LIGHT-REGULATED AND CIRCADIAN EXPRESSION OF TOMATO PHOTORECEPTORS

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INTRODUCTION
Phytochromes, cryptochromes and phototropins: the “eyes” of the plants

The ability of animals to alter their environmental surroundings through locomotion is an option lost to the majority of plants following the dispersal of pollen and seeds. To compensate for this restriction, plants adapt through alteration of their complex chemical repertoire and modifying their development. The most remarkable difference between animal and plant development is that both growth and organogenesis can continue through most of the life of plants. New organs may be developed with a more appropriate structure if current appendages are unsuitable in an altered environment. For example, to facilitate improved light catchment in a newly shaded habitat, small and dense leaves might be replaced by broader and thinner leaves, or leaves may be dropped in their entirety to favour frost-tolerant buds as the shortening daylength indicates the approach of winter.

This developmental flexibility requires the integration of multiple external signals, allowing harmonization between the growth of the plant and environmental change, thereby enabling them to compete effectively with neighbours for resources.

In addition to providing the energy for photosynthesis, light imparts crucial information regarding the surrounding environment and influences many basic physiological processes, including seed germination, seedling de-etiolation (the transition from skotomorphogenesis to photomorphogenesis), vegetative growth, organ orientation and the transition to reproductive development.

Plants have acquired the tools to precisely monitor the changing intensity and spectrum of light, its direction and, in specific cases, its plane of polarization (Kendrick and Kronenberg, 1994), through the presence of a number of photoreceptors: the red (R)/far-red(FR) – absorbing phytochromes and the blue/UV-A – absorbing cryptochromes and phototropins (Cashmore et al. 1999; Casal 2000; Christie and Briggs 2001; Nagy and Schaefer 2002; Quail 2002a, b).
In their action, the role of the photoreceptors in light signalling is somewhat analogous to vision: they provide important information that controls plant behaviour, such as the proximity of neighbouring plants or the optimal direction of elongation, in addition to the availability of solar energy.

De-etiolation is the better understood of the light-regulated developmental transitions because it can occur reproducibly and rapidly in the lab (Kendrick and Kronenberg, 1994). This is the transformation from an “etiolated” seedling, germinating without leaves and elongating rapidly through the soil towards the surface with its apex trailing upside-down for protection, into a young plant in the light, with leaves expanding from the righted apex and chloroplasts developing as quickly as possible. A significant proportion of the transcriptome is controlled by the photoreceptors at this stage, (Quail, 2002), perhaps more than at any other time. A quantitative trace in the amount of perceived light is presented by the elongation of the hypocotyl (the seedling stem) over a period of several days since extra light reduces the amount of elongation. A “blind” plant will stand out spindly and tall above those neighbours with normal light perception, making this method of detecting photoreceptor-deficient mutants amongst the easiest of genetic screens. It also gives the fastest indication as to whether a mutant plant isolated by another phenotype, such as aberrant circadian regulation, may be deficient in light signalling. The most common molecular assay tests the activation of highly expressed, light-regulated genes such as chlorophyll a/b binding protein gene (CAB) following a brief light pulse. These, and several related approaches, have determined mutants in the genes encoding the photoreceptor proteins of the 3 families mentioned above.
Figure 1. The plant photoreceptors.
Three classes of photoreceptors have been characterized from plants at the molecular level. (A) Phytochromes perceive red and far-red light of between 600 and 750 nm. The phytochrome apoprotein contains two histidine kinase related domains (HKRD1 and HKRD2) at the carboxyl terminus and two Per-Amt-Sim domains (PAS) within the HKRD1 domain that have been shown to function as protein–protein interaction domains and small ligand response modules. (B) Cryptochromes perceive blue and UVA light (320-500 nm); at the amino terminus is a photolyase related domain (PHR), and at the carboxyl terminus is DQXVP-acidic-STAEES (DAS) motif. (C) Phototropins also perceive blue and UVA light (320-500 nm). The phototropin apoprotein contains 2 chromophore binding domains (LOV1 and LOV2) as well as a Kinase domain at the carboxyl terminus. Yellow triangles represent the chromophore attachment sites in each of the photoreceptors.
Figure 2. Light-regulated development in the model plant species *Arabidopsis thaliana*. Light affects the development of *Arabidopsis* throughout its life cycle. Multiple aspects of development are regulated by the photoreceptors phytochromes (PHY), crytochromes (CRY), or phototropins (PHOT) acting alone or in combination with each-other.
PHYTOCHROMES

The discovery of physiological responses, such as the germination of lettuce seeds that is promoted by red (R) light and repressed by subsequent far-red (FR) light, led to the identification of phytochrome genes (Kendrick and Kronenberg, 1994). It has been suggested that phytochromes evolved from bacterial bilinsensory proteins, a hypothesis that is supported by the discovery of phytochrome-like proteins in photosynthetic bacteria, non photosynthetic eubacteria, and fungi (Montgomery and Lagarias, 2002). The phytochrome apoprotein is encoded by a small multigene family: in the model plant Arabidopsis thaliana this family consists of five genes (PHYA, PHYB, PHYC, PHYD and PHYE) (Sharrock and Quail, 1989). Based on their stability in the light, phytochromes have been classified into two types. Type I phytochromes (photo-labile) accumulate in etiolated seedlings and degrade rapidly upon light exposure, whereas type II phytochromes (photo-stable) are relatively stable in the light (Furuya, 1992). In Arabidopsis, PHYA is the only member of type I phytochromes; PHYB-E are type II phytochromes (Quail, 1997; Sharrock and Clack, 2002).

Phytochrome structure

All phytochromes exist as homodimers that are composed of two 125-kDa polypeptides, each carrying a covalently linked open-chain tetrapyrrol chromophore, phytochromobilin, which is synthesized in the chloroplasts from heme (Davis et al., 1999; Kohchi et al., 2001; Nagy and Schafer, 2002; Parks and Quail, 1991; Quail, 1997).

Phytochromes are composed of two functional domains: an N-terminal light-sensing domain and a C-terminal regulatory domain (Fig. 3). The N-terminal portion is necessary and sufficient for photoperception and possesses the bilin lyase activity allowing attachment of the chromophore to the apoprotein (Terry, 1997). The minimal bilin lyase domain (BLD) is actually less than 200 amino acids
long (Wu and Lagarias, 2000). The first 70 amino acids of the protein are dispensable for chromophore binding; they constitute the N-terminal extension (NTE). The NTE is poorly conserved, possibly accounting for some functional differences among PHY. Structure function analysis has revealed that in PHYA, the NTE is composed of two subdomains (Stockhaus et al., 1992; Jordan et al., 1996).

The C-terminal signaling domain is composed of a PAS (Per/Arndt/Sim)-related domain (PRD) and a histidine kinase-related domain (HKRD) (Fig. 3) (Schneider-Poetsch et al., 1991; Yeh and Lagarias, 1998).

![Domain structure of Arabidopsis phytochromes.](image)

**Figure 3.** Domain structure of *Arabidopsis* phytochromes.

NTE: N-terminal extension; BDL: Bilin lyase domain; PRD: Pas related domain; HKRD: Histidine kinase-related domain.

Each phytochrome can exist in two photointerconvertible conformations, denoted Pr (a red light-absorbing form) and Pfr (a far red light-absorbing form) (Fig. 4). Because sunlight is enriched in red light (compared with far red light), phytochrome is predominantly in the Pfr form in the light, and this can convert back to the Pr form during periods of darkness by a process known as dark reversion (Nagy and Schafer, 2002). Photoconversion back to Pr can also be mediated by pulses of far red light.
The dark reversion rates vary for different phytochromes. In *Arabidopsis* PHYB has a fast dark reversion rate, whereas PHYA is very stable in the Pfr form (Eichenberg et al., 2000; Hennig et al, 2001).

**Phytochrome localization**

In the dark, de novo synthesized phytochromes are accumulated within the cytoplasm, in the Pr form. Upon conversion to Pfr (due to the light) the five *Arabidopsis* phytochromes translocate into the nucleus (Kircher et al., 2002; Yamaguchi et al., 1999). The quality of the light is a very discriminating factor for the import into the nucleus. PHYA translocates to the nucleus in FR (Kircher et al., 2002; Nagy and Schafer, 2002), while the others accumulate in the nucleus in R or white light (Kircher et al., 2002). Moreover, the nuclear import of PHYA is much faster than that of PHYB,C,D and E (Kircher et al., 2002; Nagy and Schafer, 2002). In the nucleus they form discrete speckles (Nagy and Schafer, 2002), but the nature and the function of these subnuclear foci or nuclear body is still unclear.

**Phytochrome functions**

Phytochrome physiological responses can be divided into different groups based on the radiation energy of light, that is necessary for the response: low fluence responses (LFRs), very low fluence responses (VLFRs) and high irradiance responses (HIRs). Genetic studies of *Arabidopsis* phytochrome mutants demonstrate that PHYA is responsible for VLFR and FR-HIR responses, while
PHYB is principally involved in LFR and R-HIR responses during photomorformogenesis (Nagy and Schafer, 2002; Quail, 2002a).

The redundant and overlapping mechanisms of phytochrome action make often difficult to understand the roles of individual phytochromes in mediating plant growth. The isolation of mutants deficient in individual phytochromes and the subsequent creation of multiple mutant combinations have, therefore, been essential in the resolve of individual phytochrome functions and the dissection of functional interactions between family members.

The timing of seed germination and the consequent developmental strategy of a plant is strongly influenced by the light surroundings. Induction of Arabidopsis seed germination by R involves both PHYA and PHYB (Shinomura et al., 1994, 1996). Germination responses displaying R/FR reversibility are characteristic of the LFR response and enables buried seeds to detect proximity to the soil surface. The retention of R/FR reversible germination responses in phyAphyB double mutants implicated the participation of another phytochrome in this physiological answer, PHYE (Hennig et al., 2002).

Many seeds that have been imbibed in darkness gain acute sensitivity to light that is typical of the PHYA-mediated VLFR mode of action. It is estimated that these sensitized seeds would be induced to germinate following exposure to only a few milliseconds of daylight (Smith, 1983). Inhibition of germination following prolonged exposure to FR, most likely represents the PHYA-mediated FR-HIR response mode of phytochrome action and may be ecologically relevant as a means of delaying the germination of seeds situated under chlorophyllous vegetation or leaf litter (Casal et al., 1990).

After the induction of germination, light signals act to limit hypocotyl expansion while initiating the extension of cotyledons and the concomitant synthesis of chlorophyll. Despite showing no obvious mutant phenotype following growth under white light or R, mutants deficient in PHYA have revealed a unique role for this photoreceptor in mediating the inhibition of hypocotyl elongation growth
under FR and FR-enriched light environments (Nagatani et al., 1993; Parks and Quail, 1993; Whitelam et al., 1993). By contrast, PHYB-deficiency confers no aberrant phenotype under FR, but leads to a marked loss of seedling sensitivity to R for a wide range of de- etiolation responses (Koornneef et al., 1980; Somers et al., 1991; Reed et al., 1993). Seedlings deficient in both PHYA and PHYB display a greater insensitivity to R than monogenic PHYB seedlings (Reed et al., 1994). Thus, although PHYB plays the major role in inhibition of hypocotyl elongation in red light, PHYA can also contribute to this response. An additional minor role is performed by PHYD (Aukerman et al., 1997) whereas the contribution of PHYE to seedling de- etiolation appears insignificant (Devlin et al., 1998).

The recent identification of mutants at the PHYC locus has revealed a role for this phytochrome in the R-mediated inhibition of hypocotyl elongation (Franklin et al., 2003; Monte et al., 2003). The combined loss of PHYA and PHYC in the Ws ecotype (phyC-1) resulted in a significant increase in hypocotyl length, an effect greater than that observed in phyC-1 plants. Since loss of PHYA alone has no effect on sensitivity to R, the possibility exists that PHYA and PHYC act redundantly to regulate the R-control of hypocotyl growth (Franklin et al., 2003). The role of PHYC in this response was most pronounced at low fluence rates and not observable in the PHYB mutant background, suggesting a possible role for PHYC in modulating PHYB function (Franklin et al., 2003). No role for PHYC was identified in the inhibition of hypocotyl elongation in FR (Franklin et al., 2003; Monte et al., 2003).

The isolation and characterization of mutants deficient in cryptochromes 1 and 2 (CRY1 and CRY2) have defined roles for these photoreceptors throughout seedling development (Lin et al., 1996, 1998). Despite uncertainty over the exact nature of co-action, it is accepted that B-mediated de- etiolation involves the interaction of both phytochrome and cryptochrome signalling (Yanovsky et al., 1995; Ahmad and Cashmore, 1997; Casal and Mazzella, 1998).
A physical interaction between CRY1 and PHYA proteins has been demonstrated (Ahmad et al., 1998; Ahmad, 1999) in addition to a functional interaction between CRY2 and PHYB (Mas et al., 2000). Mutant combinations deficient in PHYC displayed elongated hypocotyls in B, an effect most evident at low fluence rates (Franklin et al., 2003). Under these conditions, it has been shown that the CRY2 function predominates in the regulation of hypocotyl elongation (Lin et al., 1998). The hyposensitivity of phyC mutants to low fluence rate of B may therefore indicate a possible functional interaction between PHYC and CRY2. There is also evidence of functional redundancy between phytochromes and cryptochromes. For example, the inhibition of hypocotyl growth by a R pulse in PHYB seedlings that have been pre-treated with white light, requires the presence of either PHYD or CRY1 (Hennig et al., 1999).

In Arabidopsis and many other plant species, lack of PHYB has a remarkable effect on the structure of the adult light-grown plant. PHYB-deficient plants show an elongated growth habit, retarded leaf development, increased apical dominance, and early flowering (Robson et al., 1993; Halliday et al., 1994; Devlin et al., 1996). This pleiotropic phenotype resembles the shade avoidance syndrome shown by wild-type plants following the perception of low R:FR ratio and suggests a predominant role for PHYB in suppressing this response under natural conditions (Whitelam and Devlin, 1997). The ability to respond to the perceived threat of shading, and therefore to execute structural changes before canopy closure, provides a crucial competitive strategy to plants growing in dense stands (Ballarè et al., 1990).

The maintenance of shade avoidance responses in phyB null mutants indicated the involvement of additional phytochromes (Robson et al., 1993; Halliday et al., 1994). Multiple mutant analyses have since revealed that the perception of low R:FR in Arabidopsis is mediated solely by PHYB, D and E, acting in a functionally redundant manner (Devlin et al., 1996, 1998, 1999;). These represent the most recently evolved members of the phytochrome family and form a distinct
subgroup (Mathews and Sharrock, 1997). It is therefore possible that competition for light may have provided the selective pressure for their evolution (Devlin et al., 1998). Adult *Arabidopsis* plants structure their leaves in a compact rosette phenotype. The elongated internodes observed in *phyAphyBphyE* triple mutant plants was the basis on which the *phyE* mutation was isolated and led to the proposal that maintenance of the rosette phenotype is regulated, redundantly, by PHYA, B and E (Devlin et al., 1998). The elongated appearance of *phyAphyBphyDphyE* quadruple mutants grown under white light, a phenotype not displayed in *phyBphyDphyE* triple mutants has supported such a proposal (Franklin et al., 2003b).

Physiological comparison of these genotypes also revealed a significant role for PHYA in the modulation of rosette leaf expansion and petiole elongation in high R:FR (Franklin et al., 2003b). Analysis of mutants deficient in PHYC revealed this phytochrome to play a similar role to PHYA in regulating rosette leaf elongation in high R:FR (Franklin et al., 2003b; Monte et al., 2003).

The phytochromes are also known to interact more directly with phototropism. For example, R, acting predominantly through PHYA is known to lead to enhancement of subsequent phototropic curvature (Parks et al., 1996; Janoudi et al., 1997). Besides, phytochromes, together with cryptochromes, are the elements of input to plant circadian clock. This aspect of phytochromes and cryptochromes action will be discussed in detail in the next chapter.
Tomato Phytochromes

In tomato five phytochrome genes have been discovered and analyzed so far: *PHYA*, *PHYB1*, *PHYB2*, *PHYE* and *PHYF* (Hauser et al., 1995). Phylogenetic analyses showed orthology between *PHYA*, *PHYE* and *PHYC/F* gene pairs in *Arabidopsis* and tomato; tomato *PHYB1* and *PHYB2* were originated by an independent duplication (Pratt et al., 1995).

Roles for *PHYA* and *PHYB1* in the mediation of de-etiolation responses to R in tomato have been demonstrated previously (van Tuinen et al., 1995a; van Tuinen 1995b). In the control of anthocyanin biosynthesis under R, *PHYA* acts predominantly at low irradiances, and *PHYB1* at higher irradiances (Kerckhoffs et al., 1997). Although the *phyAphyB1* double mutant is blind to low-irradiance R, it de-etiolated normally under white light. The phenotype of *phyAphyB1phyB2* mutants under natural daylight indicated an important role for *PHYB2* in this residual response (Kerckhoffs et al., 1999) and it also clear that *PHYB2* is also active in R-sensing (Weller et al., 2000). However, the strongly synergistic effects of *phyB1* and *phyB2* mutations indicate a high degree of functional redundancy between these phytochromes, as might be expected given their relatively recent divergence (Pratt et al., 1995). In seedling de-etiolation, effects of *PHYB2* were only seen in the absence of *PHYB1*, whereas *PHYB1* still retained substantial function in the absence of *PHYB2*.

At least one other phytochrome (PHYE or PHYF) could be active in controlling de-etiolation in tomato, but is functionally dependent on cryptochrome activity, at least in the absence of *PHYA*, *PHYB1* and *PHYB2* (Weller et al., 2000).
CRYPTOCHROMES

The cryptochromes are receptors for blue and (UV-A) light structurally related to DNA photolyases, but they don’t have photolyase activity. DNA photolyases are a group of UV-A/blue light-induced enzymes that repair UV-B-induced DNA damage by removing pyrimidine dimers from DNA (Sancar, 2003).

There are two types of DNA photolyase, which repair different types of damage: CPD photolyases repair cyclobutane pyrimidine dimers (CPDs), and 6-4 photolyases repair 6-4 pyrimidine pyrimidone photoproducts. Photolyases and cryptochromes make up a specific superfamily.

The first cryptochrome gene to be identified was *Arabidopsis CRY1* (Ahmad and Cashmore, 1993), and cryptochromes were soon found by homology in other plant species, in bacteria and animals (Brudler et al., 2003; Cashmore et al., 1999).

It was initially thought that only higher eukaryotes had cryptochromes and that prokaryotes had photolyases but not cryptochromes, but further searches of the more recently available genome databases revealed the presence of a cryptochrome gene in cyanobacteria (*Synechocystis*) (Hitomi et al., 2000). This new type of cryptochrome was referred to as CRY-DASH, to underscore its relationship with cryptochromes found in *Drosophila*, *Arabidopsis*, *Synechocystis* and *Homo* (although CRY-DASH itself is not found in *Drosophila* or humans) (Brudler et al., 2003). CRY-DASH proteins have been found not only in the photosynthetic cyanobacteria but also in non-photosynthetic bacteria, fungi, plants and animals, including *Arabidopsis*, zebrafish and *Xenopus* (Kleine et al., 2003; Dayasu et al., 2004).

**Cryptochrome structure and localization**

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 (Fig. 5). The PHR region of cryptochromes appears to bind two chromophores, cofactors that absorb light; one chromophore is flavin adenine dinucleotid (FAD) and the other 5,10-methenyltetrahydrofolate (pterin o MTHF) (Lin et al., 1995; Malhotra et al., 1995) (Fig. 5). The carboxy-terminal domains in different plant species are of variable length, but they share short stretches of homology (Lin and Shalitin, 2003). Going from the amino-terminal to the carboxy-terminal end of this extension, one finds a DQXVP motif, a stretch of acidic residues, STAES, and finally GGXVP.

**Plant cryptochrome 1-2**

![Diagram of Plant cryptochrome 1-2](image)

**Figure 5. Domain structure of plant cryptochrome 1-2.**
PHR: N-terminal photolyase related domain; DAS: C-terminal domain.

The carboxy-terminal domain of cryptochromes is generally less conserved than the PHR region (Lin and Shatilin, 2003); CRY-DASH protein has no carboxy-terminal extension and, consequently, no DAS domain (Fig. 6).

**Arabidopsis cryptochrome DASH**

![Diagram of Arabidopsis cryptochrome DASH](image)

**Figure 6. Domain structure of Arabidopsis cryptochrome DASH.**
P: N-terminal signal peptide; PHR: Photolyase related domain
Arabidopsis CRY1 and CRY2 are predominantly nuclear proteins that mediate regulation of gene expression. CRY1 and CRY2 play major roles in plant photomorphogenesis; it appears that CRY1 and CRY2 control developmental changes in plants via modifications of gene expression in response to light. CRY1 and CRY2 together are responsible for blue-light-dependent changes in gene expression of up to 10-20% of the Arabidopsis genome (Ma et al., 2001). Arabidopsis CRY-DASH protein contains a functional dual targeting signal (Fig. 6) mediating transport into chloroplast and mitochondria (Kleine et al., 2003). This is the only cryptochrome family member protein that is localized in the organelles. Arabidopsis CRY2 is 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 (Yang et al., 2000). 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 (Guo et al., 1999). In contrast, the same nuclear extract was highly enriched for CRY2 (Guo et al., 1999).

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 (Cutler et al., 2000; Guo et al., 1999; Kleiner et al., 1999; Wang et al., 2001; Yang et al., 2000). 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 (Cutler et al., 2000; Guo et al., 1999;
Although no apparent bipartite NLS is found in CRY1, the C-terminal extension has proven sufficient for nuclear/cytoplasmic trafficking of CRY1 (Wang et al., 2001; Yang et al., 2000).

**Mechanism of action**

The catalytic mechanism of cryptochromes is not still clear, but a model was proposed based on the well-described light activation of CPD photolyases, where FAD plays the main catalytic role (Park et al., 1995). In a DNA-repair reaction, CPD photolyase binds to the pyrimidine dimer, to form a stable complex with the FAD-access cavity of the enzyme. The other chromophore, which is also called the “antenna” chromophore, absorbs blue-light and transfers the excited energy to the flavin of FAD. Flavin in the excited state donates an electron to the pyrimidine dimer to split the cyclobutane ring. Subsequently, the electron moves back to flavin to regenerate the catalytic active flavin, and the DNA with the two neighbouring pyrimidines restored is released from the photolyase (Sancar, 1994).

Based on the homology with DNA photolyases, one might have expected that they also bind DNA. This has actually been demonstrated for *Arabidopsis* CRY-DASH and its *Synechocystis* homolog (Kleine et al., 2003; Brudler et al., 2003). In *Synechocystis*, CRY-DASH is directly involved in gene regulation (Brudler et al., 2003). A very recent report (Selby and Sancar, 2006) has shown that the CRY-DASH proteins of *Vibrio cholerae, Xenopus laevis, Synechocystis* and *Arabidopsis* have a clear single-stranded DNA photolyase activity. This protein is able to repair cyclobutane pyrimidine dimers in RNA and single but no double-stranded DNA. So the authors affirm that “these enzymes, which are found in bacteria, plants, and animals, and were previously designated CRY-DASH, because of the lack of significant photorepair activity on dsDNA, should be reclassified as ssDNA photolyases. It should be noted, however, that this classification does not necessarily exclude an additional non-repair function for ssDNA photolyases, as
indeed even some conventional photolyases have both repair and transcriptional regulatory functions” (Selby and Sancar, 2006).

Direct DNA binding of CRY1 and CRY2 has not been reported; however, a CRY2 carboxy-terminal extension-GFP fusion is associated to chromatin (Cutler et al., 2000).

Cryptochromes are regulated by phosphorylation. It has been shown that *Arabidopsis* cryptochromes are phosphorylated in response to blue light and that is associated with the function and regulation of the photoreceptors (Shalitin et al., 2002; Shalitin et al., 2003). An additional enzymatic activity has recently been found for CRY1. The recombinant protein, expressed in insect cells, binds ATP; this binding is steichiometric and depends on FAD binding (Bouly et al., 2003). In addition, recombinant CRY1 autophosphorylates in a light-regulated manner, but no other substrate has been found (Bouly et al., 2003; Shalitin et al., 2003). Blue light triggers CRY1 and CRY2 phosphorylation at multiple sites in vivo (Bouly et al., 2003; Shalitin et al., 2003). Some of these sites are within the carboxy-terminal extension of CRY2 (Shalitin et al., 2002). This reaction is blue light specific and fluence rate dependent (Shalitin et al., 2003; Shalitin et al., 2002). Taken together with the in vitro characterization of CRY1, one might propose that this is the result of autophosphorylation.

An earlier report has shown that PHYA can phosphorylate the cryptochromes in vitro (Ahmad et al., 1998). However, the phosphorylation state of both CRY1 and CRY2 does not appear to depend on the phytochromes in vivo (Shalitin et al., 2003; Shalitin et al., 2002).

Given that a *phyAphyBphyCphyDphyE* quintuple mutant is currently not available, the role of the functional phytochromes in cryptochrome phosphorylation cannot be fully excluded. In the case of CRY2, phosphorylation is associated with proteolytic degradation (Shalitin et al., 2002). This degradation is in part mediated by the E3 ubiquitin ligase COP1. In addition, phosphorylation of both CRY1 and CRY2 appears to be closely linked to function.
Cryptochrome signal transduction

Cryptochromes are very important during de-etiolation, the transition of a dark grown seedling living from its seed reserves to a photoautotrophically competent seedling. This developmental transition includes a massive reorganization of the transcriptional program, inhibition of hypocotyl growth, promotion of cotyledon expansion, and synthesis of a number of pigments including chlorophyll and anthocyanins (Liscum et al., 2003).

Cryptochromes are involved in mediating many, if not all, of the blue light–dependent de-etiolation responses (Cashmore et al., 1999; Lin, 2002). For example, action spectra studies demonstrated that *Arabidopsis* CRY1 and CRY2 are the major photoreceptors mediating blue light inhibition of hypocotyl elongation (Ahmad et al., 2002; Young et al., 1992).

The function of cryptochrome in mediating de-etiolation responses has also been reported in tomato (Ninu et al., 1999; Weller et al., 2001).

A photoreceptor may trigger a developmental response by amplifying 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 post-transcription regulatory apparatus to alter gene expression and developmental patterns.

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 (Parks et al., 2001; Spalding., 2000). This hypothesis may explain why *Arabidopsis* CRY1, which is the principal 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* and *cry2* mutants were similarly impaired in the blue light–induced membrane depolarization, suggesting that these two photoreceptors play a role in the regulation of blue light activation of anion channels (Folta and Spalding., 2001). Indeed, these photoreceptors may regulate leaf expansion via light-dependent control of plasma membrane anion channels, because defects in leaf expansion have been observed in cryptochrome mutants (Lin et al., 1996; Lin et al., 1998).

The hypocotyl inhibition response as measured by hypocotyl length for seedlings grown in blue light, is significantly impaired in the *cry1* mutant and slightly affected in the *cry2* mutant (Ahmad and Cashmore, 1993; Lin et al., 1998). Identification of genes encoding the specific anion channels regulated by cryptochromes would help elucidate the cellular mechanisms underlying cryptochrome-dependent growth response.

In addition to calcium’s possible involvement in the phytochrome signal transduction (Bowler et al., 1994; Neuhaus et al., 1993), it may also be used as a second message for cryptochrome signal transduction (Christie and Jenkins, 1996; Guo et al., 2001; Long and Jenkins, 1998).

An *Arabidopsis* cell culture system has been used to study how cryptochrome mediate blue/UV-A light–induced *CHS* expression (Christie and Jenkins, 1996; Long and Jenkins, 1998). 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 Ca2C-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 (Guo et al., 2001). However, a direct demonstration of whether and how cryptochromes act through calcium channels or Ca2C-ATPase
to regulate CHS gene expression or hypocotyl inhibition depends on the identification of the specific genes encoding those proteins and the corresponding mutations.

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 (Ma et al., 2001). 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 (Ma et al., 2001). The rest of the blue light–dependent gene expression change is probably mediated partly or completely by PHYA (Chun et al., 2001; Thum et al., 2001). 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 post-transcriptional processes by interacting with the respective regulatory complexes in the nucleus (Lin, 2000b).

Cryptochromes also work together with phytochromes to control photoperiodic flowering and the circadian clock. This specific role of these photoreceptors will be discussed in the next chapter.

**COP1-CRY1 Interaction**

Light-regulated protein degradation appears to be central to cryptochrome signaling.

Such a mechanism is well described for animal cryptochromes and also occurs for both CRY1 and CRY2 in Arabidopsis (Cashmore, 2003). Both cryptochromes
interact with the E3 ubiquitin ligase COP1 (Wang et al., 2001; Yang et al., 2001). The COP1 protein is required for the light-regulated degradation of several transcription factors involved in light-regulated transcription (Holm et al., 2002; Osterlund et al., 2000; Seo et al., 2003). In the dark, COP1 degrades these transcription factors including the bZIP protein HY5, but upon light perception this degradation is prevented (Holm et al., 2002; Osterlund et al., 2000; Seo et al., 2003). The constitutively de-etiolated phenotype of cop1 mutants is consistent with this model, since in those mutants a number of transcription factors (and presumably other COP1 targets) can accumulate in the absence of a light signal (Seo et al., 2003). Similarly, the light-hyposensitive phenotype of hy5 mutants can also be reconciled with this model (Holm et al., 2002; Osterlund et al., 2000; Seo et al., 2003). COP1 interacts with the cryptochromes both in the light and the dark, indicating that the light-driven electron-transfer reaction that was postulated to induce a conformation change in the cryptochromes does not disrupt this interaction (Wang et al., 2001; Yang et al., 2000; Yang et al., 2001). It was proposed that the light-driven conformational modification of the cryptochromes induces a structural modification of COP1 (Wang et al., 2001; Yang et al., 2001). Light-induced alteration of COP1 structure would release HY5 that was bound to COP1 in the dark. HY5 (and other COP1-regulated transcription factors) can then accumulate and bind to light-regulated promoter elements to initiate de-etiolation (Cashmore, 2003; Lin and Shalitin, 2003; Liscum et al., 2003) (Fig. 7).
Figure 7: Schematic mechanism of light activation proposed by Cashmore (Cashmore, 2003). The light signal modifies CRY1 conformation that leads to a conformational change of COP1. The new COP1 conformation causes the releasing of the transcription factor HY5 that can activate light-induced genes. LRE: Light Responsive Elements.

**CRY2-PHYB interaction**

*Arabidopsis* CRY2 directly interacts with PHYB (Mas et al., 2000). 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* (Mas et al., 2000). 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 co-localized with PHYB in the nuclear speckles (Mas et al., 2000). In light of the recent discovery that PHYB could mediate light regulation of transcription via its interaction with the transcription factor PIF3 (Martinez-Garcia et al., 2000; Ni et al., 1998), 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 (Ahmad et al., 1998). CRY1 may also interact with PHYB, at least indirectly, because CRY1 and PHYB can each interact with COP1 (Yang et al., 2001). The cryptochromes also interact with a number of other proteins, but the functional implications of many of these interactions are still unclear.

**Tomato cryptochromes**

In tomato (*Solanum lycopersicum*), three cryptochrome genes have been discovered so far: two *CRY1* (*CRY1a* and *CRY1b*) and one *CRY2* gene (Perrotta et al., 2000; Perrotta et al., 2001). The role of one of the *CRY1* genes, *CRY1a*, has been elucidated through the use of antisense (Ninu et al., 1999) and mutant (Weller et al., 2001) plants. *CRY1a* controls seedling photomorphogenesis, anthocyanin accumulation, and adult plant development. No effects of *CRY1a* on flowering time or fruit pigmentation have been observed. The overexpression of tomato *CRY2* causes phenotypes similar to but distinct from their *Arabidopsis* counterparts (hypocotyls and internode shortening under both low and high fluence blue light), but also several novel ones, including a high-pigment phenotype, resulting in overproduction of anthocyanins and chlorophyll in leaves and of flavonoids and lycopene in fruits (Giliberto et al., 2005).
CIRCADIAN RHYTHMS

The day and night alternation is an environmental factor which lasts since life has appeared on the Earth. This succession of light and darkness produces in the environment deep changes to which all the creatures must adapt themselves. The organisms which are able to profit by these predictable changes, have acquired an evolutionary advantage. This benefit has promoted the development of timekeeping mechanism (endogenous clocks). Thanks to this "endogenous" time measurement, they have fitted their physiological, biochemical and behavioural functions to the day and night length. The biological clocks that generate and maintain oscillations of many physiological and molecular processes with a period length close to 24 h are also referred to as circadian clocks. ( from Latin, circa, approximately and dies, day ). When placed in constant conditions and, thus, deprived of external time cues, circadian rhythms persist and “free-run” with an endogenous period that is close to but no exactly 24 hr. In the real world, of course, organism are exposed to environmental cues such as light and temperature cycles, and these cues serve to synchronize or “entrain” the endogenous organismal clock with local solar time.

The period of a circadian rhythms remains relatively constant over the range of physiologically relevant temperatures, which is referred to as temperature compensation. This means that the circadian clock maintains its pace over a range of temperatures, but does not imply that temperature changes or cycles cannot serve as potent stimuli that can entrain the clock. These three characteristics: persistence in constant conditions with an approximately 24-hr period, entrainment and temperature compensation, are the diagnostic criteria of a circadian rhythm (Johnson et al., 1998; Sweeney, 1987).
**Arabidopsis circadian clock**

Our current understanding of plant circadian clock derives mostly from genetic studies in *Arabidopsis* and rice (Hayama and Coupland, 2004). The circadian clock system is often divided into three general parts (Dunlap, 1999): an input pathway that entrains the clock, by transmitting light or temperature signals to the core oscillator; the central oscillator (the clock) that is the core of the system, responsible for driving 24-h rhythms; the output pathways that generates overt rhythms controlled by the core oscillator and represent a wide range of biochemical and developmental pathways.

**CIRCADIAN SYSTEM**

![Diagram of the circadian system]

**INPUT STIMULI**

- Light
- Temperature

**CENTRAL OSCILLATOR**

**OUTPUT PATHWAYS**

- Gene expression
- Leaf movement
- Stomatal aperture
- Flowering

Figure 8: Conceptual scheme showing simple linear information flow from input (entrainment) pathways through the central oscillator to output pathways.

Therefore, the information flow should proceed along these three components in a unidirectional way: from the input systems the information arrives to the oscillator centre and then to the pathways which give rise to the physiological answers (Fig. 8).

This scheme is only a simplification of the extraordinary complexity of the relationships and regulations which happen in the various parts of the clock, as shown by recent studies (Valverde et al., 2004).

This introduction will deviate from the linear order of figure 8 in its consideration of the *Arabidopsis* circadian system: output pathways will be considered first, the
core system will be addressed second and, finally the input pathways, in which photoreceptors are involved more directly will be analysed.

**Rhythms in mRNA quantity of the output genes**

In 1985 Kloppstech (Kloppstech, 1985) observed a circadian oscillation in mRNA abundance of a chlorophyll a/b binding protein gene (*LHCB* or *CAB*). This was the first example of a plant *clock-controlled gene* (CCG); afterwards, the list of plant CCGs has grown to considerable length (Feyes and Nagy, 1998; Kreps et al., 2000; McClung, 2000; McClung, 2001; Somers, 1999).

Oligo-based microarrays experiments, performed by Harmer et al, (Harmer et al., 2000), allowed the detection of statistically significant circadian (in continuous light) oscillations in mRNA abundance of 5-6% of the 8200 genes examined. This measurement suggests that there are at least 1275-1530 *Arabidopsis* CCGs, based on a current estimate of $\approx 25500$ *Arabidopsis*.

Among the CCGs genes, are present many genes associated with photosynthetic light harvesting oscillate, as genes encoding LHCA and LHCB proteins as well as photosystem I and II reaction centre proteins (Harmer et al., 2000; Scaffer et al., 2001). These genes showed a peak of mRNA abundance after subjective dawn (Harmer et al., 2000; Scaffer et al., 2001).

A number of *Arabidopsis* genes encoding enzymes involved in carbon metabolism and starch mobilization are clock-controlled (Harmer et al., 2000): mRNA abundance for many genes encoding enzymes of the glycolytic and oxidative pentose phosphate pathways, as well as genes encoding hexose transporters peak in the subjective afternoon; genes encoding starch kinase, $\beta$-amylase, fructose-bisphosphate aldolase and sugar transporters peak at night (Harmer et al., 2000).

Plant responses to biotic and abiotic stress responses are often gated by the circadian clock (Rikin, 1992; Rikin et al., 1993). Microarray experiments identified oscillations in mRNA abundance of several genes involved in responses to stresses (Harmer et al., 2000; Scaffer et al., 2001). Particularly remarkable is the transcript
oscillation of the \textit{DREB1a/CBF3} gene encoding a transcription factor that plays a key role in cold tolerance (Harmer et al., 2000; Thomashow et al., 2001). This could suggest that the cold tolerance process underlies a circadian rhythm.

One of the most interesting observations resulting from microarray analysis was that 23 genes encoding enzymes of phenylpropanoid biosynthesis are synchronously transcribed, oscillating with mRNA peaks about 4 hours before subjective dawn (Harmer et al., 2000).

Harmer et al. found that a number of genes implicated in cell elongation are circadian-regulated and peak coordinately around the presumptive midday. These include the auxin efflux carriers PIN3 and PIN7. Auxin promotes growth in plant stems and hypocotyls, and its relocalization plays an important role in the control of cell elongation (Taiz and Zeiger, 1998). Auxin may activate expansins (enzymes that catalyze extension of cell walls), one of which was under clock control (Harmer et al., 2000). Cell expansion is also dependent on water influx, mediated by aquaporins, into plant vacuolar compartments. They found that an aquaporin gene is under clock control and peaks 8 hours after the presumptive dawn. This aquaporin, δ-tonoplast integral protein (δ-TIP), is localized to the vacuole and in young seedlings is primarily expressed in the hypocotyl and cotyledons (Daniels et al., 1996). δ-TIP may work in concert with the PINs, the expansin, and the cell wall hydrolases to effect cell elongation in young plants (Harmer et al., 2000).

Like many plants, \textit{Arabidopsis} exhibits a circadian rhythm in stomatal aperture (Webb, 1998). Microarray analysis shows circadian transcript oscillations for a number of genes associated with Ca$^{2+}$ signalling, including genes encoding Calmodulin and a Calmodulin-like Ca$^{2+}$-binding protein, as well as a putative Ca$^{2+}$-binding EF-hand protein and a Ca$^{2+}$-transporting ATPase (Harmer et al., 2000). Ca$^{2+}$ is important in guard cell signalling (Leckie et al., 1998; Schroeder et al., 2001) and is likely to be involved in the circadian regulation of stomatal aperture and gas exchange. Ca$^{2+}$ is also implicated in red and blue light signal transduction (Frohnmeyer et al., 1998; Long and Jenkins, 1998; Guo et al., 2001) and may play a
role in entrainment of the circadian oscillator. Thus, Ca^{2+} is likely to play multiple roles in the circadian system, but none of these roles are yet well defined.

**Photoperiodism: the daylength measurement and the output gene CONSTANS**

The timing of flowering in many species is photoperiodic (Lin 2000a; Simpson et al., 1999).

Several models have been proposed to explain the mechanisms by which photoperiod information is integrated into the regulation of flowering (Yanovsky and Kay, 2003). Among them, ‘the external coincidence model’ is currently the most consistent with the genetic evidence in plants (Yanovsky and Kay, 2003; Hayama and Coupland, 2004; Putteril et al., 2004). Erwin Bunning was the first to proposed this theory (Bunning, 1936); he hypothesized that circadian timekeeping was essential for photoperiodic time measurement. In this model, light plays two crucial roles. One is resetting the circadian clock, which is important for generating the daily oscillation of a key regulatory component with peak expression in the late afternoon. The other is regulating the activity of this component. Photoperiodic responses will only be triggered when regulator levels above the threshold coincide with daylight, the external signal (Fig. 9).

![Figure 9: The external coincidence model](image)

*Figure 9: The external coincidence model.* An example of the photoperiodic flowering response in long-day (LD) plants. LD plants flower only when regulator protein levels above the threshold coincide with daylight (From Imaizumi and Kay, 2006).
In LD plants (plants which flower only in Long Day conditions: 12-16 hours of light), the function of the key regulator is to promote flowering. Given that the circadian clock always sets peak expression of the regulator in the late afternoon, coincidence with light occurs more under LD but less under SD. Thus, the regulator is most active under LD, resulting in the acceleration of flowering. In SD plants (plants which flower only in Short Day conditions: 8 hours of light), the clock-regulated factor functions as a suppressor of flowering.

Recent molecular-genetic studies of the flowering-time gene CONSTANS (CO) suggest that the interaction between circadian rhythms and light signalling may occur at the level of CO transcription and CO protein stability (Suarez-Lopez et al., 2001; Valverde et al., 2004). CO was isolated using a mutant that exhibits late flowering specifically under LDs (Putterill et al., 1995). The gene encodes a nuclear protein that contains a CCT motif and two B-box type zinc-finger domains, which were originally identified in several animal proteins and are believed to mediate protein-protein interaction. The transcript levels of this gene show a circadian rhythms under continuous light (Suarez-Lopez et al., 2001). However, CO overexpression does not alter the circadian rhythm in CAB gene expression in continuous light, suggesting that it does not have a general effect on circadian rhythms (Ledger et al., 2001), but it does result in dramatic early flowering (Putterill et al., 1995). This indicates that CO acts as a clock-output gene and mediates between the circadian clock and flowering (Suarez-Lopez et al., 2001). Moreover, CO directly induces the expression of FLOWERING LOCUS T (FT), which was originally isolated using a late-flowering mutant, and whose transcript is induced specifically under LDs (Samach et al., 2000).

Under the normal day-night cycle, CO transcripts show a diurnal rhythm. Under SDs, high levels of CO mRNA only occur during the night, whereas under LDs high CO levels occur at the end of the day and during the night (Suarez-Lopez et al., 2001). This observation suggested that CO mRNA level determines a light-sensitive phase and flowering is promoted specifically under LDs because only under these
conditions are plants exposed to light at times when CO is highly expressed. This is consistent with the external coincidence model, identifying CO as the clock-regulated factor and FT as the flowering gene (Fig. 9).

The central oscillator: the core of the circadian system

Molecular analysis of the circadian-clock in animals and cyanobacteria reveal that the core oscillator is composed of an autoregulatory transcriptional and translational negative-feedback loop (Dunlap, 1999).

In Arabidopsis, CIRCADIAN CLOCK ASSOCIATED1 (CCA1), LATE ELONGATED HYPOCOTYL (LHY), TIMING OF CAB EXPRESSION1 (TOC1), and EARLY FLOWERING4 (ELF4) are the candidate genes that may form the feedback loop (Fig. 10; Schaffer et al., 1998; Wang and Tobin, 1998; Strayer et al., 2000; Alabadi et al., 2001; Doyle et al., 2002). Molecular studies of these genes reveal that TOC1, whose mRNA abundance peaks in the evening, functions as a positive regulator to raise LHY and CCA1 transcript abundance in the morning (Alabadi et al., 2001). This idea is based on the observation that loss of TOC1 function severely reduces the transcript levels of LHY and CCA1. The strong reduction of these transcripts is also observed in elf4 mutants (Doyle et al., 2002).

Furthermore, ELF4 transcript oscillates with a phase similar to that of TOC1, which indicates that ELF4 could act together with TOC1 to induce LHY/CCA1. TOC1 belongs to a novel family of pseudo response regulators, and has a CCT (CO, COL, and TOC1) domain that may be responsible for protein-protein interaction and nuclear localization, whereas ELF4 encodes a small nuclear protein with no similarity to other proteins.

Reciprocally, overexpression of either LHY or CCA1 strongly suppresses the expression of TOC1, and lhy-cca1 double mutants exhibit increased TOC1 mRNA levels (Alabadi et al., 2001; Mizoguchi et al., 2002). LHY and CCA1 encode MYB-related transcription factors, and suppression of TOC1 by these proteins may be mediated directly through the cis-acting evening element (AAAAATCT), which
was identified in the promoter regions of several clock-controlled genes whose transcripts peak in the evening (Harmer et al., 2000; Alabadi et al., 2001). Thus, LHY/CCA1 are proposed to act as negative regulators to generate the \textit{TOC1} rhythm, with a circadian phase opposite to that of \textit{LHY/CCA1}. Therefore, as \textit{LHY/CCA1} rise in the morning, \textit{TOC1} expression falls. This eventually causes a reduction in expression of \textit{LHY} and \textit{CCA1} leading in turn to the reactivation of \textit{TOC1} in the evening, and the second cycle then begins with the activation of \textit{LHY} and \textit{CCA1} (Fig. 10).

**Input genes**

Circadian clocks, without exception, respond to light (Roenneberg and Foster, 1997) and light is the most potent and best-characterized entraining stimulus in plants (Devlin and Kay, 2001). There is considerable experimental evidence demonstrating the roles of phytochromes and cryptochromes in providing light input to the clock (Devlin and Kay, 2001). Genetic experiments with \textit{Arabidopsis} mutants have established roles for PHYA, PHYB, PHYD, PHYE, CRY1 and CRY2 in the establishment of period length (Devlin and Kay, 2000; Millar et al., 1995; Somers et al., 1998). Light-labile PHYA is the predominant photoreceptor for the clock at low intensity of red or blue light, whereas PHYB and CRY1 dominate at high intensities of red and blue light, respectively (Somers et al., 1998). Double mutant studies demonstrate a role for CRY2 in the establishment of period at intermediate intensities of blue light, although that role is redundantly specified by CRY1 (Devlin and Kay, 2000). \textit{cry1cry2} double mutant retain rhythmicity (Devlin and Kay, 2000); moreover, the quadruple \textit{phyAphyBcry1cry2} mutant retains both rhythmicity (leaf movement) and the ability to be entrained to a light-dark cycle, making it clear that others photoreceptors (PHYC-PHYE, or others), can provide light input to the clock (Yanovsky et al., 2000). Roles for PHYD and PHYE in clock input under high intensity red light are supported by period lengthening observed in triple
phyAphyBphyD and phyAphyBphyE mutants versus the phyAphyB double mutant (Devlin and Kay, 2000).

A novel family of putative photoreceptors, ZEITLUPE (ZTL) and FLAVIN-BINDING KELCH REPEAT F-BOX (FKF) has recently been identified by the mutant phenotype of altered circadian rhythms (Jarillo et al., 2001; Nelson et al., 2000; Somers et al., 2000). A third family member, LOV DOMAIN KELCH PROTEIN 2 (LKP2), was recently identified (Jarillo et al., 2001; Kiyosue and Wada, 2000). FKF mRNA abundance oscillates with an evening-specific maximum, but neither ZTL nor LKP2 mRNAs oscillate (Nelson et al., 2000; Schultz et al., 2001; Somers et al., 2000). LKP2 overexpressing plants were shown to be arrhythmic by leaf movement and gene expression in constant conditions, although a rhythm could still be driven by a light-dark cycle (Schultz et al., 2001). ztl mutants show long periods in multiple rhythms and the severity of the period lengthening displays fluence rate dependence (Somers et al., 2000), whereas fkf mutants exhibit altered waveform in CCA1 and LHCB mRNA oscillations (Nelson et al., 2000). Both ztl and fkf mutants are late flowering (Nelson et al., 2000; Somers et al., 2000).

There is considerable interaction among photoreceptors. PHYA and CRY1 directly interact at the molecular level, with CRY1 serving as a phosphorylation substrate for PHYA in vitro (Ahmad et al., 1998). In vivo, CRY1 is phosphorylated in response to red light in a far-red reversible manner (Ahmad et al., 1998). A cry1 null mutant shows lengthened period in low intensity red or white light and there is no additivity seen in the double phyAcry1 mutant (Devlin and Kay, 2000). This suggests that CRY1 acts as a signal transduction component downstream from PHYA in the low intensity light input pathway to the clock (Devlin and Kay, 2001). ZTL has also been shown in the yeast two-hybrid assay to interact physically with the photoreceptors PHYB and CRY1 (Jarillo et al., 2001). However, it is important to recall that the compartmentalization of these photoreceptors and their downstream components is regulated (Nagy et al., 2001), so it is important to confirm the
putative interaction *in vivo*. For example, PHYB and CRY2 have been shown to interact *in vivo* by Fluorescence Resonance Energy Transfer (Màs et al., 2000).

Input pathway components may themselves be encoded by *CCGs*. Microarray experiments indicate that *PHYB*, *CRY1*, *CRY2*, and *PHOT1* mRNAs oscillate (Harmer et al., 2000; Schaffer et al., 2001). *PHYB* transcription, as monitored with *PHYB::LUC* gene fusions, is rhythmic in tobacco and *Arabidopsis*, although bulk PHYB protein abundance does not oscillate (Bognár et al., 1999). *PHYA*, *PHYD*, *PHYE*, *CRY1* and *CRY2* show circadian oscillations both at mRNA abundance and transcriptional levels (Tòth et al., 2001). *PHYC* mRNA oscillates robustly, although transcription of a *PHYC::LUC* fusion is only weakly rhythmic. The clear interpretation of these data is that the clock regulates its own sensitivity to entraining stimuli through regulated expression of photoreceptors.

Genetic studies have implicated two other genes, *EARLY FLOWERING 3 (ELF3)* and *GIGANTEA (GI)*, in light signalling to the clock. *elf3* loss of function alleles yield early flowering, hypocotyl elongation, and conditional arrhythmicity in continuous light (Covington et al., 2001; Hicks et al., 1996; McWatters et al., 2000). *ELF3* is a *CCG* encoding a nuclear protein; both transcript and protein accumulation in the nucleus peak at dusk (Covington et al., 2001; Hicks et al., 2001; Liu et al., 2001). Genetic experiments suggest substantial redundancy in ELF3 and PHYB function (Reed et al., 2000). ELF3 interacts with PHYB and seems to act as a negative modulator of PHYB signalling to the clock, as *ELF3* overexpression both lengthens the circadian period and attenuates the resetting effects of red light pulses whereas loss of ELF3 function renders the plant hypersensitive to light signals (Covington et al., 2001; Liu et al., 2001; McWatters et al., 2000).

The *Arabidopsis GI* gene acts upstream of *CO*. It encodes a nucleoplasmically localized protein and functions in mediating photoperiodic flowering, controlling circadian rhythms and phytochrome signalling (Araki and Komeda, 1993; Fowler et al., 1999; Park et al., 1999; Huq et al., 2000; Suarez-Lopez and al., 2001; Curtis et al., 2002). *GI* transcript levels oscillate with a peak of expression 8-10 hours after
dawn (Fowler et al., 1999). gi mutants are altered in leaf movement and gene expression rhythms of multiple CCGs, including GI itself (Fowler et al., 1999; Park et al., 1999). The period shortening effect of gi-1 on gene expression rhythms is less severe in extended dark than in continuous light and the extension of period length seen in light of decreasing fluence is less pronounced in gi-1 than in wild type, which indicates that GI acts in light input (Park et al., 1999). However gi phenotypes are complicated. In the null gi-2 allele, the period of leaf movement is shortened but the period of gene expression rhythms gradually lengthens (Park et al., 1999).

A recent report has studied the relationship between the roles of GI in controlling circadian rhythms and promoting flowering (Mizoguchi et al., 2005). Plants overexpressing GI (35S:GI) and gi-3 mutant altered circadian rhythms under DD (continuous dark) as well as LL (continuous light), demonstrating that the effects of GI on the circadian system are not only due to its role in light signalling (Mizoguchi et al., 2005). Furthermore, under diurnal day/night cycles, 35S:GI delayed the phase of expression of circadian clock–controlled genes CCR2 and LHY, whereas gi-3 delayed the phase of CCR2 and reduced the amplitude of LHY expression. By contrast, 35S:GI and gi-3 cause early and late flowering, respectively, and their effects on the timing and amplitude of expression of the flowering-time genes CO and FT are much more dramatic than on the expression of other clock-controlled genes. Mizoguchi et al., proposed that GI plays a significant role in controlling at least a subset of circadian rhythms in light and dark with an effect on phase in diurnal cycles but that its effect on flowering is distinct from its function in regulating these circadian rhythms. In the regulation of flowering, GI is proposed to act downstream of the putative clock components LHY/CCA1 to promote the expression of CO and FT and probably other flowering-time genes (Mizoguchi et., 2005).
Figure 10: Model of the flowering circadian system of *Arabidopsis* (from Hayama and Coupland, 2004, modified).
**Aim of PhD project**

In spite of the increasing knowledge concerning the biological function of plant photoreceptors and the responses mediated by the photosensory signalling pathways which deeply impact the plant architecture, very little is known on the their potential role in entertaining the time keeping mechanisms in tomato. We have, thus, investigated the interaction network between phytochrome (*PHYA, PHYB1, PHYB2, PHYE* and *PHYF*) and cryptochrome (*CRY1a, CRY1b, CRY2* and *CRY-DASH*) photoreceptors and the tomato clock machinery, by analysing their relative expression pattern in different light conditions in wt, in a *cry1a* mutant (*cry1a-* and in a transgenic CRY2 overexpressor (*CRY2-OX*). Besides, we have isolated genomic and cDNA sequences of a putative new member of tomato cryptochrome gene family, *CRY-DASH* and we have evidenced that its mRNA is expressed in both seeds and adult organs showing diurnal and circadian fluctuations.
MATERIALS AND METHODS
Standard molecular biology protocols were followed as described in Sambrook et al., (Sambrook et al., 1989).

*Solanum lycopersicum* (cv *Moneymaker*), *cry1a*- and *CRY2-OX* plants (Weller et al., 2001; Giliberto et al., 2005) were grown in a growth chamber for 28 days in long day conditions (LD) (16 h light-25°C/8 h dark-23°C). Light intensity of about 100 µmol m⁻² s⁻¹ was provided by Osram (Munich) 11–860 daylight lamps. For continuous light (LL) experiments, plants grown as described above for 28 days were shifted to continuous light at the dawn of 29th day. The aerial parts of three plants for each genotype (wt, *cry1a*- and *CRY2-OX*) were harvested at the times shown.

Total RNA (1 µg) was retrotranscribed with oligo-dT and Superscript III (Invitrogen), according to the manufacturer’s instructions. First strand cDNA (5 ng) was used as template for quantitative real time RT-PCR (qRT-PCR). qRT-PCR assays were carried out with gene-specific primers, using an ABI PRISM 7900HT (Applied Biosystems) and the Platinum SYBR Green master mix (Invitrogen), according to manufacturer’s instructions. PCR conditions were: 50 at 95°C followed by 45 cycles at 95°C X 15” and at 58°C X 60”. Quantification was performed using standard dilution curves for each studied gene fragment and the data were normalized for the quantity of the β-actin transcript.

*In situ* hybridization was performed on seeds imbibed for 96 h and aerial parts of wt plants grown in LD conditions for 28 days as described above and harvested 12 h after the onset of illumination. Imbibed seeds and tissues (leaves and stems) excised from adult plants were fixed, dehydrated, embedded in paraffin, cut into 8 µm sections and hybridized (55°C) to a digoxigenin-labelled antisense probe as described by Canas et al., (Canas et al., 1994). A gene-specific cDNA fragment of 265 bp was used for the synthesis of the digoxigenin-labelled probe. In parallel, RNA from seeds, leaves, stems and roots was used to monitor *CRY-DASH* transcription by qRT-PCR, following the procedures described above.
RESULTS
**CRY-DASH** gene expression is under the control of the circadian clock machinery in tomato

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Abstract

Recently a new member of the blue-light photoreceptor family, CRY-DASH, was reported in Arabidopsis, though its distinctive biological functions are still unclear. We characterized the CRY-DASH gene of tomato and evidenced that its mRNA is expressed in both seeds and adult organs showing diurnal and circadian fluctuations. Moreover, the CRY-DASH transcription pattern is altered in both in a cry1a mutant and in a transgenic CRY2 overexpressor suggesting that CRY-DASH regulation must be mediated at least partially by an interaction of CRY1a and CRY2 with the timekeeping mechanism.

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Keywords: Tomato; Photoreceptors; Cryptochromes; Gene expression; Diurnal rhythms; Circadian rhythms

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1. Introduction

The ability of plants to respond to light is achieved through a number of photoreceptor families, which include red and far-red light sensing phytochromes (PHY) and blue-light specific phototropins and cryptochromes (CRY) [1].

Cryptochromes are flavoproteins that share structural similarity to DNA photolyases but lack photolyase activity [2]. Although originally identified in Arabidopsis, cryptochromes have now been found in bacteria, plants and animals [3,4]. Most cryptochrome proteins, with the exception of CRY-DASH (or CRY3), are composed of two domains, an amino-terminal photolyase-related (PHR) region and a carboxy-terminal domain (DAS) of varying size [2]. The PHR region appears to bind two chromophores; one chromophore is flavin adenine dinucleotide (FAD) and the other 5,10-methenyltetrahydrofolate (pterin or MTHF) [5,6]. The carboxy-terminal domain of cryptochromes is generally less conserved than the PHR region [2]; CRY-DASH proteins lack the DAS domain [3,7].

In Arabidopsis, three cryptochrome genes (CRY1, CRY2 and CRY-DASH) have been described so far [7–9]. Plant cryptochromes play an important role in several blue light-regulated developmental processes such as de-etiolation, flowering and flavonoid biosynthesis [10–14]. CRY1 and CRY2 are intimately connected with the circadian clock machinery: CRY1 and CRY2 transcript levels are regulated by the clock and the encoded proteins seem to be involved in the input to the clock [15–17].

In tomato (Solanum lycopersicum), three cryptochrome genes have been discovered and analyzed in detail so far: two CRY1-like (CRY1a and CRY1b) and one CRY2 gene [18,19]. The use of transgenic and mutant lines have shed light on the role of tomato cryptochromes in seedling photomorphogenesis, flavonoid and carotenoid accumulation, adult development, fruit pigmentation and flowering [12–14].

The CRY-DASH gene, recently characterized in Arabidopsis [7], shares little sequence homology with the other cryptochromes and carries an N-terminal sequence which mediates its import into chloroplasts and mitochondria. Furthermore, CRY-DASH lacks the C-terminal domain which is present in most plant cryptochromes. Though its precise physiological function remains to be elucidated, CRY-DASH is likely to function as a further blue light photoreceptor in Arabidopsis [7].

In this article, we report the characterization of an ORF of tomato which shares high similarity with Arabidopsis CRY-DASH. The tomato CRY-DASH mRNA is expressed in both seeds and adult organs and undergoes day/night cycles, with peaks of expression at dawn and dusk. Its transcription pattern is altered in a cry1a mutant and in a transgenic CRY2 overexpressor (CRY2-OX). In plants transferred for 24 h of continuous light, the CRY-DASH transcript still maintains its cycling rhythm, suggesting that it is controlled by the circadian clock machinery.

2. Materials and methods

Solanum lycopersicum (cv Moneymaker), cry1a and CRY2-OX plants [13,14] were grown in a growth chamber for 28 days in long day conditions (LD) (16 h light-25 °C/8 h dark-23 °C). Light intensity of about 100 μmol m⁻² s⁻¹ was provided by Osram (Munich) 11–860 daylight lamps. For continuous light (LL) experiments, plants grown as described above for 28 days were shifted to continuous light at the dawn of 29th day. The aerial parts of three plants for each genotype (wild-type (W), cry1a and CRY2-OX) were harvested at the times shown.

Total RNA (1 μg) was retrotranscribed with oligo-dT and SuperScript III (Invitrogen), according to the manufacturer’s instructions. First strand cDNA (5 ng) was used as template for quantitative real time RT-PCR (qRT-PCR). qRT-PCR assays were carried out with...
gene-specific primers, using an ABI PRISM 7900HT (Applied Biosystems) and the Platinum SYBR Green master mix (Invitrogen), according to manufacturer’s instructions. PCR conditions were: 5’ at 95°C followed by 45 cycles at 95°C×15 s and at 58°C×60 s. Quantification was performed using standard dilution curves for each studied gene fragment and the data were normalized for the quantity of the β-actin transcript.

In situ hybridization was performed on seeds imbibed for 96 h and aerial parts of WT plants grown in LD conditions for 28 days as described above and harvested 12 h after the onset of illumination. Imbibed seeds and tissues (leaves and stems) excised from adult plants were fixed, dehydrated, embedded in paraffin, cut into 8 μm sections and hybridized (55°C) to a digoxigenin-labelled antisense probe as described by Canas et al. [20]. A gene-specific cDNA fragment of 265 bp was used for the synthesis of the digoxigenin-labelled probe. In parallel, RNA from seeds, leaves, stems and roots was used to monitor CRY-DASH transcription by qRT-PCR, following the procedures described above.

3. Results and discussion

3.1. Isolation of the tomato CRY-DASH gene

We have isolated complete genomic and cDNA sequences of a putative new member of tomato cryptochrome gene family, CRY-DASH (GenBank Accession No. DQ222242) based on the information available in Arabidopsis [7].

Comparison of the genomic and cDNA sequences revealed the presence of a complex gene structure with 13 exons and 12 introns. Most of the exon/intron borders appear to be conserved among angiosperms (Fig. 1). As already described in Arabidopsis, the tomato CRY-DASH coding sequence contains a putative targeting sequence for import in organelles (http://www.cbs.dtu.dk/services/ChloroP/; http://urgi.ibri.org/predotar/) (Fig. 2). However, these predictions are not definitive and this aspect deserves further investigation.

Most of the amino acids putatively involved in cofactor interaction are conserved throughout the CRY-DASH subfamily (residues 333, 334, 346, 349, 355, 356, 358, 359, 392, 395, 415 and 466 in Fig. 2). All but one (residue 357) of the amino acids which appear to bind FAD in Synechocystis [3] are conserved in all plant CRY-DASH proteins (Fig. 2). Four additional residues (residues 247, 253, 261 and 477 in Fig. 2) which cluster around the FAD binding site in Synechocystis are also conserved in all species (Fig. 2).

Despite the high similarity between bacterial class I CPD photolyases and CRY-DASH, especially in the chromophore-binding domain, it is plausible that the actual FAD binding mechanism is different given the fact that two tryptophan residues (residues 393 and 459 in Fig. 2), involved in FAD binding in the Escherichia coli photolyase, are replaced with V/L and Y/F residues, respectively, in CRY-DASH proteins [21]. In the same way, FAD binding could also diverge in CRY1–CRY2 like proteins; here most of the residues putatively involved with FAD interaction are, indeed, different with respect to CRY-DASH (data not shown).

Three key tryptophans (residues 427, 480 and 503 in Fig. 2), which probably constitute an electron transfer chain from the photolyase surface to the FAD cofactor [22,23], appear to be highly conserved in CRY-DASH proteins, suggesting that, like in CRY1 and CRY2, their mechanism of action may involve intraprotein electron transfer [24].

Both Synechocystis and Arabidopsis CRY-DASH [3,7] show a non-specific DNA binding activity. In Synechocystis it has been suggested that this activity is mediated by five positively charged arginine residues conserved between CRY-DASH and photolyase (residues 347, 400, 463, 465 and 517 in Fig. 2) [3]. All the above mentioned residues are conserved in the corresponding positions of tomato CRY-DASH, suggesting that a possible DNA binding activity could also occur for the tomato protein. Further experiments are needed in order to prove the specific role of these amino acids in tomato CRY-DASH.

3.2. Tissue-specific gene expression

To determine the histological domains of CRY-DASH expression, we performed in situ hybridization with digoxigenin-labelled RNA probes. After 96 h of seed imbibition, CRY-DASH transcripts were detected both in the endosperm (Fig. 3a) and embryo (Fig. 3a–f). These results are consistent

![Fig. 1. Comparison of CRY-DASH gene structure in Solanum lycopersicum (GenBank Accession No. DQ222242), Arabidopsis thaliana (GenBank Accession No. AB062926) and Oryza sativa (GenBank Accession No. AP004744). Coding regions are boxed and introns are shown as black lines. Non-conserved intron–exon borders are indicated by dotted lines.](image-url)
with an early expression of this gene during the resumption of metabolic activity in the germinating seed. Furthermore, in the embryo, transcripts were abundant in the root meristem, along the differentiating vascular strands of the root stele and in the external layers of the root cap (Fig. 3e and f), whereas in the shoot the signal was confined to the tunica layer of Shoot Apical Meristem (SAM) (Fig. 3b). A strong signal was detected on both the abaxial and adaxial epidermis of cotyledons and...
isolated clusters of expressing cells were observed in the mesophyll tissue (Fig. 3c and d).

In adult plants, a diffuse signal was detected in the whole leaf lamina, at the level of both the palisade and spongy layers (Fig. 3g and h). As for cotyledons, highly expressing clusters of cells scattered along the leaf lamina were often observed (Fig. 3h). In the stem, CRY-DASH transcripts were preferentially accumulated in the photosynthetic cortex and at the level of vascular bundle-associated parenchyma cells (Fig. 3i and j). CRY-DASH transcripts were highly abundant in glandular trichomes (Fig. 3j). Finally, no transcripts were detected with the CRY-DASH sense RNA probe (Fig. 3k).
In parallel, using the same plants, we monitored CRY-DASH transcript levels in root, stem, leaf and seed tissues by qRT-PCR. Although CRY-DASH mRNAs were detectable in all samples, transcripts were about fivefold and twofold higher in root and leaf, with respect to seed and stem tissues, respectively (Fig. 3 l).

In principle, the wide range of tissues and organs in which CRY-DASH resulted transcriptionally active is consistent with a multiple biological role of CRY-DASH either as a possible further blue-light photoreceptor and/or as an element involved in the regulation of diurnal and circadian rhythms.

3.3. Day/night and circadian transcription fluctuation of CRY-DASH transcripts

In silico analysis of the tomato CRY-DASH promoter predicts the presence of several light-regulated transcription factor binding sites. Among these, GT-1 and GATA motifs have been shown to be very important in light-regulation of gene expression [25–27] (Fig. 4). The tomato CRY-DASH promoter also contains two CCA1 putative binding sites, both containing the AATCT core motif [27]. One of these (AAAAATCT) is a morning-phased promoter site [28] (Fig. 4). It is remarkable that just upstream of these motifs is present a light-induced-circadian G-box GCCACGTGTC [26]. Typically, all these motifs are part of light/circadian-regulated gene promoters and usually cooperate in defining the transcript oscillation properties [25,26,28] (Fig. 4).

The presence of a morning-phased CCA1 binding site is not in contrast with the biphasic CRY-DASH expression pattern, showing peaks at dawn and at dusk (see below). It should be considered that the actual transcript oscillation pattern is usually the result of the concomitant positive and negative action of different cis-acting elements [29].

In order to characterize possible patterns of light regulation and rhythmic transcript oscillations, we measured, via qRT-PCR, CRY-DASH mRNA levels at 4-h intervals in plants grown in a diurnal cycle of 16 h light/8 h dark (LD) (Fig. 5a). Plants that had been grown in an LD (16:8) cycle were put at dawn in continuous light (ZT0) and left there for 40 h measuring circadian transcript oscillation at 4-h intervals (Fig. 5b). The mRNA levels were measured in the Wt, in a cry1a mutant [13] and in a transgenic CRY2 overexpressor (CRY2-OX) [14]. Sampling time is expressed as Zeitgeber time (ZT) [30], which is the number of hours after dawn (the onset of illumination).

In the Wt, CRY-DASH transcript levels oscillate under LD conditions. They are relatively high at ZT0, decrease from ZT0 to ZT4, then increase again from ZT4 to the afternoon (ZT12) and then progressively decrease from ZT12 to ZT20. Overall, they show two peaks (at ZT0 and 12) and two troughs (at ZT4 and 20) (Fig. 5a). In cry1a and CRY2-OX plants, the peak at ZT0 is abolished, and the one at ZT12 is reduced in amplitude. CRY2-OX plants also show a delayed, lower amplitude dawn-phased peak (Fig. 5a).

Fig. 4. Graphical representation of the DNA region upstream the putative start codon of the tomato CRY-DASH gene. A number of possible targets for light-responsive elements have been identified and reported within boxes (see references in the text).
Under continuous light (LL) conditions, some similarities and some differences are observed with respect to those grown in LD conditions:

- The oscillations observed in LD (decrease from ZT16 to ZT20, subsequent increase until ZT24 and decrease until ZT28) are still observed (Fig. 5b), indicating that they are controlled by an endogenous clock.
- The expected peak at ZT36 is anticipated by 4 h (ZT32) in LL conditions (Fig. 5b), indicating that light has a partial resetting effect on the transcription rhythm.
- In cry1a and CRY2-OX plants, the peak at subjective dawn is observed in LL (ZT24), but not LD conditions (ZT0) (Fig. 5a and b), indicating that external light is able to restore a circadian signal present in the Wt but absent in these two genotypes.
- Intriguingly, the effect of the cry1a and CRY2-OX genetic backgrounds on CRY-DASH transcription is similar.

These observations allow the following conclusions:

(i) CRY-DASH gene transcription responds, directly or indirectly (e.g. through the clock machinery), to environmental light and to endogenous circadian signals.

(ii) CRY1a and CRY2 mediate part of these responses, albeit in an antagonistic way. In LD conditions, CRY1a stimulates and CRY2 represses gene transcription during the whole cycle, particularly between ZT20 and ZT24. Since no light is present at this time, these responses must be mediated at least partially by an interaction of CRY1a and CRY2 with the timekeeping mechanism.

(iii) Under LL, but not LD conditions, cry1a and CRY2-OX plants show an induction similar to the Wt between ZT20 and ZT24, suggesting that this induction is presumably mediated by a photoreceptor different from CRY1a and CRY2. Under LL, this photoreceptor is substituting for the clock function that normally produces this peak in Wt seedings.

Many authors suggest a specific role for cryptochromes 1 and 2 in light input to the circadian clock; however, the molecular mechanism that transmits light signals to the clock is not yet clear [16]. The dawn–dusk phased expression pattern of tomato CRY-DASH, especially in Wt under LL conditions is very intriguing (Fig. 5b). In fact, this pattern is consistent with a potential role for CRY-DASH in detecting the dawn and dusk transitions and, consequently, in circadian input pathways.

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References


Light-regulated and circadian expression of tomato photoreceptors

Light signals are perceived and transduced to the central oscillator via specialized photoreceptors. In plants, the photoreceptor phytochrome and cryptochrome have been shown to be involved in this process (Somers et al., 1998; Devlin and Kay, 2000; Tóth et al., 2001).

In order to identify possible interactions between photoreceptors and circadian clock machinery, we performed a detailed comparative analysis of the expression patterns of all phytochrome (PHYA, PHYB1, PHYB2, PHYE, PHYF) and cryptochrome (CRY1a, CRY1b, CRY2, CRY-DASH-discussed above) genes in wild-type (wt) tomato plants grown under a daily light cycle of 16h light/8h darkness (LD). The tomato plants were sampled every 4h in order to identify photoreceptors with transcripts regulated in a diurnal cycle. Besides, because diurnal changes in gene expression frequently reflect an underlying circadian rhythm, to determine which of these photoreceptors exhibited a circadian pattern of expression, tomato plants entrained in LD, were transferred to LL, and then genes expression was monitored for 40 h at 4h intervals.

We have also characterized the expression pattern of all tomato photoreceptors in a cry1a mutant (cry1a-) and in a transgenic CRY2 overexpressor (CRY2-OX).

To confirm, in tomato, periodic oscillations of genes known to be regulated by circadian clock (Fowler et al., 1999; Millar et al., 1999), we also monitored the transcription patterns of the putative nuclear protein GIGANTEA (GI) and of the light-harvesting chlorophyll a/b binding protein (CAB or LHC) in both LD and LL conditions.

GI is a large nuclear protein encoded by a conserved single gene found in diverse monocotyledonous (Hayama et al., 2003; Dunford et al., 2005; Zhao et al., 2005) and dicotyledonous (Curtis et al., 2002; Boxall et al., 2005; Hecht et al., 2005) plant species. GI protein is involved in circadian-clock function, red-light signaling and photoperiodic flowering (Paltiel J et al., 2006). As demonstrated in several
species (Paltiel J et al., 2006), accumulation of GI transcript displays a strong diurnal pattern, and is under circadian-clock control. 

*CAB* remains the best-studied clock-regulated plant gene, and circadian oscillation of *CAB* mRNA abundance is widespread, if not universal, among angiosperm (Fejes and Nagy, 1998; Piechulla, 1999). Curiously, this does not extend into the gymnosperms (Piechulla, 1999).

We also measured the changes in mRNA accumulation of *GI* and *CAB* genes in a *cry1a*- and in a transgenic *CRY2-OX* to determine whether (or how) the signaling pathways mediated by cryptochromes may affect the daily expression pattern of these genes and their circadian behavior in tomato.

**Day/night mRNA oscillations of tomato photoreceptor genes**

We examined the temporal transcription pattern of wt tomato phytochromes (*PHYA, PHYB1, PHYB2, PHYE, PHYF*) and cryptochromes (*CRY1a, CRY1b, CRY2*), to study if these photoreceptors undergo day/night cycles. We measured changes of the eight photoreceptor mRNA accumulations under LD by quantitative real time RT-PCR (qRT-PCR) analyses and we monitored the mRNA levels for 24 h at intervals of 4 h at the times shown (ZT-zeitgeber time is the time in hours from the start of a normal 16 h light–8 h dark cycle; Zerr et al., 1990) (Fig. 11A-D). Although with differences in amplitude, all tomato photoreceptor transcripts but *CRY1b* (Fig. 11A), exhibited maximum transcription peak in the presumptive afternoon (ZT 12), and declined dramatically in darkness (Fig. 11A-D). *CRY1a, CRY2, PHYA, PHYB2* and *PHYE* genes were expressed throughout a day, with fluctuations that suggest diurnal rhythms of mRNA levels, with peaks observed in light phase from ZT8 to ZT12 (Fig. 11B-C). In darkness, transcripts of these genes declined (from ZT16 to ZT20) and then generally started to increase before the onset of light (from ZT20 to ZT24) (Fig. 11B-C). The predawn “anticipatory” rise of expression, suggests that these genes could be under the control of an endogenous circadian clock.
PHYF gene was expressed with amplitude and fluctuations significantly reduced relative to that observed in other tomato photoreceptors (Fig. 11D), with an expression peak at ZT12. A similar pattern, though with a greater oscillation was also detected for PHYB1 (Fig. 11A).

The expression pattern of CRY1b photoreceptor was quite divergent from the other tomato photoreceptors (Fig. 11A). It didn’t show any significant fluctuation in light/dark cycles, and the abundance of the CRY1b mRNAs was uniformly high throughout the 24 h period. Only at early-morning (ZT4), CRY1b transcripts dramatically decreased (Fig. 11A). These evidences suggest that CRY1b expression is not strongly regulated by light, and it is constitutively expressed in tomato plant entrained in LD conditions.

**Day/night changes in mRNA accumulation of tomato photoreceptor genes**

In spite that comparative analysis of the diurnal expression patterns of the tomato phytochrome and cryptochrome genes underlined day/night oscillations with qualitatively comparable phase, significant changes in mRNA abundance were detected throughout the complete 24 h monitoring period (Fig. 11A-D; Tab. 1). CRY1b was the most abundant tomato photoreceptor with mRNA molecules uniformly high throughout the 24 h monitoring. CRY1b transcripts were about 3-fold higher compared to PHYB1, 6-fold higher compared to CRY2, 10 to 12-fold higher compared to CRY1a, PHYA, PHYB2 and PHYE, and more than 30-fold higher compared to PHYF (Fig. 11A; Tab. 1). Concerning the amplitude of oscillations, photoreceptor transcripts showed modest changes compared to other light-regulated and circadian genes (see CAB4 and GI), spanning from 2 (PHYF) to 9 (PHYB2) fold (Tab. 1).

At dawn (ZT0) and in darkness the quantity of all transcripts were generally low; however, the transcript amount never fell near to zero values (Fig. 11A-D; Tab. 1), as happened with other genes (i.e. GI and CAB4, see below).
Changes in mRNA accumulation of tomato photoreceptor genes in light constant conditions

The most reliable diagnostic feature of circadian rhythms is that they persist under constant conditions. Therefore, to determine whether the rhythmic fluctuation of the tomato photoreceptors observed in LD conditions were maintained in light constant conditions (LL), we measured the expression of the tomato photoreceptor genes in LL, after entraining the clock in LD. Plants were harvested at 4h intervals during a period of 40 h.

The results obtained in LL conditions showed that transcript levels of tomato cryptochromes and phytochromes continued to cycle in light constant conditions, indicating that circadian clock controls the expression of these genes (Fig. 11E-H). The sole exception was \textit{PHYB1} and \textit{PHYE} transcripts which lose any detectable oscillation under LL. However, as compared with light/dark conditions (LD) (Fig. 11A-D), we detected both similarities and differences in the phase and amplitude of the observed peaks (Fig. 11E-H). Most of circadian photoreceptor transcripts maintain an exact 24h periodicity thus they show an expression peak at ZT36 (\textit{CRY1b}, \textit{CRY2}, \textit{PHYB2} and \textit{PHYF}) (Fig. 11E-H); while \textit{CRY1a} and \textit{PHYA} genes anticipate the transcription peak at ZT32, indicating that light may have a partial resetting effect on their transcription rhythm. However the circadian oscillation when not supported by normal light/dark cycles, results in weaker transcription alterations and sometimes in slight differences in their oscillation phases.

Although \textit{CRY1b} and \textit{PHYF} transcripts didn’t show dramatic perturbations in LD, once under constant light conditions they increment their transcript levels and reach a peak at ZT36.
Effects of cryptochrome mutations and overexpression on light-induced expression of tomato photoreceptor genes in day/night cycles

To study the effect of the light signal via cryptochromes CRY1a and CRY2 on the expression profiles of tomato photoreceptors, we compared the mRNA levels changes of these genes in LD, between wt, cry1a- and CRY2-OX tomatoes. The results indicated that loss of CRY1a and overexpression of CRY2 influenced the daily transcription profiles of several tested tomato genes (Fig. 12A-C and 13A-E). However, in both cry1a- and CRY2-OX, most tomato photoreceptor transcripts continued to cycle in LD conditions, in the same phase as observed in wt plants, although with reduced or increased amplitude.

The most striking alterations involve CRY1a, CRY2, PHYA, PHYB2 and PHYF transcripts. Most notably, cry1a- stimulated CRY1a mRNAs transcription. The increase was about 2-fold from ZT4 to ZT20 with the highest levels 4h before (ZT8) with respect to wild type CRY1a transcripts (Fig. 12A).

In CRY2-OX plants, as expected, CRY2 mRNAs were present throughout the daily cycle and at each time point were about 10-15 fold more abundant relative to that observed in wt (Fig. 12C). Most notably, overexpression of CRY2 in transgenic tomato plants didn’t depress cycling of the endogenous CRY2 transcript (Fig. 12C). These data could establish both transcript and protein as components of a positive feedback circuit capable of generating a stable oscillation.

PHYA transcript levels were altered in a similar manner in both cry1a- and CRY2-OX background, consisting in a decrease of mRNA abundance especially at ZT12 (Fig. 13A). Conversely, the effect of tomato genotype was opposite for PHYB2 transcription; indeed, cry1a- increased while CRY2-OX reduced the oscillation amplitude at most the time points analyzed (Fig. 13C).

Finally, PHYF mRNAs were more abundant in cry1a- while the effect was slight in CRY2-OX genotype (Fig. 13E).
Effects of cryptochrome mutations and overexpression on light-induced expression of tomato photoreceptor genes under light constant conditions

In order to determine possible effects of the light signal via cryptochromes on the circadian expression profiles of tomato photoreceptors, we compared the changes in the mRNA levels of these genes, among wt, cry1a- and CRY2-OX plants grown under LL.

Our results underlined that loss of CRY1a photoreceptor, and overexpression of the CRY2 gene influenced the transcription profiles and the circadian regulation of a number of genes, including CRY1a, CRY2, PHYA, PHYB2 and PHYF transcripts. Under LL, the effect of the cry1a and CRY2-OX genetic backgrounds on CRY1a transcription resulted in a slight delay of the expression peak from ZT32 to ZT36 (Fig. 12D).

The cyclical pattern of CRY2 expression was not affected by CRY2 overexpression, although the transcript levels were 10-15 fold more abundant relative to that observed in wt (Fig. 12F). Furthermore, phase change was observed from ZT28 to ZT32 but the transcriptional oscillation, with an exactly 24h periodicity (ZT36), was not altered (Fig. 12F). Also in cry1a-, the waveform of CRY2 circadian expression was observed to be not altered compared to wt.

Circadian oscillations of PHYA that cycled in wt plants were repressed following the transfer of mutant and transgenic plants to LL conditions (Fig. 13F). However, in the mutant the presence of low-amplitude oscillation (ZT36) can not be excluded (Fig. 13F).

CRY2 overexpression also changed PHYB2 transcription pattern reducing the oscillation amplitude at all the time points analyzed (Fig. 13H). Circadian oscillations of PHYB2, that cycled in the wt plants, were also fully suppressed in the transgenic plants but not in cry1a- mutant (Fig. 13H). This data indicate that CRY2-OX affect the circadian regulation of PHYB2.
Finally, as already observed for LD experiments, *PHYF* transcript oscillation pattern is unclear; however in *cry1a*- background its transcripts were more abundant and oscillated with robust amplitudes (Fig. 13J).

**Diurnal oscillation of tomato *GI* and *CAB4* mRNA in day/night cycles**

To investigate the actual fluctuation of *GI* and *CAB4* transcript levels through diurnal cycle, wt plants grown under LD were analyzed. Our results confirmed that both transcripts fluctuated within a cycle, showing a direct light regulation. On the whole, *GI* transcripts cycled, with the highest amount at 12h after the onset of light (ZT12) and the lowest level at presumptive dawn (ZT0) (Fig. 14A; Tab. 1). Interestingly, the amplitude of oscillations was as high as 767-fold (Tab. 1) (Fig. 14A). Peak levels of *CAB4* transcription occurred 4 h earlier (ZT8) than *GI* (Fig. 14B) and reached trough levels 12 h later (ZT20) as *GI*, after transition to darkness (Fig. 14B). Also the amplitude of *CAB4* transcript oscillations was high, 120-fold at ZT8 over ZT20 (Fig. 14B). Interestingly, *CAB4* transcripts increased slightly in darkness from ZT20 to ZT24, showing, here again, the anticipation of “light-on” that is typical of circadian-regulated genes (Fig. 14B) (Tóth et al., 2001).

**Circadian accumulation of tomato *GI* and *CAB4* mRNA under light constant conditions**

To confirm circadian expression of *GI* and *CAB4* genes in tomato, wt plants entrained in LD were shifted to LL. In LL, *GI* mRNA levels continued to cycle in a similar phase with an exactly 24h periodicity (ZT36) (Fig. 14C), showing that this gene transcription was controlled by the endogenous circadian clock. However, in LL, peak level at ZT36 decreased, while trough levels increased compared with LD (Fig. 14A and 14C). This caused an overall decline in the amplitude of *GI* transcription levels. In addition, in subjective dark, an increase of the *GI* transcription levels was observed from ZT16 to ZT20 (Fig. 14C). As expected, also *CAB4* transcript levels were controlled by the circadian clock and its
transcription peaked during the light constant condition as in LD (Fig 14D). However light constant treatment affected the amplitude of the transcriptional oscillations in \textit{CAB4}, reducing the abundance of its transcripts (Fig. 14D). In addition, the phase of the \textit{CAB4} oscillation was also anticipated to ZT28.

**Effects of cryptochrome mutations and overexpression on light-induced expression of tomato \textit{GI} and \textit{CAB4} genes in day/night cycles**

In order to check whether tomato cryptochromes could mediate the \textit{GI} and \textit{CAB4} transcription, we examined the transcription pattern of these genes in \textit{cry1a-} and \textit{CRY2-OX}.

In \textit{cry1a-} background, \textit{GI} transcript cycled with the same phase than wt (with maximal expression at “light-on” and minimal at “light-off”) (Fig. 15A); however, loss of CRY1a (ZT12 and ZT16) caused a 2 fold reduction in peak \textit{GI} transcript levels at ZT12 and ZT16 (Fig. 15A). Furthermore, a dramatic effect was also observed for \textit{CAB4} transcription where the CRY1a loss determined not only a reduced amplitude of the transcriptional oscillations when lights were on, but also a phase alteration which anticipate the transcription peak at ZT4 (Fig. 15B). Conversely, \textit{CRY2-OX} background did not affect significantly \textit{GI} gene expression as well as \textit{CAB4} transcription pattern (Fig. 15A-B).

**Effects of cryptochrome mutations and overexpression on circadian expression of tomato \textit{GI} and \textit{CAB4} genes under light constant conditions**

The effect of CRY1a loss and \textit{CRY2} overexpression on circadian expression of tomato \textit{GI} and \textit{CAB4} genes were considered also under LL (Fig. 15C-D). Our results showed that the CRY1a and CRY2 are involved in their circadian regulation (Fig. 15C-D). Indeed, in \textit{cry1a-} plants, \textit{GI} transcript levels continued to cycle in the same phase as observed in wt plants (Fig. 15C), while in \textit{CRY2-OX} circadian expression of \textit{GI} was disrupted (Fig. 15C).
Loss of CRY1α produced a negative regulation of \textit{CAB4} gene expression in LL and a very weak alteration of rhythmicity, if any, from ZT24 to ZT40 (Fig. 15D). In transgenic tomato, \textit{CAB4} transcription changes were less evident (Fig. 15D).
Figure 11. Abundance of the mRNAs of cryptochrome and phytochrome genes analyzed by qRT-PCR in wild type (wt) tomato plants grown in LD (A-D) and LL (E-H) conditions. Results are presented as a proportion of the highest value after normalization with respect to β-actin expression levels. Yellow, dark and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitberg time-ZT). Each experiment was done at least twice with similar results.
Figure 12. Effect of cry1a- mutation and CRY2 overexpression on tomato cryptochrome genes under LD (A-C) and LL (D-F) conditions. The abundance of the mRNAs of cryptochrome genes were measured by qRT-PCR. Results are presented as a proportion of the highest value after normalization with respect to β-actin expression levels. Yellow, dark and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitberg time-ZT). Each experiment was done at least twice with similar results.
Figure 13. Effect of cry1a- mutation and CRY2 overexpression on tomato phytochrome genes under LD (A-E) and LL (F-J) conditions. The abundance of the mRNAs of cryptochrome genes were measured by qRT-PCR. Results are presented as a proportion of the highest value after normalization with respect to β-actin expression levels. Yellow, dark and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitberg time-ZT). Each experiment was done at least twice with similar results.
Figure 14. Abundance of the mRNAs of *Gi* and *CAB4* genes analyzed by qRT-PCR in wild type (wt) tomato plants grown in LD (A-B) and LL (C-D) conditions. Results are presented as a proportion of the highest value after normalization with respect to β-actin expression levels. Yellow, dark and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitberg time-ZT). Each experiment was done at least twice with similar results.
Figure 15. Effect of cry1a- mutation and CRY2 overexpression on tomato GI and CAB4 genes under LD (A-B) and LL (C-D) conditions. The abundance of the mRNAs of cryptochrome genes were measured by qRT-PCR. Results are presented as a proportion of the highest value after normalization with respect to β-actin expression levels. Yellow, dark and hatched bars along the horizontal axis represent light, dark and subjective night periods, respectively; these are measured in hours from dawn (zeitberg time-ZT). Each experiment was done at least twice with similar results.
Table 1. Day/night oscillations in mRNA relative quantity of *GI, CAB4, cryptochrome and phytochrome* genes. Relative mRNA abundance of peaks and troughs are normalized with respect to β-actin expression levels.

<table>
<thead>
<tr>
<th>GENE</th>
<th>Relative mRNA abundance peak</th>
<th>Relative mRNA abundance trough</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAB4</td>
<td>8.975 (ZT8)</td>
<td>0.075 (ZT20)</td>
<td>120</td>
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<tr>
<td>GI</td>
<td>3.833 (ZT12)</td>
<td>0.005 (ZT0)</td>
<td>767</td>
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<td>0.016 (ZT4)</td>
<td>6</td>
</tr>
<tr>
<td>PHYA</td>
<td>0.052 (ZT12)</td>
<td>0.011 (ZT4)</td>
<td>5</td>
</tr>
<tr>
<td>CRY1a</td>
<td>0.047 (ZT12)</td>
<td>0.009 (ZT4)</td>
<td>5</td>
</tr>
<tr>
<td>PHYB2</td>
<td>0.064 (ZT12)</td>
<td>0.007 (ZT20)</td>
<td>9</td>
</tr>
<tr>
<td>CRY-DASH</td>
<td>0.061 (ZT12)</td>
<td>0.015 (ZT4)</td>
<td>4</td>
</tr>
<tr>
<td>PHYE</td>
<td>0.059 (ZT12)</td>
<td>0.018 (ZT20)</td>
<td>3</td>
</tr>
<tr>
<td>PHYF</td>
<td>0.019 (ZT12)</td>
<td>0.005 (ZT0)</td>
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</table>
DISCUSSION
Isolation of the tomato \textit{CRY-DASH} gene and tissue-specific expression

We have isolated complete genomic and cDNA sequences of a putative new member of tomato cryptochrome gene family, \textit{CRY-DASH} based on the information available in \textit{Arabidopsis} (Kleine et al., 2003).

To determine the histological domains of \textit{CRY-DASH} expression, we performed \textit{in situ} hybridization in both seeds and adult organs of tomato wt plants. \textit{CRY-DASH} transcripts were detected in the seed endosperm and embryo, indicating a possible role of this gene in the resumption of metabolic activity in the germinating seed. In adult plants, a diffuse signal was detected in the whole leaf lamina, in the stem and in glandular trichomes (Fig. 3 in \textit{CRY-DASH} results section). The wide range of tissues and organs in which \textit{CRY-DASH} resulted transcriptionally active is consistent with a multiple biological role of CRY-DASH either as a possible further blue-light photoreceptor and/or as an element involved in the regulation of diurnal and circadian rhythms.

**Day/night and circadian transcription fluctuation of CRY-DASH transcripts**

In the wt, \textit{CRY-DASH} transcript levels oscillate under LD conditions with two peaks (ZT0 and ZT12) and two troughs (ZT4 and ZT20) (Fig. 5a in \textit{CRY-DASH} result section). In \texttt{cry1a-} and \texttt{CRY2-OX} plants, the peak at ZT0 is abolished, and the one at ZT12 is reduced in amplitude (Fig. 5a in \textit{CRY-DASH} results section). Under LL conditions, the oscillation observed in LD are still observed (Fig. 5b in \textit{CRY-DASH} results section), indicating that they are controlled by an endogenous clock.

These results indicate that \textit{CRY-DASH} gene transcription responds directly or indirectly (e.g through the clock machinery), to environmental light and to endogenous circadian signals. Moreover, CRY1a and CRY2 mediate part of these response, albeit in antagonistic ways. In LD conditions, CRY1a stimulates and CRY2 represses gene transcription during the whole cycle, particularly between ZT20 and ZT24. Besides, the dawn-dusk phased expression pattern of tomato
CRY-DASH, especially in wt is very interesting: this pattern is consistent with a potential role for CRY-DASH in detecting the dawn and dusk transitions and, consequently, in circadian input pathways.
Cryptochrome and Phytochrome expression in wt plants under LD and LL conditions

Previous genetic experiments in *Arabidopsis* have established the important role of phytochromes and cryptochromes in providing light input to the plant circadian clock (Devlin and Kay, 2001). It has been demonstrated that both light and circadian clock control the promoter activity of all *CRY* and *PHY* genes in *Arabidopsis* and this regulation is reflected at the level of mRNA accumulation (Tòth et al., 2001).

Our analysis of tomato phytochrome and cryptochrome gene expression in wt plants, under LD and LL conditions, showed some similarities and some differences with respect to *Arabidopsis* counterpart. As it occurs in *Arabidopsis* (Tòth et al., 2001), tomato *PHY* and *CRY* genes followed a diurnal rhythm and exhibited maximum expression in the light phase (Fig. 11A-D). However, in tomato, photoreceptor genes appeared to be synchronized with a peak of expression at presumptive afternoon (ZT 12), except for *CRY1a*, whose transcripts were uniformly high throughout the LD cycle monitored (Fig. 11A-D). This finding shows a very different situation from *Arabidopsis* where the photoreceptor genes coding for relatively light-stabile proteins (PHYC, PHYD, and PHYE) are intensively transcribed at the beginning or in the first half (PHYB and CRY1) of the light phase, and the expression of the *PHYA* and *CRY2* genes, coding for photolabile receptors, reaches maximum close to the end of the light interval. The massive synthesis of tomato photoreceptors transcripts in late afternoon could be interpreted as a physiological adaptation which prepares the plant to the following day light stimuli.

It is very interesting to note that, in their LD cycle of expression, all the cryptochromes and also the phytochrome A presented a minimum level of transcript quantity early in the morning (ZT4), just after dawn (Fig. 11A-B). This expression trough could act as a “light-on” signal that, transferred by the photoreceptors to the clock machinery, may result in a clock reset signal.
The temporal regulation of \textit{CRY1b} expression was quite different from that of the other \textit{CRY} genes (Fig. 11A and 11E). This gene didn’t show remarkable fluctuation during the day, and the quantity of \textit{CRY1b} mRNA was the most abundant among the photoreceptors analysed (Tab. 1). This data could suggest a different role for this gene in light perception process of tomato plants.

Moreover, our data showed close similarities in the expression pattern of \textit{PHYA}, \textit{CRY1a} and \textit{CRY2} genes both in LD and LL (Fig. 11B and 11F). They presented high levels of expression in the second part of luminous phase (ZT8-16) and very low transcript abundance during the night; this overlap of their transcription trends could be the effect of a possible cooperation in their physiological roles.

Our results concerning photoreceptor expression pattern under LL conditions, demonstrated that all tomato cryptochromes and \textit{PHYA}, \textit{PHYB2} and \textit{PHYF} kept the oscillations observed in LD conditions, following a period close to 24 hours (Fig. 11E-H). This proves that a circadian clock regulates the expression of these photoreceptors, as already reported in \textit{Arabidopsis} (Harmer et al., 2000; Tòth et al., 2001). However, as compared with LD, we detected minor changes in the phase of the peaks in LL: indeed, \textit{CRY1a} and \textit{PHYA} transcripts reached their maximum 4 hours earlier (Fig. 11F). In LL conditions, the transcription patterns of \textit{PHYB1} and \textit{PHYE} lost rhythmicity, showing that these two genes are not regulated by the circadian clock (Fig. 11E and 11G).

Our data concerning the expression trends (both in LD and LL) of \textit{PHYB1} and \textit{PHYB2} differ from previous results published by Hauser et al., (Hauser et al., 1997; Hauser et al., 1998). These authors reported that in tomato both \textit{PHYB1} and \textit{PHYB2} are expressed at similar level in most plant parts, except for the fruit in which the expression of \textit{PHYB2} is substantially elevated relative to \textit{PHYB1} (Hauser et al., 1997). In a second report the authors showed a clear difference in diurnal rhythms of expression of \textit{PHYB1} and \textit{PHYB2}, which are out of phase by approximately 10 hours (Hauser et al., 1998).
Our experiments showed that in leaves, \textit{PHYB1} transcript levels were definitely more abundant than \textit{PHYB2} at all time points up to 15 fold (Tab.1). Moreover in LD experiments the expression peak of the two genes was overlapped at ZT12 and there were no major phase differences; finally, in LL conditions, \textit{PHYB1} lost the oscillation whereas \textit{PHYB2} maintained its rhythmicity (Fig. 11A, 11C, 11E and 11G). These divergent features of \textit{PHYB1} and \textit{PHYB2} could reflect a divergence of function that is not evident in previous reports (Weller et al., 2000).

When observing the photoreceptors expression pattern under LL conditions (Fig. 11E-H), we can detect a very remarkable trend: when the LL cycle started, in the beginning of presumptive night (ZT20), it was generally evident an increase of transcript abundance with respect to the correspondent LD point. This difference may be explained with a sort of direct activation driven by light. The actual transcript levels, increased in response to the light stimulus, appear to be soon after restored to the “normal” light/dark oscillation, possibly caused by a feedback action mediated by the clock machinery. The proposed mechanism of interaction between photoreceptors and circadian clock is totally consistent with the model proposed by Töth et al., in \textit{Arabidopsis} (Töth et al., 2001): the photoreceptors send the “light-on” signal to the clock core and the core regulates their expression, forming a regulatory loop. This regulatory loop ensures maximal efficiency in the perception of the resetting light signals at the right times and neutralization of signals from non-predictable environmental cues, which could cause resetting of the circadian clock (Fig. 16).
CAB4 and Gi expression in wt plants under LD and LL conditions

Diurnal and circadian rhythms at the mRNA level were first reported for the CAB genes of photosynthetic light-harvesting complexes (Kloppstech, 1985). The CAB proteins are among the major proteins associated with the thylakoid membranes, and are encoded by a nuclear, multigene family. In Arabidopsis plants, grown in LD conditions, the abundance of CAB-family mRNA peaks before the middle of the light period and begins to decrease well before “lights-off” (Millar, 1999).

Our expression experiments showed that the putative CAB4 tomato gene peaked in the middle of the light phase (ZT8) and it exhibited a minimum of expression in the middle of the night (Fig. 14B), showing a very high oscillation (120-fold) (Tab. 1). The oscillations persisted under constant light, indicating that CAB4 gene expression is controlled by a circadian clock (as in Arabidopsis; Millar, 1999)(Fig. 14D). However, in constant light the amplitude of the oscillations were reduced and the expression peak was shifted to an earlier time (Fig. 14D).

GIGANTEA is a large nuclear protein which is in Arabidopsis is involved in circadian-clock function, red-light signalling and photoperiodic flowering.
Moreover, accumulation of GI transcript under LD conditions displays a strong diurnal pattern, with a maximum level of expression in the presumptive afternoon and a minimum at dawn (Fowler et al., 1999).

In tomato wt plants, grown in LD conditions, GI transcript levels cycled, with the highest level 12 hours into the light (ZT12) and the lowest level at dawn (ZT0) (Fig. 14A), with a big amplitude of oscillation (about 760-fold) (Tab. 1).

In LL, GI transcripts continued to cycle with a period close to 24 hours, proving that also in tomato a circadian clock regulates the expression of the gene (Fig. 14C). At ZT20 was present a second peak of expression that could be due to a direct effect of the light on the GI expression, as reported for phytochrome and cryptochrome genes (Fig. 14C).

In LL, the amplitude of the oscillations, as occurred for CAB4 and other tested genes, was much lower than in LD experiments. These results showed that the mRNA quantity of both tomato CAB4 and GI was more affected by a continuous high fluence light treatment than observed in Arabidopsis (Fowler et al., 1999; Millar, 1999) (Fig. 14D).

**Cryptochrome and Phytochrome expression in cry1a- plants under LD and LL conditions**

CRY1a is involved in many physiological responses to blue- light (hypocotyls elongation, anthocyanin accumulation etc. etc.) and it can interact with the PHYA, PHYB1 and PHYB2 (Weller et al., 2001).

In order to individuate the possible interactions between this cryptochrome and the other tomato photoreceptors, we have monitored the expression patterns of the tomato phytochrome and cryptochrome genes in LD and LL conditions, in wt and cry1a- genotypes.

Our data showed that the loss of CRY1a photoreceptor modifies the expression trends of many of the photoreceptors in both LD and LL conditions (Fig. 12-13).
In mutant plants, grown in LD condition, the lack of a CRY1a functional protein often resulted in a weak anticipation of the mRNA peak of CRY1a, CRY2, PHYB1 and PHYE (Fig. 12A, 12C, 13B and 13D). Interestingly, the expression of CRY1a gene was up-regulated in cry1a- plants (Fig. 12A); we infer that this is the effect of some auto-regulatory feedback mechanism caused by the absence of a CRY1a functional protein.

Moreover the point of minimum expression of CRY1a and CRY2 genes occured 4 hours earlier than in wt plants, at dawn (ZT0) (Fig. 12A and 12C).

Under LD conditions PHYB2 mRNA quantities were slightly increased, whereas PHYA expression was down-regulated in mutants with respect to wt plants, and the expression of PHYF was strongly up-regulated in cry1a- tomato plants (Fig. 13A, 13C and 13E). These findings suggest that CRY1a may play an important role of transcriptional regulator of some phytochrome and cryptochrome genes. However, the most evident effect of the loss of CRY1a function remain the down-regulation of PHYA. In Arabidopsis there is evidence for a direct interaction between PHYA and CRY1, with PHYA mediating a light-dependent phosphorylation of CRY1 (Ahmad et al., 1998). Besides, Devlin and Kay (Devlin and Kay, 2000) demonstrated a role of Arabidopsis CRY1 as a signal transduction component downstream of PHYA in light input to the clock. Our results showed that, under high fluence white light, there was an epistatic effect of CRY1a on PHYA expression. This is a further prove of the complexity of the interactions between phytochromes and cryptochromes.

Under LL conditions, there were any significant expression changes in mutants plant with respect to LD cry1a- and LL wt patterns except for CRY1a and PHYA transcripts (Fig. 12D and 13F). In fact, in cry1a- plants, the expression peak of CRY1a was 4 hours delayed (ZT 36) (Fig. 12D), and the one of PHYA was strongly reduced in amplitude and delayed by 4 hours as well, with respect to wt plants (Fig. 13F). Therefore, also in LL conditions was evident an effect of the lack of CRY1a on the transcription of PHYA. In wt plants the circadian oscillation of this
gene was quite clear, while in cry1a- plants this oscillation was almost totally abolished. However, the molecular mechanisms by which CRY1a regulates PHYA expression remain unknown.

**GI and CAB4 expression in cry1a- plants under LD and LL conditions.**

Under LD conditions, the mRNAs of GI in cry1a- plants cycled with the same phase than wt, but the amplitude of the oscillation was much lower than in wt plants; in fact cry1a- mutation caused a 2 fold reduction in GI maximum transcript level that occurs at ZT 12 (Fig. 15A).

Under LL conditions, GI transcripts continued to cycle in the same phase observed in wt plant, with no major changes (Fig. 15C).

A very recent report on new roles of GIGANTEA in Arabidopsis and Medicago truncatula (Paltiel et al., 2006) showed that the daily exposure to white/blue light at dawn up-regulates dramatically At/MtGI expression in the morning. Moreover, the authors demonstrated that Arabidopsis plants in which both CRY1 and CRY2 photoreceptors are mutated, displayed a severely reduced GI response to blue light at dawn. According to these features, our results showed that CRY1a protein plays a main role in the activation of tomato GI, under high fluence white light. If we accept the hypothesis of GIGANTEA as the “factor Y” in an interlocked feedback loop through light affecting TOC1 expression (Locke et al., 2005), we may then assume that CRY1a must have a direct influence on the tomato circadian core system.

Also the expression pattern of the photosynthesis-related gene CAB4 was affected by the loss of CRY1a functional protein. In LD conditions, CAB4 expression peak at ZT8 was strongly reduced and the maximum expression of the transcript occurred 4 hours earlier than in wt plants (ZT4) (Fig. 15B). In LL, in cry1a- plants, the rhythmicity of CAB4 expression was altered, showing a second peak of expression in the presumptive night (ZT20) (Fig. 15D). This transcription pattern
could provide a further cue of the influence of this cryptochrome on the tomato circadian clock.

**Cryptochrome and phytochrome expression in CRY2-OX plants under LD and LL conditions.**

A recent report has demonstrated that the overexpression of tomato *CRY2* gene causes a delay in flowering, observed under both short and long day conditions (Giliberto et al., 2005). This result could be due to a specific effect of CRY2 on the input elements (phytochromes and cryptochromes) of the circadian system that regulate tomato flowering time.

In order to determine the possible effects of the overexpression of *CRY2* gene on the diurnal and circadian transcription pattern of tomato photoreceptors, we have monitored transcript level fluctuations of these genes in wt and *CRY2-OX* tomato plants, under LD and LL conditions.

Under LD the mRNA levels of *PHYA* and *PHYB2* were strongly reduced with respect to wt plants (Fig. 13A and 13C). We didn’t observe remarkable differences in the expression patterns of the other analysed photoreceptors.

Under LL, the expression trends of *PHYA* and *PHYB2* continued to be strongly affected by *CRY2* overexpression (Fig. 13F and 13H). In fact both genes lost their circadian oscillations that were evident in wt tomato plants; moreover, the mRNA quantity of these two genes was sensibly decreased. It is not so easy to explain the loss of circadian oscillations of these two genes, because of the overexpression of *CRY2*. We could hypothesize that the presence of constitutive protein CRY2 disrupts the specific pace-maker mechanism of the core that normally causes the oscillations of these two phytochromes in wt plants. The fact that this effect was present only in *PHYA* and *PHYB2* expression is probably the result of a CRY2 direct control on these two genes, as hinted by the down-regulation of their expression in *CRY2-OX* plants under LD conditions (Fig. 13A and 13C).
GI and CAB4 expression in CRY2-OX plants under LD and LL conditions.

Previous molecular experiments in Arabidopsis (Paltiel et al., 2006) showed that CRY2 levels are not a limiting factor in AtGI expression. On the contrary, in tomato, the overexpression of CRY2 seems to cause the loss of circadian oscillations of GI expression, under LL conditions (Fig. 15C). We can speculate that this effect, if true, could be a consequence of the PHYB2 loss of circadian rhythm in CRY2-OX plants. It is reasonable to hypothesize that CRY2 controls the transcription pattern of PHYB2 that, at its turn, regulates the expression of GI. This model could be consistent with the experimental evidences in Arabidopsis in which GI is a positive mediator of PHYB signalling to the clock (Huq et al., 2000; Mizoguchi et al., 2005).

Under LD conditions no changes in tomato CAB4 expression were detected in transgenic CRY2-OX plants (Fig. 15B); in LL the peak of expression at ZT28 was slightly reduced in amplitude with respect to wt plants, showing a small effect of CRY2 on CAB4 circadian oscillation (Fig. 15D).

Figure 17. A model of genetic interactions among tomato CRY1a and CRY2 proteins and PHYA, PHYB2, PHYF, CAB4 and GI genes. The arrows represent a stimulatory effect, and the lines terminated with a bar represent an inhibitory effect.
CONCLUSIONS
In the frame of the Doctorate-Research Program in Plant Biology at the Università della Calabria, we have identified a putative new member of tomato cryptochrome gene family, CRY-DASH. We have characterized the expression pattern of this gene in different plant tissues and organs (both in seeds and adult organs) and under different light condition. Our results showed that this cryptochrome is expressed very early in the plant development and in a wide range of tissues and organs. Moreover its transcription pattern shows diurnal and circadian fluctuations and is altered in both in cry1- and CRY2-OX tomatoes. However, further experiments are needed in order to elucidate the exact physiological functions of CRY-DASH and its role as a plant photoreceptor.

We have performed an extensive analysis of all tomato phytochrome and cryptochrome transcripts in wt, cry1- and CRY2-OX plants under both LD and LL conditions. We have demonstrated that the expression of most of tomato photoreceptors is light-regulated and under the control of an endogenous circadian clock. Notably we report that, under LD and LL, the transcription pattern of several photoreceptors (CRY1a, CRY2, PHYA, PHYB2 and PHYF) is regulated by both CRY1a and CRY2. Our data pointed out that, at least in tomato, the influence of 1-2 type cryptochromes over circadian transcript oscillations is certainly broader since it also involves major perturbations on other non photoreceptor genes like GI and CAB4 transcripts.

In our knowledge, these data provide a first contribute to the characterization of possible interactions between photoreceptors and the time keeping machinery in tomato.
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