CRYPTOCHROME STRUCTURE AND SIGNAL TRANSDUCTION

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Abstract Cryptochromes are photosensory receptors mediating light regulation of growth and development in plants. Since the isolation of the Arabidopsis CRY1 gene in 1993, cryptochromes have been found in every multicellular eukaryote examined. Most plant cryptochromes have a chromophore-binding domain that shares similar structure with DNA photolyase, and a carboxyl terminal extension that contains a DQXVP-acidic-STAES (DAS) domain conserved from moss, to fern, to angiosperm. In Arabidopsis, cryptochromes are nuclear proteins that mediate light control of stem elongation, leaf expansion, photoperiodic flowering, and the circadian clock. Cryptochromes may act by interacting with proteins such as phytochromes, COP1, and clock proteins, or/and chromatin and DNA. Recent studies suggest that cryptochromes undergo a blue light–dependent phosphorylation that affects the conformation, intermolecular interactions, physiological activities, and protein abundance of the photoreceptors.

CONTENTS
INTRODUCTION ..................................................... 469
CRYPTOCHROME STRUCTURE .................................. 470
The Photolyase/Cryptochrome Gene Family .................. 471
The Structure-Function Relationships of Plant Cryptochromes ........................................... 477
CRYPTOCHROME SIGNAL TRANSDUCTION ............... 481
Mechanisms of Cryptochrome Signal Transduction ........... 481
Cryptochrome-Interacting Proteins .......................... 484
Cryptochrome Phosphorylation ............................ 487
CONCLUSIONS AND PERSPECTIVES ...................... 489

INTRODUCTION

Cryptochromes are flavoproteins in plants and animals that share structural similarity to DNA photolyase but lack photolyase activity. Most cryptochromes act as blue/UV-A light receptors, although whether mammalian cryptochromes also act...
as photoreceptors remains controversial (20, 64, 96). The meaning and definition of cryptochrome have undergone an interesting evolution in the past three decades. Cryptochrome was initially dubbed as a laboratory nickname for plant blue light receptors with action spectra comprising two peaks, one in the UV-A light region (∼320–400 nm), and the other with fine structures in the blue light region (∼400–500 nm) (35, 98). It was so called because blue light responses appeared prevalent in cryptogams (an old primary division of plants comprising those without true flowers and seeds, including fern, moss, algae, and fungi), and the molecular nature of blue light receptors was frustratingly cryptic at the time (97). It is intriguing that, prior to the isolation of the first cryptochrome gene, DNA photolyase was suspected to be a likely prototype of plant blue light receptors (71). It was also recognized at the time that different blue/UV-A light receptors might be evolutionarily unrelated, and therefore, a single nomenclature of cryptochrome might not be able to cover all the blue light receptors (98). Indeed, after the term cryptochrome was claimed for the photolyase-like blue light receptors (2, 20, 68), the molecular nature of another type of blue light receptors—the LOV domain–containing protein kinases that mediate phototropic responses—was identified (48). It is a bit ironic that although phototropism was one of the most prominent blue light responses at which our earlier hunt for cryptochromes was largely aimed (97), a different name, phototropin, had to be invented for the blue light receptors mediating this response (14, 16). It seems clear now that plants have at least two types of blue light receptors, phototropins that mediate photomovement responses including phototropism, chloroplast relocation, and stomatal opening (15, 63); and cryptochromes that act concurrently with phytochromes to mediate photomorphogenetic responses such as inhibition of stem elongation, stimulation of leaf expansion, control of photoperiodic flowering, entrainment of the circadian clock, and regulation of gene expression (20, 64).

Since isolating the first cryptochrome gene in 1993 (2), studies of cryptochromes have been extensively reviewed and commented on (1, 3, 16, 17, 19, 20, 26, 41, 46, 53, 61, 62, 64, 67, 96, 100, 111, 116). Some of these review articles provide detailed accounts of the general molecular characteristics and possible mode of actions of cryptochromes (20), the interesting history of the study of plant blue light receptors (16), and the diverse functions of these photoreceptors (64). In this article, we focus on some of the recent discoveries of cryptochromes, especially that of the two cryptochromes in Arabidopsis for which most studies of plant cryptochromes have been carried out. We also try to address two basic questions: What are the structural characteristics of cryptochromes, and what is our current understanding of the signal transduction mechanism of plant cryptochromes?

CRYPTOCROME STRUCTURE

Our current understanding of cryptochrome structure derives mostly from analyses of cryptochrome sequences, studies of cryptochrome mutations, and investigation of recombinant cryptochromes in vitro or in transgenic plants. Sequence analyses
show that most plant cryptochromes have two domains, an N-terminal photolase-related (PHR) domain that shares sequence homology to DNA photolyase, and a C-terminal extension that is unrelated to photolyase. The PHR domain of cryptochrome is the chromophore-binding domain, whereas the C-terminal extension is important for the nuclear/cytosol trafficking and protein-protein interactions. The crystal structure of cryptochromes has not been solved, but the structure of photolyases is known. Given the sequence similarity between cryptochrome and photolyase, it is likely that the structure of at least the PHR domain of a cryptochrome may resemble that of a photolyase.

The Photolyase/Cryptochrome Gene Family

DNA PHOTOLYASES Members of the photolyase/cryptochrome gene family encode either light-dependent DNA repairing enzymes (photolyases) or photosensory receptors (cryptochromes). DNA photolyases are \(~55–65\text{-kD}\) flavoproteins widely found in prokaryotes and eukaryotes, and they are thought to be the evolutionary progenitors of cryptochromes. DNA photolyases catalyze blue/UV-A light-dependent repair of DNA damages resulting from exposure to high-energy short-wavelength UV light (95, 109). There are two types of structurally related DNA photolyases: The photolyase or CPD photolyase repairs cyclobutane pyrimidine dimers, and the 6–4 photolyase repairs pyrimidine-pyrimidine 6–4 photoproducts (95, 107). Based on sequence similarity, the CPD photolyases are further divided into two classes, type I photolyase (mostly found in unicellular organisms) and type II photolyase (found in both unicellular and multicellular organisms) (95).

The reaction mechanism of photolyase has been well characterized (95, 96). All the photolyases studied contain two chromophores, a light-harvesting antenna chromophore, being either a folate (methenyltetrahydrofolate) or a deazaflavin, and a catalytic chromophore that is fully reduced flavin adenine dinucleotide (FADH\textsuperscript{−}). Using the antenna chromophore allows photolyases to repair UV-damaged DNA with high photon efficiency (95). A photolyase recognizes and binds to the CPD-containing DNA. The light-harvesting chromophore absorbs blue light and transfers the excited energy in the nonradiative form to the FADH\textsuperscript{−}. The excited FADH\textsuperscript{−} passes an electron to the CPD substrate, and the resulting negative charge induces splitting of the cyclobutane ring of the CPD pyrimidine dimer. Subsequently, the electron moves back to flavin to regenerate the catalytic active flavin, and the DNA with the two neighboring pyrimidines restored is released from the photolyase (95). The crystal structure has been reported for photolyases from *Escherichia coli* and the cyanobacterium *Synechocystis sp PCC6301* (previously called *Anacystis nidulans*) (88, 105). These two photolyases, which share only \(~30\%\) amino acid sequence identity and use different light-harvesting chromophores (pterin or deazaflavin), have similar structures that are almost superimposable.

THE STRUCTURE OF PHOTOLYASE/CRYPTOCHROME One prominent feature of a photolyase structure is a positively charged groove running through the enzyme,
which is believed to be the DNA-binding site (88) (Figure 1). There is a cavity in the center of the groove opposing the flavin-binding site, and the CPD of a bound DNA can fit into the hole to accept the electron from the flavin. Despite the lack of the cryptochrome’s crystal structure, computational protein modeling showed that the structure of a cryptochrome is similar to that of a photolyase (107). A structural model of the PHR domain of Arabidopsis cry2 is shown in Figure 1 (H. Nakamura, personal communication). Based on this model, the PHR domain of Arabidopsis cry2 is relatively hydrophobic, which appears to be consistent with the observation that Arabidopsis cry2 or PHR domain purified from heterologous expression systems tends to aggregate and precipitate (X. Yu & C. Lin, unpublished data). This hypothetical model shows that the positively charged groove found in photolyase is partially conserved in cry2, although the groove in cry2 appears shorter, wider, and with a relatively lower surface electrostatic potential than that of the photolyase (Figure 1). Such a positively charged groove structure may allow cry2 to bind to DNA. There also seems to be a cavity located in the predicted groove of cry2, which is larger in size compared to the CPD-binding cavity in photolyase (Figure 1). The concentrated positive charge around the relatively larger cavity in cry2 indicates that it is likely an important interface for interaction with other molecules such as DNA or proteins. It has been proposed that a redox reaction(s) is likely associated with photochemistry of cryptochromes (20). The cavity in cry2, like that in a photolyase, is positioned toward the FAD-binding site, which may allow an intermolecular redox reaction to occur. It is not clear how inclusion of the C-terminal extension may affect this structure model.

EVOLUTION OF THE PHOTOLYASE/CRYPTOCHROME GENE FAMILY  Most plant species studied have multiple members of the photolyase/cryptochrome gene family. For example, Arabidopsis has at least four members of the photolyase/cryptochrome gene family, including two cryptochromes, one type II photolyase, and one 6-4 photolyase (20). Since the discovery of the first cryptochrome gene in Arabidopsis (2), cryptochromes have also been found in animals including Drosophila (30), fish (58), Xenopus (126), chicken (10), and human (47, 96, 108). An early phylogenetic analysis suggested that ancestral cryptochrome genes may have emerged before the divergence of prokaryotes and eukaryotes (53). Based on the observation that animal cryptochromes are more closely related to 6-4 photolases, including the Arabidopsis 6-4 photolyase, than to the plant cryptochromes, and the lack of report of a prkayotic cryptochrome at the time, it was argued that plant and animal cryptochromes might have arisen independently after the divergence of the two lineages in evolution (20). Recent phylogenetic studies and the discovery of a putative cryptochrome from a prokaryote appear to be more consistent with the earlier view (43, 58, 84, 107).

The photosynthetic cyanobacterium Synechocystis sp PCC6803 contains two photolyase-like sequences, slr0854 and sll1629 (also called phrA and phrB, respectively) (43, 84). Neither gene product contains a C-terminal extension, but both noncovalently bind to partially oxidized FAD. A phylogenetic analysis suggests
slr0854 and sll1629 are more closely related to the type I CPD photolyase or animal cryptochromes (and 6-4 photolyases), respectively. The recombinant slr0854 gene product showed CPD photolyase activity in vitro, and expression of slr0854 rescued the photolyase-deficient *E. coli* mutant in a light-dependent manner. The wild-type cyanobacterial cells showed a white light-dependent resistance to UV-light irradiation, but red light irradiation had little effect. The cyanobacterial mutant disrupted in slr0854 was over a million times more sensitive to UV light than the wild type, confirming that the slr0854 gene product is a photolyase. In contrast, the sll1629 gene product showed no photolyase activity, in vitro or in *E. coli*, and the cyanobacterial mutant disrupted in sll1629 showed little defect in UV sensitivity (43, 84). The double mutant missing both slr0854 and sll1629 genes was only slightly more sensitive to UV than the monogenic slr0854 mutant. Because the sll1629 gene product had little photolyase activity, it may represent the first prokaryotic cryptochrome identified. A study of the genome-wide gene expression changes resulting from the sll1629 mutation suggests that sll1629 may regulate gene expression in *Synechocystis* (K. Hitomi & E. Getzoff, personal communication).

It has been proposed that at least four gene duplication events may have occurred in evolution to give rise to the present-day photolyases and cryptochromes (107). The first gene duplication produced the ancestral type I CPD photolyase and type II CPD photolyases. The ancestral type I photolyase gene duplicated again to become the present-day type I photolyase and the progenitor of cryptochrome/6-4 photolyase. The progenitor cryptochrome/6-4 photolyase later duplicated a third time. One copy evolved to become the present-day cryptochromes in higher plants, whereas the other copy duplicated again to give rise to the 6-4 photolyases as well as the cryptochromes in the animal lineage.

**PLANT CRYPTOCHROMES** Cryptochromes have been found throughout the plant kingdom (Figures 2 and 3) (Table 1), including angiosperm, fern, moss, and algae (2, 11, 44, 50, 51, 54, 56, 69, 86, 90, 101, 115). Most plant cryptochromes are 70–80-kD proteins with two recognizable domains, an N-terminal PHR domain that shares sequence homology with photolyases, and a C-terminal extension that has little sequence similarity to any known protein domain (Figure 2) (Table 1). Most residues known to be important for flavin-binding in photolyase are conserved in cryptochromes, whereas residues of photolyases that are critical for the binding of the second chromophore and DNA lesions are less well conserved in cryptochromes (2, 51, 108) (Figure 2).

Arabidopsis has two cryptochrome genes, *CRY1* and *CRY2* (2, 44, 66). Tomato has at least three cryptochrome genes, *CRY1a*, *CRY1b*, and *CRY2* (90, 91). Fern and moss have at least five and at least two cryptochrome genes, respectively (49, 51, 54). The amino acid sequences of tomato CRY1 (CRY1a or CRY1b) and CRY2 are more similar to their Arabidopsis counterparts than to each other (90). As shown in Table 1, the PHR domain of tomato CRY1 is ~85% identical to the PHR domain of Arabidopsis CRY1, but only ~63% identical to that of the tomato CRY2. Similarly, the C-terminal extension of tomato CRY1 is ~50% identical to
that of Arabidopsis CRY1, but only ~15% identical to the C-terminal extension of tomato CRY2 (Table 1). In comparison, the PHR domain and the C-terminal extension of Arabidopsis CRY1 and CRY2 share only 58% or 13% amino acid sequence identity, respectively. This observation indicates that the gene duplication event resulting in plant CRY1 and CRY2 occurred at least 100 million years ago, before the divergence of Brassicaceae (e.g., Arabidopsis) and Solanaceae (e.g., tomato) (59, 90). However, it is not clear whether orthologs of both Arabidopsis CRY1 and CRY2 exist in other major branches of the plant kingdom. Two putative cryptochrome genes were found from a search of the newly completed rice genome (Figure 3) (Table 1) (34, 124). But both cryptochrome genes found in the rice genome are more similar to Arabidopsis CRY1 than CRY2 (Table 1). Although the putative cryptochrome sequences are also found in other monocot species, including maize and barley, most of those sequences are partial sequences derived from ESTs. A more detailed analysis of cereal cryptochromes would likely answer this question.

Due to its low abundance in general, the chromophore composition of cryptochromes has never been studied in holoproteins purified from any host organism. However, studies of recombinant cryptochromes expressed and purified from heterologous expression systems demonstrated that cryptochromes noncovalently bind to flavin (FADH\(^{-}\)) and possibly pterin [methenyltetrahydrofolate (MTHF)], and that the PHR domain is the chromophore-binding domain (68, 76). No photolyase activity was found for any plant cryptochromes tested (43, 51, 68, 76).

Different cryptochromes share a much higher sequence similarity in the PHR domain than in the C-terminal extension (51, 69, 90) (Table 1). For example,

Figure 2 An amino acid sequence alignment of four representative plant cryptochromes. The four cryptochromes were aligned using ClustalW. Black-boxed and gray-boxed letters represent identical or similar residues, respectively. The characters F/f and M/m above sequences indicate residues known to interact with FAD or methenyltetrahydrofolate (MTHF), respectively (53). Bolded letters F, f, M, and m indicate residues that are conserved between cryptochromes and photolyases; nonbold letters indicate those that are not conserved between cryptochromes and photolyases. Letters F and M indicate the positions in which the corresponding residues of E. coli photolyase form direct H bonds with the chromophores; f and m indicate positions in the photolyase that form water-mediated H bonds with the chromophores. The arrowhead indicates junctions between PHR domains and the C-terminal extensions (e.g., residue 494 in At-CRY1) used to calculate sequence similarities in Table 1. Underlines mark the DAS domain in the C-terminal extension. At CRY1: Arabidopsis thaliana CRY1, a representative of dicotyledon plants (accession AAB28724); Rice 1: Oryza sativa CRY1a, a representative of monocotyledon plants (BAB70686); Fern 4: Adiantum capsillus-veneris CRY4, a representative of ferns (BAA88425); Moss 1a: Physcomitrella patens CRY1a, a representative mosses (BAA83338).
Arabidopsis CRY1 and CRY2 are 59% identical in the PHR domain. However, the C-terminal extension of Arabidopsis CRY1 and CRY2 is only ~13% identical, which is lower than that between the PHR domain of either Arabidopsis cryptochromes compared with the *E. coli* photolyase (~30% identical) (Table 1). The size of the C-terminal extension of cryptochromes also varies significantly, from ~380 amino acids long in algae (*Chlamydomonas*), 181 amino acids and 123 amino acids in Arabidopsis CRY1 and CRY2, respectively, to almost no C-terminal extension in the SH-PHR of the white mustard *Sinapis alba* (11, 76) and AcCRY5 of the fern *Adiantum capsillus-veneris* (51).
Despite the lack of overall structural similarity in the C-terminal extensions of different cryptochromes, there are three recognizable motifs in this region of cryptochromes in most plants examined (64, 69). These motifs are (a) DQXVP near the amino end of the C-terminal extension, (b) a region containing a varying number of acidic residues (E or D), and (c) STAES followed by another conserved sequence, GGXVP, at the carboxyl end. Because these three motifs and their linear order are well conserved in plant cryptochromes from moss to Arabidopsis, the region of cryptochrome C-terminal extension containing these motifs is referred to as DAS (Figure 2) (64). The presence of the DAS sequence in cryptochromes from moss to angiosperm suggests that the evolutionary history of cryptochromes in plants is likely over 400 million years old, dating back to before the wide spread of vascular plants on the earth (55). It appears that the ancestral plant cryptochrome emerged from a gene fusion of a photolyase sequence to a DAS-containing sequence, which may or may not be lost during evolution. Genetic studies indicate that the DAS domain is important for cellular localization, intermolecular interaction, and physiological functions of a cryptochrome (Figure 4).

### The Structure-Function Relationships of Plant Cryptochromes

Researchers have used two approaches to investigate the structure-function relationship of cryptochromes: one to analyze how mutations of individual amino acids may affect cryptochrome function, and the other to investigate how a fusion protein containing a marker enzyme fused to a partial or a full-length
Figure 4  Structure-function relationships of cryptochromes. (A) Distribution of mutations found in Arabidopsis CRY1 and CRY2 genes. Arabidopsis cryptochromes are roughly divided into the PHR domains—and the C-terminal extensions—as indicated. The approximate FAD- and MTHF-binding regions and the DAS domains are shown by brackets. Circles and squares denote missense and in-frame-deletion mutations found in CRY1 (7) and CRY2 (H. Guo & C. Lin, unpublished data), respectively. (B) A summary of experiments showing how recombinant proteins of Arabidopsis CRY1 and CRY2 affect cellular localization, protein degradation, and blue light inhibition of hypocotyl elongation in transgenic plants. “1”: degradation detected; “2”: nd (not defined); “3”: +/− means weak phenotypes detected; “4”: CCT1 localizes in the nucleus in dark and in the cytoplasm in light. See references listed for details.

cryptochrome sequence may behave in planta. Figure 4 summarizes the results of some of the studies of Arabidopsis CRY1 and CRY2 with respect to how a cryptochrome’s structure may affect its cellular localization, stability, and activity.

When the first approach was used to study Arabidopsis CRY1 (7) and CRY2 (H. Guo & C. Lin, unpublished data), missense mutations were found throughout
the coding sequence of both cryptochromes (Figure 4A). In addition to the expected mutations found in the PHR chromophore-binding domain, some of the missense mutations were found in the DAS domain of CRY1 and CRY2. These DAS-domain mutations have a similar effect on CRY1 function as that of the mutations in the chromophore-binding PHR domain, indicating the functional importance of the DAS domain. No specific region of the cryptochrome sequence was found to accumulate mutations at a significantly higher frequency. In this regard, cryptochromes seem different from the phytochrome for which a relatively higher frequency of mutations was found in a 160-residue region of the C-terminal domain (93). The lack of an apparent mutation-prone region of cryptochromes may simply be because the mutagenesis of cryptochromes has not been saturating. Alternatively, the overall protein conformation may be more important for the function of a cryptochrome.

NUCLEAR LOCALIZATION Arabidopsis cry1 and cry2 are nuclear proteins (20, 36, 57). It appears that Arabidopsis cry2 is more or less constitutively imported to the nucleus regardless of light treatment. However, Arabidopsis cry1 may be imported to the nucleus in the dark but may be exported or remain in the cytosol in response to light. It was found that the GUS-CCT1 (CRY1 C-terminus) fusion protein was mostly located in the nucleus in root hair cells of dark-grown transgenic plants, but the fusion protein was mostly cytosolic in the light-grown transgenic plants (119). Consistent with the notion that cry1 may be largely cytosolic in light-grown plants, the relative amount of cry1 detected in the nuclear extract obtained from the green tissue of light-grown Arabidopsis was significantly lower than that detected in the total protein extract (36). In contrast, the same nuclear extract was highly enriched for cry2 (36).

Although one may expect that the PHR domain of a cryptochrome would contain the nuclear localization signal (NLS), because DNA photolyase, the presumed ancestor of the PHR domain of cryptochromes, has to move into the nucleus to carry out its DNA-repairing function, the C-terminal extension is sufficient to direct nuclear transportation for both cryptochromes in Arabidopsis (24, 36, 57, 114, 119). A putative bipartite nuclear localization signal was found within the DAS domain of cry2, and fusion proteins of β-glucuronidase (GUS) to the C-terminal extension of cry2 are constitutively nuclear (24, 36, 57). Although no apparent bipartite NLS is found in CRY1, the C-terminal extension has proven sufficient for nuclear/cytoplasmic trafficking of CRY1 (114, 119).

The function of the C-terminal extension in directing subcellular localization of a cryptochrome is conserved in plants from fern to angiosperm. Among the five cryptochromes that have been identified in the fern Adiantum, two (CRY3 and CRY4) are apparently nuclear proteins (51). CRY3 is imported to the nucleus in dark or in red light, but it is mostly cytosolic in blue light, whereas CRY4 accumulates constitutively in the nucleus. Studies of the fusion proteins of GUS and the C-terminal extension of CRY3 and CRY4 indicate that, like Arabidopsis cryptochromes, the C-terminal extension also contains
signals regulating nuclear/cytosol trafficking of the fern cryptochromes (51).

**CRYPTOCHROME DEGRADATION**  The Arabidopsis cry2 protein undergoes a rapid blue light–induced degradation (5, 36, 69). In etiolated seedlings exposed to blue light, the level of cry2 decreased to a new steady state in about 15 min after blue light exposure, which is less than 10% that of dark-grown seedlings (99). Both the PHR domain and the C-terminal extension appear to be important for the blue light–induced degradation of cry2 (Figure 4B). The GUS-CRY2C (residue 480-612 of cry2) fusion protein was stable in transgenic plants exposed to blue light, suggesting that the C-terminal extension of cry2 by itself is insufficient to mediate its degradation (36). However, using a domain-swap approach, both the C1C2 fusion protein (the PHR domain of CRY1 fused to the C-terminus of CRY2) and the C2C1 fusion protein (the PHR domain of CRY2 fused to the C-terminus of CRY1) were degraded in response to blue light (5) (Figure 4B). This result is consistent with a proposition that blue light–induced conformational change of cry2 may play an important role in triggering its degradation, and both the PHR domain and the C-terminal extension are required for appropriate turnover of Arabidopsis cry2.

It is not clear whether ubiquitination is associated with cry2 degradation. However, application of a proteosome inhibitor to Arabidopsis seedlings suppresses blue light–dependent cry2 degradation in vivo, indicating the involvement of this general protein degradation apparatus in the blue light regulation of cry2 turnover (D. Shalitin & C. Lin, unpublished data). It is worth pointing out that Drosophila cryptochrome has been reported to undergo a light-induced and proteosome-dependent degradation and that the light-dependent conformational change appears to be responsible for the degradation of the Drosophila cryptochrome (70).

**CHROMATIN INTERACTION**  A recent report showed that Arabidopsis cry2 may be associated with chromosomes (24). When transgenic Arabidopsis plants expressing random GFP-cDNA fusions were examined for the subcellular localization of individual fusion proteins, one transgenic line that expressed GFP-CRY2C (CRY2 C-terminal extension) was identified. Interestingly, the GFP-CRY2C fusion protein was found to bind to all five chromosome pairs in a mitotic cell (24) (http://deepgreen.stanford.edu/). The cryptochrome-chromatin interaction can also be detected using the chromatin immunoprecipitation (ChIP) method (M. Ong & C. Lin, unpublished data). However, it remains to be determined whether the cryptochrome-chromatin interaction is involved with a direct cryptochrome-DNA interaction and whether cryptochromes bind to chromosomes via protein-protein interactions. It is now clear that cryptochromes can physically interact with other proteins associated with light signal transduction, for which the C-terminal extension of Arabidopsis cryptochromes often acts as the protein-protein interaction domain.
CRYPTOCHROME SIGNAL TRANSDUCTION

A major difficulty in addressing this question has been that, except for cryptochrome mutants, extensive genetic screens in Arabidopsis have not turned up other blue light–specific mutants (7, 9, 18, 73) (H. Guo & C. Lin, unpublished data). This result may be explained if genetic redundancy is associated with most genes involved in cryptochrome signaling. Alternatively, it may reflect a significant convergence between the signal transduction of cryptochromes and phytochromes. Consistent with the latter hypothesis, cryptochromes have been found to interact with phytochromes and other proteins such as COP1 known to be associated with blue light responses as well as phytochrome-mediated photomorphogenetic responses.

Mechanisms of Cryptochrome Signal Transduction

Based largely on genetics and photophysiology studies, it has been established that, in at least Arabidopsis, cryptochromes can mediate blue light inhibition of hypocotyl elongation, blue light modification of gene expression, photoperiodic flowering, and regulation of the circadian clock. Readers are referred to recent review articles and references for more detailed analyses of physiological roles of cryptochromes (16, 20, 40, 61, 62, 64, 82, 89, 96). In the following sections, we briefly discuss possible modes of action of cryptochromes to mediate various light responses and then describe molecular aspects of cryptochrome signal transduction.

DE-ETIOLATION

De-etiolation refers to a collection of light-dependent developmental changes in germinating young seedlings, including inhibition of stem elongation, stimulation of leaf expansion, changes in gene expression, and induction of chloroplast development (31, 112). Cryptochromes are involved in mediating many, if not all, of the blue light–dependent de-etiolation responses (20, 64). For example, action spectra studies demonstrated that Arabidopsis cry1 and cry2 are the major photoreceptors mediating blue light inhibition of hypocotyl elongation (4, 123). The function of cryptochrome in mediating de-etiolation responses has also been reported in tomato (86, 115).

There are at least two ways a photoreceptor may trigger a developmental response. A photoreceptor may amplify a light signal via cytosolic second messages that provoke other cellular activities including regulation of gene expression. Alternatively, a nuclear photoreceptor may directly interact with a transcription or posttranscription regulatory apparatus to alter gene expression and developmental patterns. Currently, evidence exists for the involvement of both mechanisms in the cryptochrome-mediated de-etiolation responses.

Based on analyses of blue light effects on plasma membrane depolarization, anion channel activity, and growth inhibition kinetics, it was proposed that cryptochromes activate anion channel activity, resulting in plasma membrane
depolarization, and the inhibition of cell elongation (89, 104). This hypothesis may explain why Arabidopsis cry1, which is the primary blue light receptor mediating blue light inhibition of hypocotyl elongation, is exported to the cytosol in response to light, where it may regulate cytosolic or plasma membrane proteins. It was shown recently that the Arabidopsis cry1, cry2, and phot1 mutants were similarly impaired in the blue light–induced membrane depolarization, suggesting that all three photoreceptors play a role in the regulation of blue light activation of anion channels (33). Indeed, all three photoreceptors may regulate leaf expansion via light-dependent control of plasma membrane anion channels, because defects in leaf expansion have been observed in not only cryptochrome mutants but also in phototropin mutants (65, 69, 94). On the other hand, blue light inhibition of hypocotyl elongation appears more complicated. The hypocotyl inhibition response as measured by hypocotyl length for seedlings grown in blue light, is significantly impaired in the cry1 mutant, slightly affected in the cry2 mutant, but almost unaffected in the phot1 mutant (2, 69, 72). However, a high-resolution growth kinetics analysis showed that both cry1 and cry2 are similarly impaired in the slow phase (30–120 min after light on) of growth inhibition whereas phot1 mutant is defective in the rapid phase (<30 min after light on) of growth inhibition (33). It was proposed that phot1 might be associated with the initiation of blue light inhibition of growth, whereas cryptochromes are required for the maintenance of the growth inhibition (33, 89). Identification of genes encoding the specific anion channels regulated by cryptochromes and/or phototropins would help elucidate the cellular mechanisms underlying cryptochrome-dependent growth response.

In addition to calcium’s possible involvement in the phytochrome signal transduction (13, 83), it may also be used as a second message for cryptochrome signal transduction (22, 37, 74). For example, when Arabidopsis cell culture was used to monitor blue/UV-A light–induced chalcone synthase (CHS) expression, blue light promoted calcium efflux in the cytosol, and compounds that inhibit voltage-gated calcium channel or Ca2+-ATPase significantly altered blue/UV-A light–induced CHS expression (22, 74). An Arabidopsis cell culture system has been used to study how cryptochrome mediate blue/UV-A light–induced CHS expression (22, 74). In this system, cryptochrome-mediated CHS expression correlates with blue light promotion of calcium efflux in the cytosol. The involvement of calcium homeostasis in cryptochrome-mediated CHS expression was indicated by the observation that compounds that inhibit voltage-gated calcium channel or Ca2+-ATPase significantly altered blue/UV-A light–induced CHS expression. A possible role of calcium homeostasis in cryptochrome signaling is consistent with a recent study of the Arabidopsis SUB1 gene, which encodes a calcium-binding protein that acts downstream from cryptochromes in the hypocotyl inhibition response (37). However, a direct demonstration of whether and how cryptochromes act through calcium channels or Ca2+-ATPase to regulate CHS gene expression or hypocotyl inhibition depends on the identification of the specific genes encoding those proteins and the corresponding mutations. Moreover, like the anion channel hypothesis discussed above, the role of calcium in cryptochrome function is also
complicated by phototropins. Using Ca\(^{2+}\) microelectrodes or transgenic plants expressing the calcium-dependent luminescent protein aequorin targeted to specific cellular compartments, it has been found that blue light stimulates a transient increase of cytosolic calcium (8, 12). However, the blue light–induced elevation of the cytosolic calcium is impaired only in the phot1 and phot1/phot2 mutants but not in the cry1 or cry2 mutants (8, 12). Since phot1 and phot2 are not the major photoreceptors regulating hypocotyl inhibition or CHS expression in response to blue light, the phototropin–dependent change in calcium homeostasis is not likely directly involved in those cryptochrome-dependent responses. It remains to be examined whether there is a more subtle change of calcium homeostasis associated with cryptochrome function.

Recent studies demonstrate that gene expression regulation is a major signaling mechanism underlying cryptochrome action. In Arabidopsis, cry1 and cry2 are known to regulate sets of similar genes in a partially redundant manner. A DNA microarray analysis demonstrated that the expression of about one third of Arabidopsis genes change in response to blue light, and cryptochromes are the major photoreceptors mediating these gene expression alterations (75). More than 71% of blue light–induced gene expressions and more than 40% of blue light–suppressed gene expressions are affected in etiolated cry1cry2 double mutants exposed to blue light, suggesting the two photoreceptors regulate expression of these genes in response to blue light (75). The rest of the blue light–dependent gene expression change is probably mediated partly or completely by phyA (23, 106). It is unclear which genes regulated by cryptochromes are directly involved in individual reactions of the de-etiolation responses and how cryptochromes regulate gene expression. One possibility is that cryptochromes regulate transcriptional or posttranscriptional processes by interacting with the respective regulatory complexes in the nucleus (62). A direct test of this hypothesis requires identification of transcriptional or posttranscriptional regulators associated with cryptochrome-mediated light responses and a demonstration of how these regulators interact with cryptochromes.

**PHOTOPERIODIC FLOWERING AND THE CIRCADIAN CLOCK** Cryptochromes also work together with phytochromes to control photoperiodic flowering and the circadian clock (61). Genetic studies of the Arabidopsis cry2 mutant have demonstrated that cry2 is a major photoreceptor regulating photoperiodic flowering in Arabidopsis (38). Taking together a large number of studies by various laboratories, it was proposed that cryptochromes and phytochromes act in both an antagonistic and redundant manner to regulate floral initiation (61, 80, 81). The molecular mechanism underlying cry2 promotion of floral initiation is not clear, although it may regulate expression of important flowering-time genes such as FT and CO (38, 82, 119a). Photoperiodic responses rely on photoreceptors to perceive changes of day length and on the circadian clock to memorize such changes. Investigations of how Arabidopsis cryptochrome and phytochrome mutants affect the circadian clock–controlled transcription demonstrated that phyA, phyB, cry1, and cry2 are all involved in the regulation of the circadian clock in Arabidopsis (102, 121, 125).
However, unlike mammalian cryptochromes, plant cryptochromes may not be the essential part of the central oscillator (27, 120). It remains to be examined how cryptochromes and phytochromes regulate the circadian clock.

Although each of the five phytochromes and two cryptochromes may potentially convey the day-length signal in Arabidopsis, cry2 and phyA are the only two photoreceptors for which a photoperiod-dependent change has been shown (29, 64, 80). Like other photoreceptors in Arabidopsis, the transcription of cry2 and phyA are regulated by the circadian clock (39, 110). But cry2 and phyA are the only ones that are known to be degraded in light, and their protein level in plants grown in photoperiod showed a largely diurnal rhythm. The cellular levels of cry2 and phyA proteins decline in daytime but increase in the evening, a phenomenon likely associated with their light-dependent degradation. More importantly, the diurnal rhythm of cry2 and phyA protein abundance is dependent on photoperiods: The daily rhythmic change of cry2 and phyA abundance is more pronounced in short days than in long days. The functional relevance of the diurnal rhythm of cry2 is clearly demonstrated in a recent study (29). In this elegant study, the early day–length insensitive (EDI) quantitative trait loci (QTL) of the Cvi accession (ecotype), collected in the tropical Cape Verde Islands, was mapped and cloned. The EDI locus, which is dominant and largely responsible for the photoperiod-insensitive early flowering trait of the Cvi accession, turned out to be the CRY2 gene. CRY2-Cvi encodes the CRY2 protein with a methionine substitution for the valine at position 376 (V367M). Val1376 was completely conserved among eight different cryptochrome genes compared except for CRY2-Cvi. Transgenic plants expressing the mutated CRY2-Ler (CRY2 gene of Ler accession) with the V367M substitution flowered earlier than Ler, whereas plants expressing the mutated CRY2-Cvi with a M367V substitution flowered later than Cvi. This experiment confirmed that the single V367M substitution in the CRY2-Cvi protein is the cause of the photoperiod-insensitive early flowering of the Cvi ecotype. Moreover, the V367M substitution of the CRY2 protein resulted in a change in the photoperiod-dependent diurnal cycling of CRY2 expression. The CRY2-Cvi type protein with the V367M substitution had a reduced amplitude of the diurnal rhythm of the cry2 abundance in short day, suggesting that the reduced diurnal rhythm of CRY2-Cvi protein abundance in response to photoperiods is responsible for the reduced day-length insensitivity and early flowering of Cvi plants.

**Cryptochrome-Interacting Proteins**

Direct protein-protein interaction(s) has been associated with almost every well-characterized signal transduction system. Cryptochrome signal transduction, despite the current lack of full understanding of its mechanism, is no exception. A convincing demonstration of the involvement of protein-protein interaction in cryptochrome signal transduction requires at least two pieces of evidence: identification of the interacting partner(s) and demonstration of the involvement of the interacting proteins in the biological processes mediated by cryptochromes. Both lines of evidence have been reported for Arabidopsis cryptochromes.
CRYPTOCHROME-COP1 INTERACTION  It may be expected that if cryptochrome function is dependent on a direct protein-protein interaction, overexpression of the protein-protein interaction domain of a cryptochrome would result in competitive inhibition of the endogenous cryptochrome and dominant phenotypic changes in the transgenic plants. Indeed, transgenic overexpression of fusion proteins of GUS and cryptochrome C-terminal domains confer a dominant positive phenotype (119). Transgenic plants expressing GUS-CCT1 (CRY1 C-terminus) or GUS-CCT2 (CRY2 C-terminus) fusion proteins showed short hypocotyls, opened cotyledons, and increased anthocyanin accumulation when they were grown in dark or in light regardless of wavelength of illumination (119). These phenotypes are reminiscent of those found for the cop/det/fus mutants, although cop/det/fus mutants are recessive and often lethal, whereas the GUS-CCT expression caused a dominant but not lethal phenotype (21, 25, 119). Mutations in the CRY1 C-terminal domain, including E515K, E531K, and R576K, which had previously been shown to affect cry1 activity (7), eliminated the ability of fusion proteins to confer the cop/det/fus phenotype. Interestingly, transgenic plants expressing the GUS-CRY2C fusion protein, which contains seven additional residues at the amino terminus of CRY2C (CRY2 C-terminal domain) than GUS-CCT2 does, did not show the constitutive photomorphogenic phenotype (36). This could be due to the relatively lower level of expression of GUS-CRY2C compared to GUS-CCT2, or because the two different fusion proteins possess significantly different conformations due to the additional seven residues in GUS-CRY2C. Nevertheless, it was proposed that cryptochromes interact with COP1 or other COP/DET proteins in a light-dependent manner to suppress the activity of COP/DET proteins in wild-type plants (119). This hypothesis satisfactorily explains the phenotypes of the cop1 mutant, the cryptochrome mutants, and the GUS-CCT transgenic plants (114, 119, 118). Several lines of evidence support this hypothesis. Full-length CRY1, CCT1, or GUS-CCT1 fusion proteins interact with COP1 in yeast two-hybrid assays (114, 118). Further evidence of the COP1-CRY1 interaction came from the observation that onion cells coexpressing GFP-CCT1 and COP1 exhibited fluorescent nuclear speckles, which were detected when GFP-COP1 was expressed alone but not when GFP-CCT1 was expressed alone (114). The cry1-COP1 interaction does not seem to be dependent on light. Therefore, protein-protein interaction alone is insufficient to explain cryptochrome-mediated responses. It is also noted that the recruitment of GFP-CCT1 to the nuclear speckles by coexpression of COP1 does not necessarily suggest that cry1 is associated with nuclear speckles because neither this study nor another study discussed later detected GFP-CRY1 in the nuclear speckles (78).

Cry2 may also interact with COP1. It was found that GUS-CCT2 interacted with COP1 in both yeast two-hybrid and coimmunoprecipitation assays (114), although CRY2-COP1 interaction was not detected using a similar yeast two-hybrid assay in another study (118). Transgenic plants expressing GUS-CCT1 or GUS-CCT2 fusion proteins accumulated more of the bZIP transcription factor HY5 in the dark, a finding that is similarly observed in the cop1 mutant (87). Moreover, genome-wide gene expression profiles of dark-grown GUS-CCT1 and
GUS-CCT2 transgenic seedlings are also similar to that of blue light–grown wild-type seedlings or the dark-grown cop1 mutant (114).

COP1 is a zinc-finger and WD40-repeat protein that has a light-regulated nucleocytoplasmic partitioning pattern similar to CRY1 in that both are enriched in the nucleus in the dark but are enriched in the cytosol in light (113, 119). COP1 has been proposed to act as a subunit of an E3-ubiquitin ligase complex associated with degradation of the bZIP transcription factor HY5 in the dark (87). These observations are consistent with a transcription regulation model for the cryptochrome-mediated de-etiolation response (114, 119). According to this model, COP1 interacts with HY5 in the dark to facilitate its degradation, ensuring the off status of light-induced gene expression and thus etiolated development. In light, photoactivated cry1 is excluded, together with COP1, from the nucleus, allowing an accumulation of the transcription factor HY5 and transcription activation for genes required for photomorphogenesis. It was further hypothesized that, although cryptochrome-COP1 interaction may be light-independent, absorption of light by the cryptochromes may result in an intramolecular redox reaction and a change of the cryptochrome conformation, which in turn leads to an alteration of the conformation or activity of the COP1 protein and developmental responses (118).

CRYPTOCHROME-PHYTOCHROME INTERACTION

Arabidopsis cry2 directly interacts with phyB (78). The cry2-phyB interaction was shown by both yeast two-hybrid assays and coimmunoprecipitation tests. In addition, using fluorescent resonance energy transfer (FRET) microscopy, an intermolecular energy transfer was shown to occur between cry2-RFP and phyB-GFP fusion proteins, indicating that these two photoreceptors interact in vivo (78). Further evidence that cry2-phyB interaction is essential for the function of cry2 came from a finding that CRY2-RFP, but not CRY1-RFP, was colocalized with phyB in the nuclear speckles (78). In light of the recent discovery that phyB could mediate light regulation of transcription via its interaction with the transcription factor PIF3 (77, 85), the direct interaction between phyB and cry2 suggests that alteration of phytochrome-mediated regulation of transcription may be an important mechanism of cryptochrome signal transduction. In addition, cry1 has also been reported to interact, via its C-terminal domain, with phyA in a yeast two-hybrid assay (6). cry1 may also interact with phyB, at least indirectly, because cry1 and phyB can each interact with COP1 (118).

CRYPTOCHROME-ZTL/LKP1/ADO1 INTERACTION

COP1 may not be the only protein that can interact with both phytochromes and cryptochromes. Arabidopsis CRY1 and PHYB both interact with ZTL/LKP1/ADO1 in yeast two-hybrid assays and in vitro pull-down tests (52, 56, 103). ZTL/LKP1/ADO1 is a PAS domain–containing protein that also possesses an F-box and six Kelch repeats that were originally identified in a study of the circadian clock in Arabidopsis (103). ZTL/LKP1/ADO1 plays an important role in regulating the
circadian clock and photoperiodic flowering in Arabidopsis. Mutations or over-expression of the \textit{ZTL/LKP1/ADO1} gene caused an alteration of the free-running period of clock-controlled transcription, hypocotyl elongation, leaf movement, and photoperiodic flowering \((52, 56, 103)\). The demonstration of direct interactions between cry1/phyB and \textit{ZTL/LKP1/ADO1} indicates that the input pathway from photoreceptors to the central oscillator may be short even though neither photoreceptor appears to be an integral part of the central oscillator.

\section*{About the Nuclear Speckles}

It is intriguing that cry2, phyB, and COP1 not only physically interact with one another, but also associate with nuclear speckles \((78, 114)\). Speckles are poorly defined nuclear substructures that are even less well understood in plant systems. In animal cells, nuclear speckles are most often associated with spliceosomes, although transcription factors, proteins associated with RNA export, and translation factors have also been found in the nuclear speckles \((60, 79)\). Apparently, many intriguing questions remain to be addressed with respect to the function of nuclear speckle in plant cells and their association with photoreceptor functions. For example, what are the basic structure and function of plant nuclear speckles? Do nuclear speckles in plant cells share the similar protein composition and function as their counterparts in animal cells? And if so, what is the function of cryptochromes in nuclear speckles and how may transcription, RNA splicing, or RNA export be associated with cryptochrome regulation of plant growth and development?

\section*{Cryptochrome Phosphorylation}

Light-dependent protein phosphorylation has been associated with almost every class of photoreceptors, including rhodopsins, phytochromes, and phototropins \((16, 32, 45, 122)\). The first attempt to investigate whether cryptochromes may undergo light-dependent protein phosphorylation was reported in 1998 \((6)\). In this study, a recombinant CRY1 protein was shown to be phosphorylated in vitro by the recombinant oat phyA protein. The in vitro phosphorylation of cry1 by phyA occurs similarly under either red light \((30 \mu\text{mole m}^{-2} \text{s}^{-1})\) or blue light \((30 \mu\text{mole m}^{-2} \text{s}^{-1})\), and to a lesser degree in the dark. The C-terminal domain but not the PHR domain of cry1 was phosphorylated by phyA in vitro. The recombinant C-terminal domain of some cry1 mutants, including that of \textit{hy4-9}, \textit{hy4-22}, and one containing a deletion in the STAES motif showed decreased phosphorylation. The cry1 phosphorylation was also reported in vivo in transgenic plants overexpressing CRY1 in a red light–dependent and far red light–reversible manner, again suggesting an involvement of phytochromes in the phosphorylation of cry1.

Another attempt to investigate cryptochrome phosphorylation was reported recently \((99)\). In this study, Arabidopsis cry2 underwent a blue light–dependent phosphorylation in vivo using both \text{P}^{32}\text{-label and SDS-PAGE migration shift assays. The cry2-phosphorylation is dependent on blue light, because cry2}
phosphorylation was not detected in etiolated seedlings, or in seedlings transferred to dark following the blue light treatment. In contrast to the study described previously for cry1 (6), the cry2 phosphorylation was not detected in seedlings exposed to red light. Moreover, normal cry2 phosphorylation was found to occur in every phytochrome mutant tested, including phyA, phyB, phyAB, phyABD, phyBDE, and hy1. A similar study showed that cry1 underwent a blue light-dependent phosphorylation. Similar to the cry2 phosphorylation, the cry1 phosphorylation was not affected by phytochrome mutations (D. Shalitin & C. Lin, unpublished data). Therefore, it appears that phytochromes are not responsible for the blue light–dependent cryptochrome phosphorylation. Recently, a mouse cryptochrome (mCRY1) was shown to undergo a phosphorylation catalyzed by the Casein kinase Iε (28). It would be interesting to test whether CKI-like protein kinases are involved in cryptochrome phosphorylation in Arabidopsis.

The lack of effect of phytochrome mutations on the cryptochrome phosphorylation is somewhat surprising because phytochrome has demonstrated protein kinase activity (32, 117, 122) and phytochromes can physically interact with crytochromes (6, 78). However, phytochrome involvement in the cryptochrome phosphorylation can not be completely excluded. This is because, first, as far as the red light–induced in vivo cryptochrome phosphorylation is concerned, different experimental conditions employed in different studies, such as genotypes, age of plants, total fluence, etc., may explain the different results observed. Second, it is possible that there is a complete redundancy among the five different phytochromes in Arabidopsis, and any residual phytochrome activity may be sufficient to catalyze cryptochrome phosphorylation. This possibility could be tested when a phytochrome null mutant lacking all five phytochromes becomes available.

A kinetics analysis showed that cry2 phosphorylation is dependent on both the fluence rate of blue light and the time period in which the seedlings were exposed to blue light. The relative level of phosphorylated cry2 increased initially when etiolated seedlings were exposed to blue light, but decreased when the total fluence increased to a certain level. This result suggests that the phosphorylated cry2 may be degraded because it has been shown previously that cry2 is degraded in blue light, especially in high-fluence blue light (5, 36, 69). This hypothesis was supported by the observed correlation between an increase in the relative abundance of phosphorylated cry2 and a decrease in cry2 degradation in the cop1-6 mutant that was impaired in cry2 degradation (99).

Protein phosphorylation affects not only cry2 regulation but also cry2 function. When the transgenic plants overexpressing GUS-CCT2 fusion protein were analyzed, it was found that, unlike the endogenous cry2 that is phosphorylated in response to blue light, the GUS-CCT2 fusion protein was constitutively phosphorylated (99). Given that the transgenic plants overexpressing GUS-CCT2 fusion protein exhibited a cop/det/fus-like constitutive photomorphogenic phenotype (119), it was suggested that the constitutively phosphorylated GUS-CCT2 fusion protein can constitutively trigger a photomorphogenic response. Based on these
A hypothetical model for the action and regulation of Arabidopsis cry2.

CONCLUSIONS AND PERSPECTIVES

The past decade has witnessed exciting progresses in the study of plant blue light responses and blue light receptors. Characterization of cryptochromes in Arabidopsis and other plant species have revealed a general picture of the structural characteristics and likely signal transduction mechanisms of plant cryptochromes. At least in Arabidopsis, cryptochromes are nuclear proteins that function by interacting with other proteins to regulate gene expressions. Further investigation of the intermolecular interactions of cryptochromes, examination of how light affects these interactions, and characterization of the proteome composition and functions of nuclear speckles would likely shed more light on the mechanism of cryptochrome signal transduction. It is also expected that studies of cryptochromes, phytochromes, COP/DET/FUS proteins, and the circadian clock will converge to bring about a better understanding of the molecular mechanism of photomorphogenesis.
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Figure 1  Structure of E. coli photolyase and a model structure of Arabidopsis cry2. 
(A) Structure of E. coli photolyase is shown in two forms, a ribbon diagram (top) and a solvent accessible surface and electrostatic surface potential diagram (bottom). (Atomic coordinates were from the Protein Data Bank, Accession number 1DNP). (B) A hypothetical structure of the Arabidopsis cry2 PHR domain (residue 5–490) modeled based on the crystal structure of E. coli photolyase. The amino acid sequence alignment and tertiary model construction was carried out by Nakamura using the method described previously (42). Colors indicate the surface electrostatic potential being above 0.1 Volt (blue), neutral (white), or below −0.1 Volt (red), or the solvent accessible surface that is hydrophobic (yellow).
# CONTENTS

Frontispiece—Lloyd T. Evans  xii  

**Conjectures, Refutations, and Extrapolations**, Lloyd T. Evans  1  

**Understanding the Functions of Plant Disease Resistance Proteins**, Gregory B. Martin, Adam J. Bogdanove, and Guido Sessa  23  

**Protein Phosphatases in Plants**, Sheng Luan  63  

**Plant Peroxiredoxins**, Karl-Josef Dietz  93  


**Biosynthesis and Metabolism of brassinosteroids**, Shozo Fujioka and Takao Yokota  137  

**The COP9 Signalosome: Regulating Plant Development Through the Control of Proteolysis**, Giovanna Serino and Xing-Wang Deng  165  

**Iron Transport and Signaling in Plants**, Catherine Curie and Jean-François Briat  183  

**From Bacterial Glycogen to Starch: Understanding the Biogenesis of the Plant Starch Granule**, Steven G. Ball and Matthew K. Morell  207  

**The Plant Cell Cycle**, Walter Dewitte and James A.H. Murray  235  

**Phospholipid-Based Signaling in Plants**, Harold J.G. Meijer and Teun Munnik  265  

** Gibberellins and Flowering of Grasses and Cereals: Prizing Open the Lid of the “Florigen” Black Box**, Rod W. King and Lloyd T. Evans  307  

**Photosynthesis of Overwintering Evergreen Plants**, Gunnar Öquist and Norman P.A. Huner  329  

**Structure of Linkage Disequilibrium in Plants**, Sherry A. Flint-Garcia, Jeffry M. Thornsberry, and Edward S. Buckler IV  357  

**Single-Nucleotide Mutations for Plant Functional Genomics**, Steven Henikoff and Luca Comai  375
CONTENTS

HOW DO CELLS KNOW WHAT THEY WANT TO BE WHEN THEY GROW UP? LESSONS FROM EPIDERMAL PATTERNING IN ARABIDOPSIS,
John C. Larkin, Matt L. Brown, and John Schiefelbein 403

TRANSFER CELLS: CELLS SPECIALIZED FOR A SPECIAL PURPOSE,
Christina E. Offer, David W. McCurdy, John W. Patrick, and Mark J. Talbot 431

CHLOROPLAST MOVEMENT, Masamitsu Wada, Takatoshi Kagawa, and Yoshikatsu Sato 455

CRYPTOCHROME STRUCTURE AND SIGNAL TRANSDUCTION,
Chentao Lin and Dror Shalitin 469

MEMBRANE-BOUND DIIRON CARBOXYLATE PROTEINS,
Deborah A. Berthold and Pål Stenmark 497

LIGNIN BIOSYNTHESIS, Wout Boerjan, John Ralph, and Marie Baucher 519

APOMIXIS: A DEVELOPMENTAL PERSPECTIVE, Anna M. Koltunow and Ueli Grossniklaus 547

MOLECULAR MECHANISMS AND REGULATION OF K+ TRANSPORT IN HIGHER PLANTS, Anne-Aliénor Very and Hervé Sentenac 575

PERCEPTION AND SIGNAL TRANSDUCTION OF CYTOKININS,
Tatsuo Kakimoto 605

FUNCTIONAL GENOMICS OF P450S, Mary A. Schuler and Daniele Werck-Reichhart 629

METABOLOMICS IN SYSTEMS BIOLOGY, Wolfram Weckwerth 669

REMODELING THE CYTOSKELETON FOR GROWTH AND FORM: AN OVERVIEW WITH SOME NEW VIEWS, Geoffrey O. Wasteneys and Moira E. Galway 691

INDEXES

Subject Index 723
Cumulative Index of Contributing Authors, Volumes 44–54 753
Cumulative Index of Chapter Titles, Volumes 44–54 758

ERRATA

An online log of corrections to Annual Review of Plant Biology chapters (if any, 1997 to the present) may be found at http://plant.annualreviews.org/