370   | Hypocotyl    | 1.18    | 0       |
| Glyma07g09220   | Hypocotyl    | 1.21    | 0       |
| Glyma16g03280   | Hypocotyl    | 1.23    | 0       |
| Glyma11g01350   | Hypocotyl    | 1.3     | 0       |
| Glyma10g12060   | Hypocotyl    | 1.34    | 0       |

\(a. \) base 2 logarithm of fold change (FR/dark), positive log2FC value means up-regulation of the gene, while negative log2FC value means down-regulation.
TABLE II. Annotation of identified FRc responsive genes

| Glycine max Identifier | Arabidopsis Homolog | Annotation |
|------------------------|---------------------|------------|
| Glyma02g42500          | AT3G55990            | Encodes ESK1 (Eskimo1). A member of a large gene family of DUF231 domain proteins whose members encode a total of 45 proteins of unknown function. ESK1 functions as a negative regulator of cold acclimation. Mutations in the ESK1 gene provide strong freezing tolerance. |
| Glyma13g37320          | AT4G28290            | unknown protein |
| Glyma01g38590          | AT3G26300            | CYP71B34 (cytochrome P450, family 71, subfamily B, polypeptide 34); oxygen binding |
| Glyma06g14170          | AT5G24460            | hydrolase |
| Glyma11g03850          | AT4G16780            | ATHB-2 (ARABIDOPSIS THALIANA HOMEobox PROTEIN 2); DNA binding / transcription factor |
| Glyma08g28730          | AT1G67265            | DVL3/RTFL21 (ROTUNDIFOLIA LIKE 21) |
| Glyma15g16190          | No hits              | No hits |
| Glyma11g36210          | AT5G10180            | AST68 (Sulfate transporter 2.1) |
| Glyma07g21150          | AT2G26500            | cytochrome b6f complex subunit (petM), |
| Glyma02g04170          | AT1G60790            | similar to unknown protein; contains InterPro domain Protein of unknown function DUF231, plant (InterPro:IPR004253) |
| Accession | GeneID | Description |
|-----------|--------|-------------|
| Glyma18g05720 | AT2G30520 | RPT2 (ROOT PHOTOTROPISM 2) |
| Glyma11g08850 | AT4G35680 | similar to unknown protein; contains InterPro domain Protein of unknown function DUF241, plant (InterPro:IPR004320) |
| Glyma08g45310 | No hits | No hits |
| Glyma05g34870 | AT1G14870 | Identical to Uncharacterized protein At1g14870 contains InterPro domain Aspartic acid and asparagine hydroxylation site (InterPro:IPR000152); contains InterPro domain Protein of unknown function Cys-rich (InterPro:IPR006461) |
| Glyma02g13930 | No hits | No hits |
| Glyma09g05180 | AT4G02570 | ATCUL1 (CULLIN 1) |

| Accession | GeneID | Description |
|-----------|--------|-------------|
| Glyma20g28890 | AT3G22840 | ELIP1 (EARLY LIGHT-INDUCABLE PROTEIN); chlorophyll binding |
| Glyma12g13290 | AT4G28400 | protein phosphatase 2C, putative / PP2C, putative |
| Glyma17g03350 | No hits | No hits |
| Glyma20g38030 | AT1G09100 | RPT5B (26S PROTEASOME AAA-ATPASE SUBUNIT RPT5B); ATPase/ calmodulin |
| Glyma15g09150 | AT1G63310 | similar to oxidoreductase, acting on NADH or NADPH |
| Glyma10g33370 | AT2G33360 | Similar to unknown protein |
| Glyma07g09220 | AT4G28940 | catalytic |
| Glyma16g03280 | No hits | No hits |
| Glyma11g01350 | AT5G13930 | ATCHS/CHS/TT4 (CHALCONE SYNTHASE); |
naringenin-chalcone synthase
Glyma10g12060  AT5G06900  CYP93D1 (cytochrome P450, family 93, subfamily D, polypeptide 1); oxygen binding

TABLE III. Over-represented GO term in organ specific genes. Go term annotation were associated to each transcripts by determining the GO annotation for its Arabidopsis homolog (e-value cutoff: 1e-6) by TAIR. P-value was calculated by hypergeometric method. FDR was controlled to 0.05.

| GO term                        | GO ID        | Organ-specific genes with the GO ID | Whole probe sets with the GO ID | p-value   |
|-------------------------------|--------------|-------------------------------------|---------------------------------|-----------|
| chloroplast thylakoid membrane | GO:0009535   | 7.32%                               | 0.74%                           | 3.04E-18  |
| chloroplast ribulose          | GO:0009573   | 0.91%                               | 0.03%                           | 1.03E-04  |
| oxygen evolving complex       | GO:0009654   | 1.22%                               | 0.09%                           | 2.08E-04  |
| cysteine protease inhibitor activity | GO:0004869 | 0.91%                               | 0.05%                           | 6.33E-04  |
| cytochrome b6f complex        | GO:0009512   | 0.61%                               | 0.02%                           | 2.25E-03  |
| biological_process            | GO:0008150   | 9.72%                               | 1.10%                           | 1.41E-05  |
| endomembrane system           | GO:0012505   | 16.67%                              | 5.74%                           | 7.68E-04  |
| ubiquitin ligase complex      | GO:0000151   | 4.17%                               | 0.33%                           | 1.71E-03  |
| DNA primase activity          | GO:0003896   | 1.39%                               | 0.00%                           | 2.74E-03  |
| cell wall                     | GO:0005618   | 3.90%                               | 0.32%                           | 5.00E-08  |
| metabolic process             | GO:0008152   | 3.90%                               | 1.07%                           | 9.22E-04  |
| oxidoreductase activity       | GO:0016491   | 2.16%                               | 0.40%                           | 2.28E-03  |
TABLE IV. Novel FRc-responsive and organ specific cis regulatory elements revealed by Sift.

| co-regulated gene set          | motif sequence | Co-regulated gene set containing the motif | Reference gene set containing the motif | P-value  | FDR  |
|-------------------------------|----------------|-------------------------------------------|----------------------------------------|----------|------|
| FRc positive regulated genes  | TGNGCNANT      | 80.0%                                     | 20.6%                                  | 2.15E-08 | <0.05|
| cotyledon specific genes      | CNACGTGG       | 13.0%                                     | 4.3%                                   | 1.28E-10 | <0.001|
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