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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.

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

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 (*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

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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″ and at 58 °C × 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. [20]. A gene-specific cDNA fragment of 265 bp was used for the synthesis of the digoxygenin-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.infobiogen.fr/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

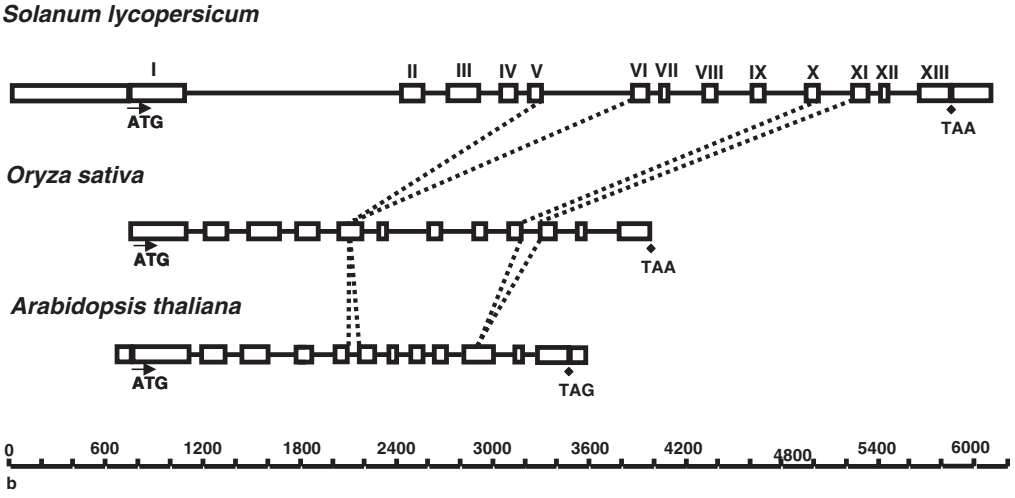

Fig. 1. Comparison of *CRY-DASH* gene structure in *Solanum lycopersicum* (GenBank Accession No. DQ222242), *Arabidopsis thaliana* (GenBank Accession No. AB062926) and *Oryza sativa* (GenBank Accession No. AP004744). Coding regions are boxed and introns are shown as black lines. Non-conserved intron–exon borders are indicated by dotted lines.