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The cryptochrome gene family in pea includes two differentially expressed *CRY2* genes

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Abstract

The cryptochromes are a family of blue light photoreceptors that play important roles in the control of plant development. We have characterised the cryptochrome gene family in the model legume garden pea (*Pisum sativum* L.). Pea contains three expressed cryptochrome genes; a single *CRY1* orthologue, and two distinct *CRY2* genes that we have termed *CRY2a* and *CRY2b*. Genomic southern blots indicate that there are unlikely to be more *CRY* genes in pea. Each of the three genes encodes a full-length CRY protein that contains all the major domains characteristic of other higher plant cryptochromes. Database searches have identified *Medicago truncatula* expressed sequence tags (ESTs) corresponding to all three genes, whereas only a single *CRY2* is represented in EST collections from the more distantly related legumes soybean and *Lotus japonicus*. The proteins encoded by the pea and Medicago *CRY2b* genes are distinguished from other CRY2 proteins by their shorter C-terminus. Expression analyses have identified marked differences in the regulation of the three genes, with *CRY2b* expression in particular distinguished by high-amplitude diurnal cycling and rapid repression in seedlings transferred from darkness to blue light.

Introduction

Light is one of the most important factors influencing plant growth and development, and plants possess several photoreceptor systems that allow them to sense and respond to their light environment. In general, plant responses to red and far-red light are mediated by the phytochrome family of photoreceptors, whereas two other photoreceptor families, the cryptochromes and phototropins, specifically mediate responses to blue light. Cryptochromes are flavoproteins that share homology with the photolyase family of light-dependent DNA repair enzymes, but do not themselves possess photolyase activity (Cashmore, 2003; Lin and Shalitin, 2003). They are found in plants, animals and cyanobacteria, and appear to have arisen independently from different branches of the photolyase family in plants and animals (Brudler *et al.*, 2003). Plant cryptochromes have been characterized most thoroughly in the model species Arabidopsis, which possesses two cryptochromes (CRY1 and CRY2) that function in blue light perception (Lin and Shalitin, 2003). An

additional *CRY* gene that belongs to the newly characterised cryptochrome-DASH class has also been identified in Arabidopsis, although its biological role is not yet known (Brudler *et al*., 2003).

The Arabidopsis CRY1 and CRY2 proteins comprise two distinct domains; a highly conserved N-terminal domain and a divergent C-terminal extension. The N-terminal domain shares many features with microbial photolyases and includes regions that are proposed to bind the chromophores flavin adenine dinucleotide (FAD) and methenyltetrahydrofolate (MTHF). This region has been designated the photolyase-related or PHR domain (Lin and Shalitin, 2003). In contrast, the C-terminal extensions of Arabidopsis CRY1 and CRY2 proteins have only limited similarity to each other and do not show significant homology with any known protein. Both CRY1 and CRY2 are constitutively nuclear proteins (Guo *et al*., 1999; Kleiner *et al*., 1999).

Several recent reports indicate that the C-terminal extension plays an important role in cryptochrome signalling (Yang *et al*., 2000, 2001). Overexpression of the C-terminal extension from either CRY1 or CRY2 causes Arabidopsis seedlings to develop with the morphology of a light-grown seedling even when grown in the dark (Yang *et al*., 2000). This result suggests that in the native cryptochrome molecule, the activity of the C-terminal extension is suppressed by the unexcited PHR domain, and that light absorption by the PHR domain somehow removes this suppression. This intramolecular signalling may involve phosphorylation, because native CRY1 and CRY2 molecules undergo blue-light dependent phosphorylation, whereas the C-terminal extension when expressed alone is constitutively phosphorylated (Shalitin *et al*., 2002, 2003). The C-terminal extension of CRY1 has been shown to interact with the COP1 protein, a E3 ubiquitin ligase that promotes degradation of several transcription factors including the bZip protein HY5 (Wang *et al*., 2001). Suppression of COP1 activity by CRY1 may contribute to photomorphogenesis by enabling HY5 and other transcription factors to activate expression of light responsive genes (Cashmore, 2003).

Mutant studies in Arabidopsis have shown that the cryptochromes play a central role in a number of distinct blue light regulated developmental processes, including de-etiolation and photoperiodic flowering. Studies of *cry1* mutants have shown that CRY1 mediates de-etiolation responses under high-irradiance blue light (Ahmad and Cashmore, 1993). Adult *cry1* mutant plants grown under white light also exhibit changes in development, including altered stem elongation and leaf size (Jackson and Jenkins, 1995). Arabidopsis CRY2 also contributes to blue-light mediated de-etiolation, but this contribution is relatively subtle and is restricted to lower irradiances because the CRY2 protein is subject to degradation under higher irradiances of blue light (Lin *et al*., 1998). CRY2 has a more prominent role in the photoperiodic control of flowering, and *cry2* mutants flower considerably later than wild-type (WT) plants under long days, with reduced photoperiod responsiveness (Guo *et al*., 1998). CRY1 also contributes to the promotion of flowering in long days, but to a much lesser extent than CRY2 (Mockler *et al*., 1999).

Although considerable progress has been made in understanding cryptochrome function in Arabidopsis, comparative studies are less advanced. Cryptochrome genes have so far been identified in only a small number of plant species. Most higher plants so far examined appear to have at least one *CRY1* and one *CRY2* gene (Perrotta *et al*., 2000). The *CRY1* gene appears to have been duplicated in several species, including rice and barley (Perrotta *et al*., 2001; Matsumoto *et al*., 2003) and an additional expressed *CRY1* gene (*CRY1b*) has also been reported in tomato (Perrotta *et al*., 2001) although it has a truncated C-terminal extension and is possibly non-functional. A *CRY2* gene has also been identified in tomato and barley (Perrotta *et al*., 2000). The fern *Adiantum capillus-veneris* contains five cryptochromes, which comprise two distinct groups that cluster separately from higher plant cryptochromes (Kanegae and Wada, 1998), and two very closely related *CRY* genes have been described from the moss *Physcomitrella patens* (Imaizumi *et al*., 2002). Disruption of the *Physcomitrella CRY* genes affects the photocontrol of protonemal branching and gametophore induction (Imaizumi *et al*., 2002), showing that cryptochromes also play important developmental roles in lower plants. Mutant analyses have shown that tomato CRY1 regulates de-etiolation in a manner similar to Arabidopsis CRY1, while in plants grown under white light it also influences branching, internode length, and the chlorophyll content of leaves and fruit (Weller *et al*., 2001b). Apart

from these examples, data on the function of cryptochrome genes in species other than Arabidopsis is limited to preliminary transgenic studies (Matsumoto *et al*., 2003; Giliberto *et al*., 2005).

Our earlier analyses of phytochrome-deficient mutants in the garden pea (*Pisum sativum* L.) have revealed important roles for a blue light photoreceptor system in both de-etiolation and photoperiodic flowering (Weller *et al*., 2001a). In this paper, we describe the members of the cryptochrome gene family in pea, and investigate their transcriptional regulation. We show that pea contains three expressed full-length cryptochrome genes, including a single *CRY1* and two differently regulated *CRY2* genes.

Materials and methods

Gene isolation

Degenerate primers were designed from alignments of Arabidopsis and tomato CRY1 and CRY2 sequences using the Blockmaker and CODEHOP programs (Henikoff, 1995; Rose *et al*., 1998) and tested on cDNA isolated from *Pisum sativum* L. cultivar Torsdag. Primers 5′-TTTTCCTGTTTTTATTTGGTGTCCNRARGARGA-3′ (forward, target amino acid sequence FPVFIWCPEEE) and 5′-CCCAAAAATACTTCATTCCCCANYKCCANGG-3′ (reverse, target amino acid sequence PWRWGMKYFWD) yielded fragments of *CRY2a* and *CRY2b*. Primers 5′-GAGCTTGGTCWCCWGGNTGG-3′ (forward, target amino acid sequence RAWSPGW) and 5′-CCARAARTAYTTCATYCCCC-3′ (reverse, target amino acid sequence WGMKYFW) yielded fragments of *CRY1* and *CRY2a*. Additional primers 5′-TGGAGCAATGCGAACAAG-3′ (forward) and 5′-GCCAACCAGAAGCCCACAA-3′ (reverse) were designed to specifically amplify a 420 bp fragment of *CRY1* used as a probe for library screening. Fragments of the pea cryptochrome genes were labelled using the DIG-ChemLink Labelling and Detection Set (Roche Molecular Biochemicals, Castle Hill, NSW, Australia) and used to screen approximately 2×10^5 plaque colonies from a pea cv. Alaska shoot cDNA library (Clontech, Palo Alto, CA, USA). In each case several positive clones were confirmed and purified in secondary and tertiary screens. 3′-RACE was performed as described by Frohman *et al*. (1988).

Mapping

For each of the *CRY* genes, variant alleles in parents of available recombinant inbred line (RIL) populations were identified by sequencing genomic DNA, and CAPS markers were developed to distinguish appropriate variants. The *CRY2a* and *CRY2b* genes were mapped in a RIL population derived from cultivars Torsdag and Térèse (Laucou *et al*., 1998). For *CRY2a*, the primers 5′-CTTATTCCGTGCTGATATAC-3′ (forward) and 5′-TCGAAACTCGGCCCGGATAA-3′ (reverse) amplified a 360 bp genomic fragment containing a diagnostic HphI site. For *CRY2b*, primers 5′-GTTCAAGCTACAAAAGTAGTGTTTAATC-3′ (forward) and 5′-CATGTCCACTTTCGTCGAATA-3′ (reverse), amplified a 332 bp genomic fragment differentially digested by NsiI. Scoring of markers in the mapping population and two-point linkage analysis was kindly performed by Dr. Catherine Rameau (INRA, Versailles, France). The *CRY1* gene showed no convenient polymorphism between these two parents and was instead mapped in a RIL population JI281 × JI399 (Hall *et al*., 1997). Primers 5′-ATTCGTCGTTGGTCTTTCAG-3′ (forward) and 5′-AACATGACAGAGAAGCGAGTAA-3′ (reverse) amplified a 253 bp product digested with HpaI in JI281.

Sequence analysis and phylogenetic relationships

Multiple sequence alignments were performed by manual adjustment of output from ClustalX software (Thompson *et al*., 1997) and analysed using GeneDoc (http://www.psc.edu/biomed/genedoc (Nicholas *et al*., 1997). Phylogenetic trees were constructed from alignments using both distance and parsimony methods in PAUP* 4.0b10 (Swofford, 1993). Sequences used in these analyses and not previously mentioned in the text are available in GenBank under the following accession numbers; Arabidopsis CRY1 (AAB28724) and CRY2 (AAD09837), tomato CRY1a (AAF72555), CRY1b (AAL02093) and CRY2 (AAF72557), rice CRY1a (BAB70686), CRY1b (BAB70688) and CRY2 (BAC56984), *Sorghum bicolor* CRY1 (AAN37909), *Orobanche minor* CRY1 (AAR08429), *Armoracia*

rusticana CRY2-1 (BAC67176), *Sinapis alba* PHR (CAA50898), *Adiantum capillus-veneris* CRY1 (BAA32810), CRY2 (BAA32808), CRY3 (BAA32809), CRY4 (BAA88423) and CRY5 (BAA88424), *Physcomitrella patens* CRY1a (BAA83338) and CRY1b (BAB70665), *Chlamydomonas reinhardtii* CPH1 (AAC37438).

Gene expression

All plants were grown in growth cabinets at 20 °C employing standard light sources as described by Reid *et al.* (2002) with the exception that blue light was provided by Nichia NSPB510S WF3 Super Blue diodes (Nichia Corp., Singapore). Plants used to monitor expression in different plant parts were grown in continuous white light at 100 μmol m^{-2} s^{-1} for 12 days. For the continuous dark/light comparison plants were grown for 12 days in continuous darkness, white light (100 μmol m^{-2} s^{-1}) or blue light (20 μmol m^{-2} s^{-1}) before the whole shoot was harvested for analysis. For transfer experiments plants were grown for 7 days in dark and transferred to blue light at 20 μmol m^{-2} s^{-1}. In these experiments, 2–3 pools of 6–8 plants were harvested for each data point. To monitor diurnal changes in expression, plants were grown under 12 h-light/12 h-dark cycle for 21 days and apices were harvested every 3 h for two consecutive days, in either the same light-dark cycle, or after transfer to continuous light.

RNA was extracted from approximately 100 mg of tissue using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA was quantified by spectrophotometry and cDNA was synthesised from 4.5 μg of RNA with Superscript II (Invitrogen, USA) according to the manufacturers instructions. cDNA was diluted and duplicate RT-qPCR reactions were carried out using Dynamo SYBR Green Master Mix (Finnzymes, Espoo, Finland) on a Rotorgene 2000 Real-Time Cycler (Corbett Research, Mortlake, Victoria, Australia). Reactions were conducted in 20 μl with 100–200 pmol of each prihmer under the following conditions; 94 °C for 15 min, followed by 60 cycles of (15 s at 94 °C, 20 s at 58–59 °C and 30 s at 72 °C), and a final 15 min at 72 °C. Primers for actin, 18S rRNA and ubiquitin reference genes have been described previously (Albrecht *et al.*, 1999; Ozga *et al.*, 2003; Foo *et al.*, 2005). Primers for RT-qPCR amplification of pea *CRY* genes were designed to flank an exon-exon boundary using Primer Express software; CRY1F (5′-GGTATAGGAGCCACTTCACCTTACTT-3′), CRY1R (5′-TGTATATAATGTGAGTTTATCCAGTTTGAGGTA-3′), CRY2aF (5′-ACAGTGTTCTTCATCTTCGAATCTAAAA-3′), CRY2aR (5′-AACCTCATGTTGGCCCATCTT-3′), CRY2bF (5′-CTCAAGACCGTTTTTAAGAATCTTGA-3′), CRY2bR (5′-CCCTCCTAAACCAAACAATAGTCCTA-3′).

Results

Isolation of pea cryptochrome genes

We initially attempted to isolate *CRY*-related sequences from pea using a PCR approach. Degenerate primers were designed from alignments of published Arabidopsis and tomato CRY sequences with publicly available *Medicago truncatula* EST sequences. These primers amplified three distinct cDNA fragments; one corresponding to *CRY1* and two different products corresponding more closely to *CRY2*, which we designated *CRY2a* and *CRY2b*. Probes derived from these partial sequences were then used to screen a pea cv. Alaska shoot cDNA library. Full-length cDNA clones were isolated for *CRY1* and *CRY2b*. For *CRY2a* an incomplete cDNA was isolated, and the missing sequence was subsequently obtained by 3′-RACE.

The *PsCRY1*, *PsCRY2a* and *PsCRY2b* genes encode conceptual proteins of 682, 629 and 598 amino acids, respectively. The difference in size between the pea CRY proteins is mainly due to differences in the length of the extreme C-terminus. Overall amino acid identities derived from a ClustalX alignment of full-length CRY apoproteins show that the predicted PsCRY1 is clearly much more similar to CRY1 proteins from tomato (80%) and Arabidopsis (75%) than it is to the CRY2 proteins from these species or from pea itself (45–47%). The two predicted pea CRY2 proteins showed stronger similarity to each other (73%) than to CRY2 sequences from other dicotyledonous species (59–65%).

We also determined the cDNA sequence of all three genes from our standard WT line, cv. Torsdag (GenBank accession numbers AY508969,

AY508972, AY508974). No polymorphisms between cv. Torsdag and Alaska were observed in the coding regions of *CRY1* or *CRY2a* genes, but for *CRY2b*, several polymorphisms were identified, all within the C-terminal domain. Relative to the cv. Alaska sequence, three single nucleotide substitutions in the Torsdag sequence are predicted to direct amino acid substitutions F482L, K492E and C589S, while a 3-bp insertion causes the substitution of H500 with an EQ residue pair.

To define the extent of the *CRY* gene family in pea, full-length cDNA clones for each of the three *CRY* genes were used to probe genomic Southern blots. Figure 1 shows that when blots were hybridized with *CRY1* probe at low stringency, a single strongly hybridizing band was detected in each lane. In contrast, multiple bands per lane were detected in low stringency hybridizations with either *CRY2a* or *CRY2b*. Comparison with high stringency hybridizations (data not shown) revealed that all the bands detected on low stringency *CRY2* blots were specific for either *CRY2a* or *CRY2b* (Figure 1). Together with the fact that the *CRY1* probe only detected a single band even under low stringency conditions, this suggests that there are unlikely to be any additional *CRY1*- or *CRY2*-related genes present in pea.

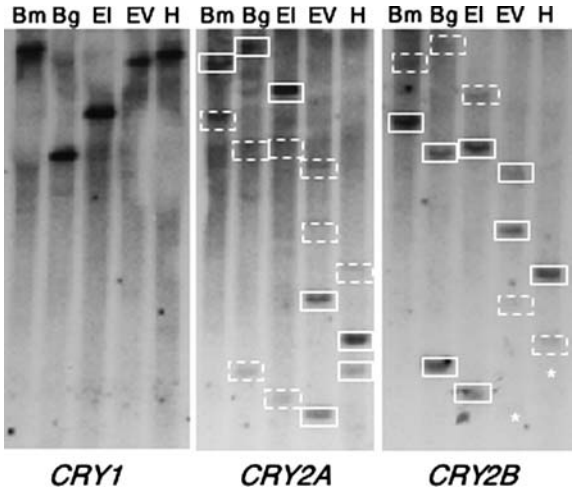

Figure 1. Southern blots of pea cv. Torsdag genomic DNA probed with *CRY1*, *CRY2a* and *CRY2b* under low stringency conditions. Bands indicated by solid boxes were also detected under high stringency conditions, while those marked with dashed boxes represent cross-hybridizing bands and were only present under low stringency conditions. Asterices indicate bands present in *CRY2a* blots and not seen in *CRY2b* blots. Enzymes used for restriction digests Bm – *Bam*HI, Bg – *Bgl*II, EI – *Eco*RI, EV – *Eco*RV, H – *Hin*dIII.

We next obtained genomic sequence corresponding to each of the *PsCRY* genes from cv. Torsdag (GenBank accession numbers AY508970, AY508973, AY508975). Comparisons of genomic and cDNA sequences revealed four introns in each gene. Figure 2 shows that the positions of the first three introns in the coding sequence are common to all three pea genes, and are also conserved in other higher plant *CRY* genes. *PsCRY1* and *PsCRY2a* each have an additional intron near their 3′ end that is also found in most other *CRY* genes, with the exception of *AtCRY2* (Perrotta *et al.*, 2001). *PsCRY2b* also lacks this intron, but like *AtCRY2*, has an additional intron in the region corresponding to the 5′-untranslated region of the mRNA.

Polymorphisms in non-coding regions were used to locate each of the pea genes on the pea genetic map (Figure 3). *PsCRY1* was mapped using a set of recombinant inbred lines derived from the cross JI281 × JI399 (Hall *et al.*, 1997) and is located in the top half of linkage group 1, less than 1 cM from the Tps1/145+ RAPD marker (Laucou *et al.*, 1998). *PsCRY2a* and *PsCRY2b* were mapped using a set of recombinant inbred lines derived from a cross between cv. Térèse and K586, a mutant in the cv. Torsdag background (Laucou *et al.*, 1998). These genes are located 6 cM apart in linkage group 2, between *LF* and *PHYA*.

Relationships to cryptochromes from other species

Full-length CRY apoprotein sequences have been reported from only a small number of plant species. Figure 4A shows a phylogram derived from alignment of the three predicted pea CRY sequences with these other plant cryptochromes and rooted with the *Chlamydomonas reinhardtii* cryptochrome CPH1 as an outgroup. This analysis supports previous observations that higher plant cryptochromes fall into distinct CRY1 and CRY2 clades and that each cluster separately from lower plant sequences (Perrotta *et al.*, 2000; Lin and Shalitin 2003). In addition it demonstrates that the two cryptochrome sequences previously described from rice and designated as *CRY1* and *CRY2* (Matsumoto *et al.*, 2003) in fact both encode CRY1-like proteins, whereas a third rice *CRY* sequence retrieved from GenBank (accession

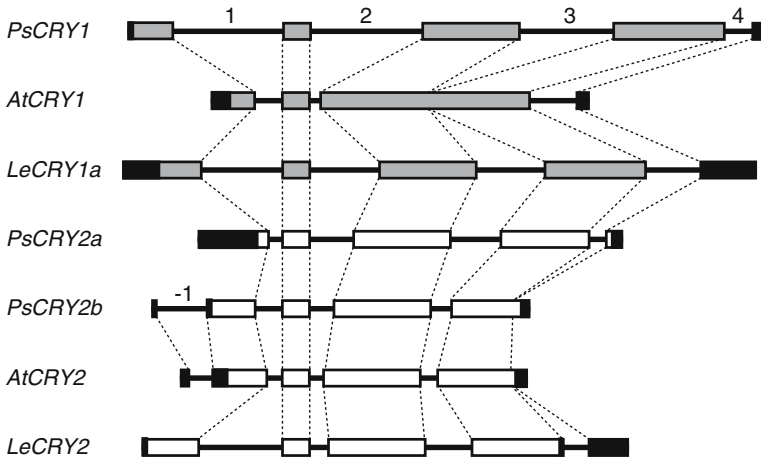

Figure 2. Comparison of *CRY* gene structure in pea, tomato and Arabidopsis. Coding regions are depicted by grey boxes (*CRY1*) or white boxes (*CRY2*), and untranslated regions are shown as black boxes. Introns are shown as solid lines. The conserved intron/exon boundaries are connected by dashed lines. *Ps – Pisum sativum*, *Le – Lycopersicon esculentum*, *At – Arabidopsis thaliana*.

number BAC56984) encodes a *bona fide* CRY2. The presence of both *CRY1* and *CRY2* sequences in rice and in three diverse dicotyledonous species indicates that the *CRY1*/*CRY2* duplication is relatively ancient, and is likely to predate the divergence of the monocots and eudicots some 200 million years ago (Soltis and Soltis, 2003). Within the CRY2 clade, pea CRY2a and CRY2b are clearly more closely related to each other than either is to tomato CRY2 or Arabidopsis CRY2, indicating that they most likely have arisen from a duplication that occurred after the divergence of these three groups. However, the two pea CRY2 sequences are also relatively divergent from each other, and with an amino acid identity of 72% they are less similar than CRY2 proteins from the two closely related species *Armoracia rusticana* and Arabidopsis (91% identity).

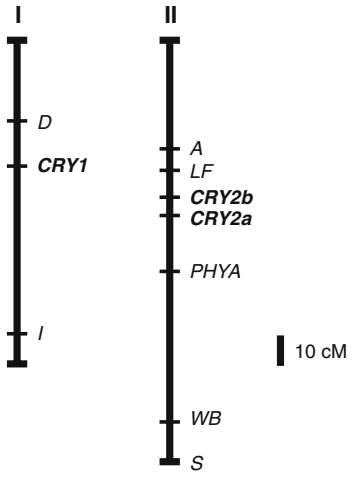

Figure 3. Positions of *CRY* genes in pea linkage groups I and II.

In an attempt to better define the history of the *CRY2* duplication in the legume lineage, we looked for *CRY2*-homologous sequences within the Medicago, soybean and Lotus EST collections. BLAST searches (http://tigrblast.tigr.org/tgi) of the Medicago EST database identified contigs corresponding to each of the three pea genes; *CRY1* (TC89497), *CRY2a* (TC78737, TC79042) and *CRY2b* (TC86685). Genomic sequence corresponding to TC86685 was also identified (AC122171) through the MtGenome facility at UC Davis (http://mtgenome.ucdavis.edu/), allowing the conceptual translation of the full-length Medicago CRY2b protein. The map position of the *MtCRY2b* gene (linkage group 1/18.1 cM) corresponds to the position determined for *PsCRY2b* in linkage group 2 according to the syntenic relationship recently described (Kaló *et al*., 2004). BLAST searches of the soybean EST database identified only a single *CRY2* contiguous sequence (TC190841) but at least two distinct contigs for *CRY1* at both the 5′ (TC183546 and TC193237) and 3′ (TC193238 and TC177386) ends. In Lotus databases, both *CRY1* and *CRY2* are represented by several non-overlapping ESTs or EST contigs (*CRY1*, TC15430 and AV424420; *CRY2*, AV417531, AV424018, AV766989). This information shows that two distinct *CRY2* genes are

expressed in Medicago, and at least two distinct *CRY1* genes are expressed in soybean. In addition, it suggests that soybean and Lotus each transcribe only a single *CRY2*-related sequence. The phylogram in Figure 4B is derived from alignment of a 196 amino acid N-terminal fragment common to Medicago and soybean EST contigs and shows that *Gm*CRY2 clusters basally to pea and Medicago CRY2 sequences.

Sequence comparisons within the cryptochrome family

All previously described plant cryptochromes consist of two domains; the N-terminal photolyase-homologous PHR domain of approximately 500 amino acids and a shorter, highly variable C-terminal extension. The PHR domain is relatively well conserved across all plant cryptochromes. It contains a number of highly conserved regions including several motifs proposed to bind or interact with the FAD and MTHF chromophores (Lin *et al*., 1998; Perrotta *et al*., 2000), and a conserved TGYP motif around which a number of *cry1* mutations are found (Ahmad *et al*., 1995). These motifs are all represented in each of the three pea cryptochromes.

In contrast to the PHR domain, the C-terminal extension is very poorly conserved across the cryptochrome family, and is highly variable in length across species (Lin, 2002; Lin and Shalitin, 2003). The low degree of conservation makes it difficult to align in a meaningful way, but a number of conserved motifs have nevertheless been described (Lin and Shalitin, 2003). The two most prominent motifs in the C-terminal extension are the so-called DQXVP and STAESS motifs, which are separated by a region rich in acidic residues. These three features have been referred to

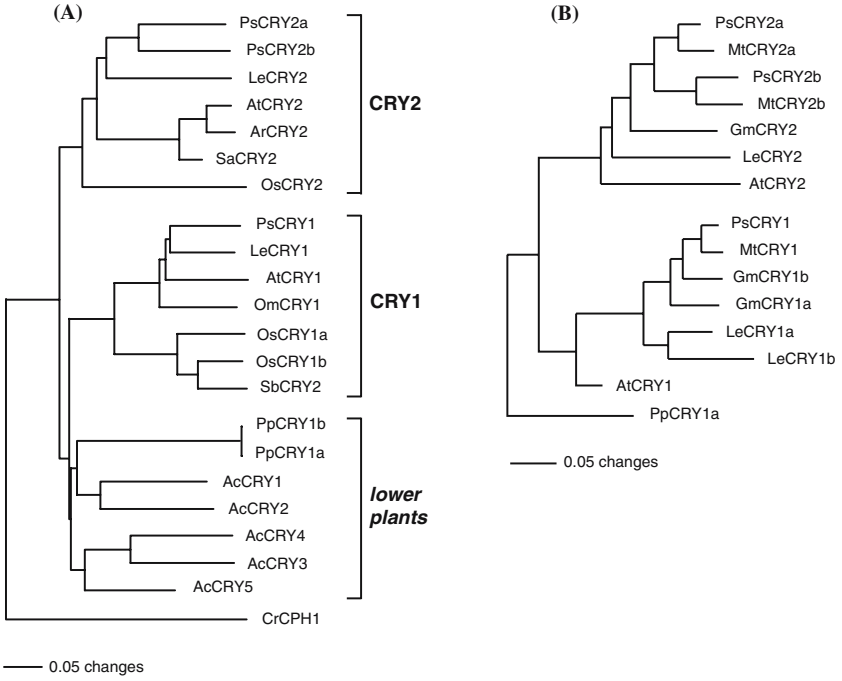

Figure 4. Neighbour-joining phylogram depicting relationships among plant cryptochrome proteins. (A) Tree constructed from an alignment of full-length plant cryptochrome sequences (B) Tree constructed from alignment of a 196 residue N-terminal sequence block (extending from residues 14 to 204 in pea CRY1) common to CRY-homologous EST and genomic sequences from Medicago and soybean. Ps – *Pisum sativum*, Le – *Lycopersicon esculentum*, Ar – *Armoracia rusticana*, At – *Arabidopsis thaliana*, Os – *Oryza sativa*, Om – *Orobanche minor*, Sb – *Sorghum bicolor*, Ac – *Adiantum capillus-veneris*, Pp – *Physcomitrella patens*, Cr – *Chlamydomonas reinhardtii*, Mt – *Medicago truncatula*, Gm – *Glycine max*, Sa – *Sinapis alba*. Both alignments were performed using ClustalX and adjusted manually.

collectively as the DAS region (Lin, 2002). The importance of the DAS region is implied by the conservation of all three features in moss and fern CRY proteins, and the fact that several loss-of-function mutants in Arabidopsis CRY1 or CRY2 carry missense mutations in this region (Lin and Shalitin, 2003). Figure 5 shows pairwise sequence identities calculated separately for the PHR domain and C-terminal extension across a number of higher plant CRY proteins. Overall, the C-terminal extension is somewhat better conserved in the CRY1 subfamily (50–60% identity for dicots) than in the CRY2 subfamily, where the highest identity for a cross-family comparison is 32% between tomato CRY2 and pea CRY2a (Figure 5).

Figure 6 shows an alignment of the C-terminal extension from a number of higher plant CRY proteins including the three pea sequences and the putative CRY proteins encoded by EST contigs or genome sequence from Medicago and soybean. This alignment confirms that all three features of the DAS region are present in legume sequences, and illustrates the variation within each motif across species and between the CRY subfamilies. The DQXVP motif is perfectly conserved across all higher plant CRY proteins. The STAESS motif is not absolutely conserved across all CRY proteins, but nevertheless clearly constitutes a cluster of serine residues that shows only minor variation across all the higher plant sequences. In addition to being serine-rich itself, the STAESS motif forms the first part of a larger region at the extreme C-terminus that is also relatively rich in serine residues. The proportion of serine residues in this region ranges from 15% in pea CRY1 to 50% in pea CRY2b (Figure 6). Rice CRY2 is quite different from dicot CRY2 sequences, with a much longer intervening acidic region between the DQXVP and STAESS motifs that is also relatively serine-rich. Figure 6 also shows that the GGXVP motif previously noted to follow the STAESS motif in a number of higher plant CRY (Lin and Shalitin, 2003) is only conserved in CRY1 sequences, and is not present in CRY2. The presence of a putative nuclear localization signal between the DQXVP and STAESS motifs has previously been observed for rice CRY1a (Matsumoto *et al*., 2003) and Arabidopsis CRY2 (Guo *et al*., 1999; Kleiner *et al*., 1999), and similar residue clusters are present in the same region of pea CRY2a and CRY2b (Figure 6)

The most distinctive feature of pea CRY2b is the truncation of the C-terminal region following the STAESS motif, which consists of approximately 50aa in CRY2a but only 20aa in CRY2b. This same truncation is also seen in the predicted CRY2b from Medicago (Figure 6). The single CRY2 sequence represented in soybean EST collections contains a longer C-terminal extension typical of pea and Medicago CRY2a and other dicot CRY2 (Figure 6). The predicted soybean CRY2 shares features of both pea CRY2a and CRY2b, but overall is closer to CRY2a than CRY2b. Out of 175 residues that are imperfectly conserved across these three CRY, 72 (41%) are

C-terminal extension

	Ps 1	Le 1	At 1	Os 1a	Sb 2	Ps 2a	Ps 2b	Le 2	At 2	Ar 2.1	Os 2
Ps 1		57	52	37	38	10	11	13	10	10	10
Le 1	90		50	39	42	12	11	12	12	12	10
At 1	86	86		35	35	11	11	13	12	12	13
Os 1a	75	76	74		63	9	9	10	9	9	10
Sb 1	74	76	76	84		11	10	9	7	7	8
Ps 2a	64	64	60	62	62		42	32	26	26	16
Ps 2b	60	60	57	59	58	83		31	24	25	16
Le 2	61	63	58	60	59	77	74		23	23	15
At 2	60	61	57	59	58	74	70	71		82	11
Ar 2.1	59	60	57	59	58	74	69	71	94		11
Os 2	59	60	57	59	58	68	65	68	66	65	

N-terminal PHR domain

Figure 5. Pairwise amino acid identities (%) for the N-terminal PHR domain (lower left) and the C-terminal extension (upper right) of higher plant cryptochrome proteins. Values are derived from a manually adjusted ClustalX alignment. Species abbreviations are as in Figure 4.

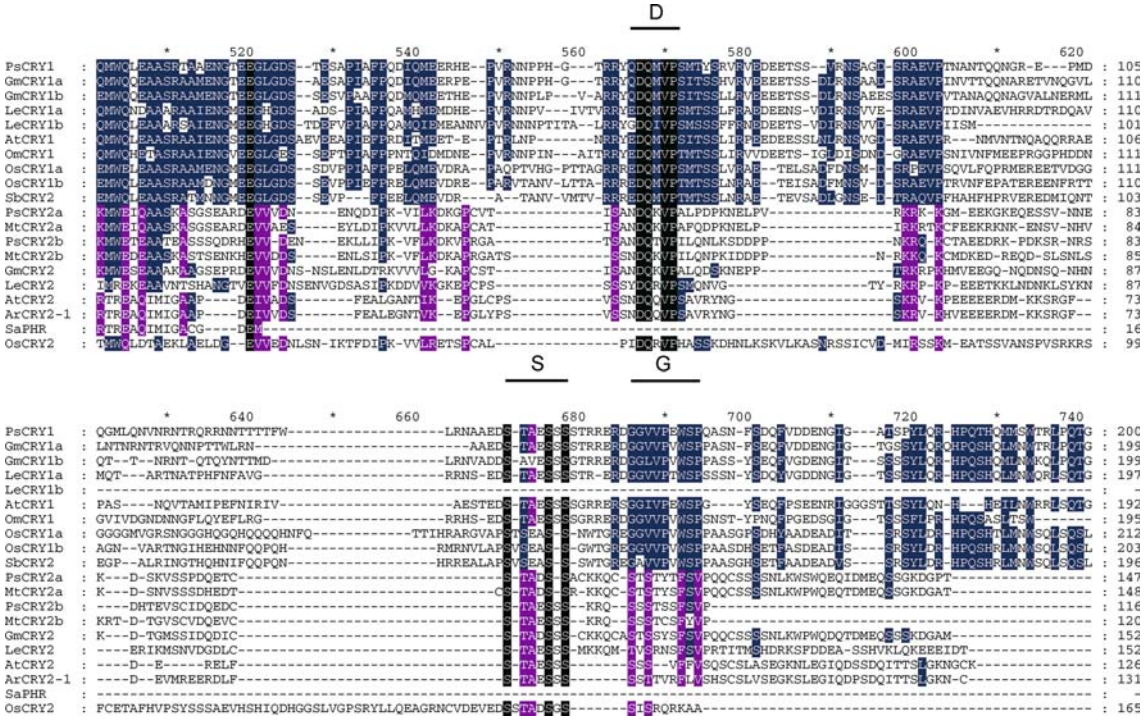

Figure 6. Amino acid alignment of the C-terminal extensions of higher plant cryptochromes. Residues conserved across all sequences are shaded in black. Residues conserved across CRY1 but not CRY2 sequences are shaded in blue, residues conserved across CRY2 but not CRY1 sequences are shaded in purple. D – DQXVP motif, S – STAESS motif, G – GGXVP motif. Species abbreviations are as in Figure 4. The alignment was performed using ClustalX and adjusted manually.

identical in soybean CRY2 and pea CRY2a, but only 16 (9%) are identical in soybean CRY2 and pea CRY2b.

Expression of pea cryptochrome genes

It is possible that functional differences among the pea *CRY* genes might be reflected in differences in their patterns of expression, and we therefore examined whether transcript levels of the pea cryptochrome genes were regulated in a tissue-specific manner or in response to different light treatments. The results of this analysis are shown in Figure 7.

Each of the *CRY* genes was expressed to a detectable level in all seedling tissues that were examined (Figure 7A). *CRY1* transcript levels were somewhat elevated in leaf compared to other tissues and the *CRY2a* gene was expressed equally in all tissues examined. In contrast, *CRY2b* transcript levels were relatively high in shoot apex and root tip tissues compared to the levels in leaf and internode tissue.

Each of the *CRY* genes was expressed at a lower level in whole shoots of seedlings grown in continuous white or blue light relative to seedlings grown in complete darkness (Figure 7B). The levels of both *CRY1* and *CRY2b* transcripts were 5- to 10-fold lower in plants grown in continuous white or blue light than in the dark-grown seedlings. *CRY2a* transcript levels were also somewhat reduced in these light-grown seedlings. To further characterize the regulation of *CRY2b* expression by blue light we examined *CRY2b* transcript levels in a *phyA* null mutant. Figure 8A shows that the down-regulation of *CRY2b* expression is greatly reduced in the *phyA* mutant, to the extent that the *CRY2b* transcript level in blue-light grown phyA seedlings did not significantly differ from the level in dark-grown seedlings.

CRY gene expression was also monitored in whole shoots of 7-day-old seedlings over 24h following transfer from complete dark to continuous blue light (Figure 7C). A substantial and rapid reduction in *CRY2b* transcript level was

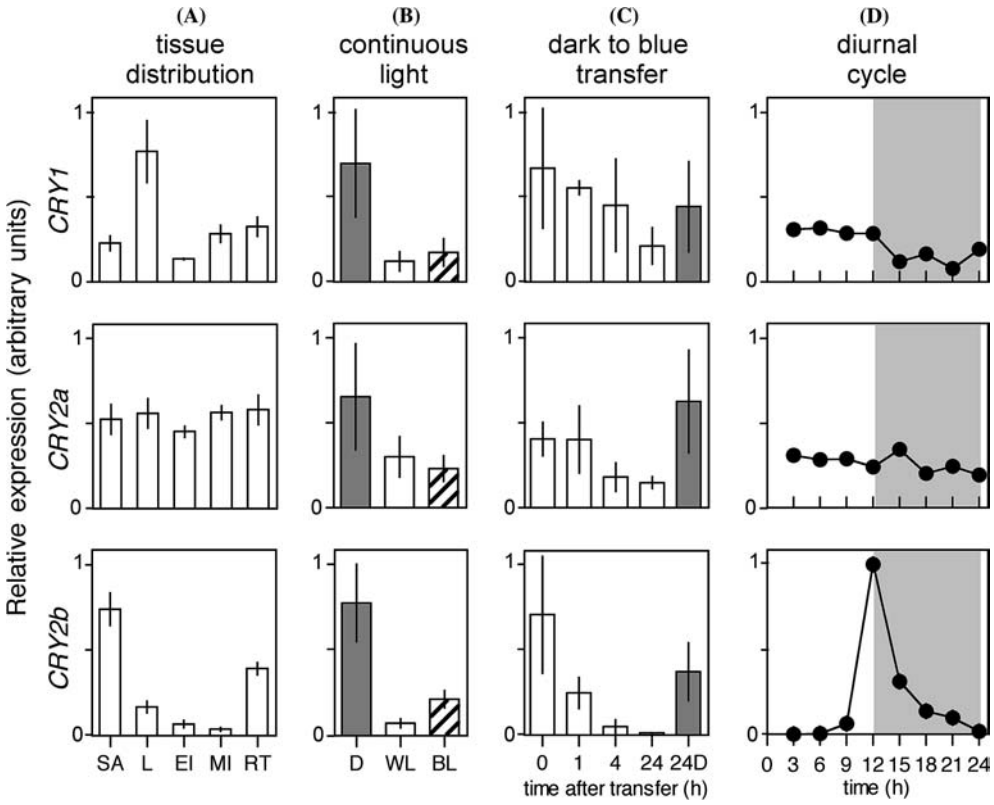

Figure 7. Expression analysis of pea *CRY* genes. The expression levels of *CRY1*, *CRY2a* and *CRY2b* were determined by RT-qPCR and normalized to expression of an appropriate reference gene. Values represent arbitrary units and are only comparable within each panel. (A) Relative expression in different plant tissues. SA – shoot apex, L – leaves, EI – elongating internode, MI – mature internode, RT – root tip. Values are normalised to actin expression and represent mean ± se, $n = 3$. (B) Relative expression in whole shoots of plants grown under different light regimes. Plants were grown for 12 days from sowing in continuous dark (D), white light (100 μmol m^{-2} s^{-1}, WL) or blue light (20 μmol m^{-2} s^{-1}, BL). Values are normalised for 18sRNA expression and represent mean ± se for $n = 3$. (C) Time-course for expression in whole shoots following transfer of seedlings from darkness to light. Plants were grown for 7 days in darkness and transferred to blue light (20 μmol m^{-2} s^{-1}). 24D – plants maintained for an additional day in darkness before harvest. Values are normalised for 18S rRNA expression and represent mean ± se for $n = 3$. (D) Relative expression over a diurnal cycle. Plants were grown under a 12 h W/12 h D cycle, and shoot apices harvested every 3 h beginning at lights-on on day 22. Values are normalised for ubiquitin expression. Data shown are for one cycle only, but for *CRY2b* a similar pattern was observed over a second daily cycle.

observed after transfer to blue light. This reduction could be detected as early as 1 h after transfer, and by 24 h *CRY2b* transcript levels had fallen to less than 2% of the level in dark-grown seedlings. In general, *CRY1* and *CRY2a* transcript levels were relatively stable following transfer into blue light, showing at most a small reduction over the 24 h after transfer.

Finally, we monitored diurnal changes in expression of the *CRY* genes in the apices of three-week-old plants grown under a daily light cycle of 12h light/12h darkness (Figure 7B). Both *CRY1* and *CRY2a* transcript levels were relatively stable over a 24h period. In contrast, *CRY2b* expression showed strong diurnal regulation with an amplitude of more than 400-fold, rising to a maximum level at the end of the day and falling during the night to a minimum level around dawn. Diurnal changes in gene expression in many cases reflect an underlying circadian rhythm, and to check if this was the case for *CRY2b*, we again grew seedlings under 12 h light/12 h dark cycles, and monitored *CRY2b* expression over a period of 48 h following the transfer of these seedlings to continuous light. Figure 8B shows that rhythmic expression of *CRY2b* expression is maintained under continuous light, confirming a circadian component to *CRY2b* regulation. However, the

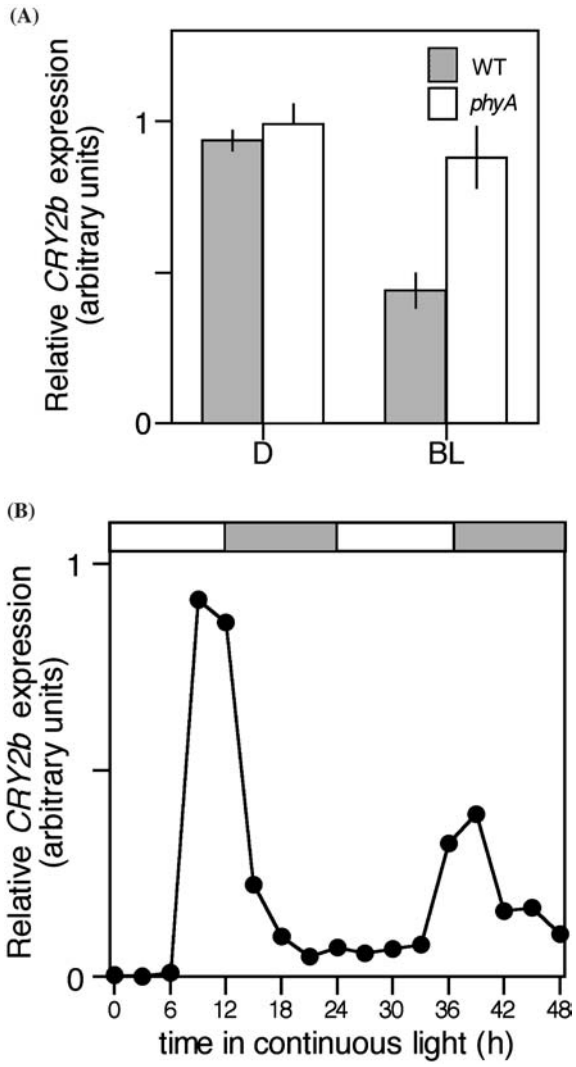

Figure 8. Further characterization of *CRY2b* expression. Expression levels of *CRY2b* were determined by RT-qPCR and normalized to expression of an appropriate reference gene. Values represent arbitrary units and are only comparable within each panel. (A) Relative *CRY2b* expression in shoot tips (apical bud and uppermost expanded leaf) of plants grown under different light regimes. WT and *phyA* seedlings were grown for 12 days from sowing in continuous dark (D) or blue light (20 μmol m^{-2} s^{-1}, BL). Values are normalised for 18sRNA expression and represent mean ± se for $n = 3$. (B) Relative *CRY2b* expression following transfer of seedlings from light/dark cycles to continuous light. Plants were grown under a 12 h W/12 h D cycle, and transferred to continuous white light (150 μmol m^{-2} s^{-1}) at 21 days of age. Shoot tips (apical bud and uppermost expanded leaf) were harvested every 3 h for 48 h. Shaded bars at top indicate subjective night. Values are normalised for ubiquitin expression.

amplitude in the second cycle was much lower than in the first, suggesting that the rhythm is subject to rapid damping under the continuous light conditions used.

Discussion

Most of what we currently know about the function of the cryptochrome photoreceptor family in higher plants has come from studies in Arabidopsis, which has two cryptochrome genes (Lin and Shalitin, 2003). Orthologues of Arabidopsis *CRY1* and *CRY2* have been reported in a range of different species and it appears that most higher plants probably contain both *CRY1* and *CRY2* (Perrotta, *et al*., 2001). However, recent data is beginning to reveal variation in the number of *CRY* genes present and in how their expression is regulated. Two *CRY1* genes are present in both rice and barley (Perrotta *et al*., 2001; Izawa *et al*., 2003), suggesting that a *CRY1* duplication may be relatively common among monocots. Two expressed *CRY1* genes have also been described from tomato (Perrotta *et al*., 2001). However, the isolation of two distinct *CRY2* genes reported here for pea is the first instance of duplication in the *CRY2* lineage. The relatively low amino acid identity of the proteins encoded by the pea *CRY2a* and *CRY2b* genes (72%), and the fact that they both have orthologues in Medicago (Figure 3B) together show that the duplication is not particularly recent and already existed in the last common ancestor of these two species. The apparent presence of only one *CRY2* gene in the more distantly-related legumes Lotus and soybean does suggest that the *CRY2* duplication is specific to the Fabaceae and may be restricted to the hologalegoid group of temperate legumes, which are estimated to have arisen approximately 40 mya (Wojciechowski, 2003; Choi *et al*., 2004). However, denser sampling of legume lineages and more thorough gene isolation efforts will clearly be necessary in order to refine this picture. Studies of this nature may also provide more information about the relative divergence rates of the duplicated *CRY2* genes. Our limited sequence data does hint that *CRY2b* may exhibit a higher allelic diversity than either *CRY1* or *CRY2a*, and it will be interesting to examine this possibility in a broader range of pea accessions.

Relatively little attention has so far been given to the transcriptional regulation of higher plant *CRY* genes. Neither of the Arabidopsis *CRY* genes are strongly photoregulated in experiments comparing dark-grown and light-grown seedlings (Ma *et al*., 2001) or dark-grown and light-transferred seedlings, while other studies have provided equivocal evidence for the light-regulation of *CRY2* (Ma *et al*., 2001; Folta *et al*., 2003). A weak up-regulation of tomato *CRY1* and *CRY2* in response to extended growth under continuous light has also been reported (Perrotta *et al*., 2000). The strong down-regulation of pea *CRY1* and *CRY2b* mRNA expression in response to white and blue light (Figure 7) is therefore unprecedented in higher plants, and suggests that these *CRY*, particularly *CRY2b*, might be subject to autoregulated expression in a manner analogous to *PHYA* (Cantón and Quail, 1999) and may also be regulated by phytochrome. In the absence of pea *cry* mutants, we are not yet able to test the first possibility. However, we have clearly demonstrated that *CRY2b* expression is regulated at least in part by phyA, as *phyA* mutants grown in continuous blue show substantially elevated levels of *CRY2b* transcript compared to WT (Figure 8A). An elevated level of CRY2b in *phyA* mutant seedlings could explain in part the unusual photomorphogenic response previously described for *phyA* mutant pea seedlings, in which high-irradiance B is actually more effective for inhibition of stem elongation in *phyA* mutants than in WT (Weller *et al*., 2001a).

The strong diurnal regulation of *CRY2b* expression is also intriguing. Some degree of diurnal variation in expression might be expected given the rapid down-regulation on transfer from dark to light (Figure 7C). However, a direct effect of light is clearly insufficient to explain the rise in expression anticipating dusk or the subsequent drop in expression during the night (Figure 7D), and it seems more likely that the *CRY2b* diurnal rhythm reflects at least in part a true circadian regulation. Our preliminary examination confirms that this is the case, as *CRY2b* expression continues to cycle under constant light conditions (Figure 8B) with a peak expression at subjective dusk corresponding to that observed under the L/D cycles. It is not yet clear whether the apparent rapid damping under constant light is a feature inherent to *CRY2b* or whether it merely reflects the relatively high irradiance used, and further more detailed examinations of clock-regulated gene expression in pea will clearly be necessary to resolve this matter. In any case, the dusk-phased expression pattern is consistent with a potential role for *CRY2b* in detecting differences in day-length, and it will be interesting to examine how *CRY2b* expression may differ under short and long photoperiod regimes. In Arabidopsis, circadian regulation of *CRY1* and *CRY2* mRNA levels has been reported in the Columbia ecotype, but this regulation appears relatively weak (Harmer *et al*., 2001). A much stronger diurnal rhythm peaking early in the day has been reported for *CRY2* expression in the Landsberg *erecta* ecotype under both long and short photoperiods (El-Assal *et al*., 2003). Diurnal rhythms have also been reported for *CRY1*::*LUC* and *CRY2*::*LUC* reporter genes in the WS background under a 12L/12D cycle (Toth *et al*., 2001). The *CRY2* expression rhythm in these plants is much weaker than observed by El-Assal *et al*. (2003) but with a peak towards the end of the day it more closely approximates the pattern we observe for pea *CRY2b*.

More attention in Arabidopsis has been given to differences in CRY1 and CRY2 regulation at the protein level. Arabidopsis CRY2 protein level shows a strong diurnal rhythm under short photoperiods, accumulating during the night before being rapidly degraded after the beginning of the light period (El-Assal *et al*., 2003; Mockler *et al*., 2003). The CRY2 protein also undergoes rapid degradation after exposure of etiolated seedlings to blue light (Lin *et al*., 1998; Shalitin *et al*., 2003). We do not yet know if either of the pea CRY2 proteins are degraded in response to light, but in view of the complex relationship between transcript and protein level regulation in Arabidopsis, this information is likely to be important for understanding the function of these two pea cryptochromes.

In Arabidopsis, CRY1 is mainly active in regulating de-etiolation responses while CRY2 has a more prominent role in the regulation of photoperiodic flowering. Roles for specific blue-light photoreceptors in both these processes have been identified in pea (Weller *et al*., 2001a), Given the strong diurnal regulation of *CRY2b* expression, we can also speculate that this photoreceptor may also contribute to photoperiod responses. However, it is clear that a definitive

assignment of physiological roles to specific cryptochromes awaits the isolation of pea cryptochrome mutants.

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