Journal of Plant Physiology 213 (2017) 108–121

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Original article

Identification and expression analysis of photoreceptor genes in kiwifruit leaves under natural daylength conditions and their relationship with other genes that regulate photoperiodic flowering

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ARTICLE INFO

Article history:
Received 24 November 2016
Received in revised form 17 January 2017
Accepted 13 March 2017
Available online 18 March 2017

Keywords:
A. chinensis var. *deliciosa* 'Hayward'
Flowering
Photoperiod
Phytochromes
Cryptochrome

ABSTRACT

Kiwifruit (*Actinidia chinensis* var. *deliciosa* (A. Chev) A. Chev.) is a dioecious vine highly dependent on pollination, which is limited by a lack of synchrony of flowering time between male and female plants. In many plant species, the regulation of the timing of flowering depends largely on seasonal cues such as photoperiod, which is detected by photoreceptors. In this report, we determined the full sequences of the *PHYB* (*AcPHYB*) and *PHYA* (*AcPHYA*) genes and a partial sequence of the *CRY2* (*AcCRY2*) gene in kiwifruit. Next, we monitored the expression patterns of these photoreceptor genes (*AcPHYA*, *AcPHYB* and *AcCRY2*) as well as other genes involved in flowering regulation (*AcCO*-like and *AcFT*) in the leaves of kiwifruit plants grown under natural photoperiods in the field. The annual expression patterns of *AcPHYB*, *AcPHYA* and *AcCRY2* genes showed that they were significantly highly expressed from late flower development until full bloom and fitting with floral evocation, closely matching the peaks of expression detected for the *AcFT* and *AcCO*-like genes. In addition, the daily expression patterns of *AcPHYB*, *AcPHYA* and *AcCRY2* were analyzed in leaves collected under different daylength conditions. Under long-day (LD) conditions, maximum expression levels were detected in the middle of the day in April (before full bloom), while their expression lost their daily rhythmic patterns in June (after full bloom) and were consistently expressed at low levels. Under short-day (SD) conditions, *AcPHYB*, *AcPHYA* and *AcCRY2* gene expression patterns were the opposite of those observed in April. With respect to *AcFT*, no expression was detected in SD conditions. In contrast, the *AcCO*-like gene oscillated for all daylength conditions with the same daily rhythm. Our results seem to indicate the involvement of photoreceptor genes in kiwifruit flowering regulation. The different daily expression patterns detected for *AcPHYA*, *AcPHYB*, *AcCRY2* and *AcFT* under different daylength conditions suggest that photoperiod regulates their expression, while the uniform expression of the *AcCO*-like gene is in agreement with its reported regulation by the circadian clock.

1. Introduction

Kiwifruit (*Actinidia chinensis* var. *deliciosa* (A. Chev) A. Chev.) is a dioecious plant, morphologically hermaphrodite but physiologically unisexual (Biasi and Costa, 1984). Thus, it presents female plants with pistillate flowers and abortive pollen and male plants with staminate flowers and rudimentary pistils (Coimbra et al., 2004). The main female cultivar is 'Hayward', both due to the quality of its fruits and because it is the most extensively grown fruiting cultivar in commercial orchards. In kiwifruit, studies on flowering regulation are extremely important due to the high dependence of this species on pollination, especially since fruit size is directly related to the number of seeds formed (Costa et al., 1993; González et al., 1998). Two factors are known to limit pollination and thus the economic value of the crop (González et al., 1998; Howpage et al., 1998): the short effective pollination period (EPP) of the cultivar 'Hayward' (González et al., 1995a,b) and the lack of efficient male pollen donors due to their lack of synchrony in flowering with 'Hayward' (González et al., 1994).

Flowering is one of the most important events in a plant's life cycle (Endo and Nagatani, 2008). The regulation of the timing of flowering is necessary to ensure that sexual reproduction occurs at an appropriate time and to ensure that flowering is coordinated to enable cross-pollination. A comparison of many tropical trees has shown that the annual timing of flowering year after year has a

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http://dx.doi.org/10.1016/j.jplph.2017.03.007
0176-1617/

precision of 10–15 days within the annual cycle (Lüttge and Hertel, 2009).

In many plant species, the timing of flowering depends largely on seasonal cues, such as photoperiod and temperature, so changes in daylength (photoperiod) are a common cue that many plant species utilize to coordinate their flowering time (Golembeski and Imaizumi, 2015). The photoperiod coordinates flowering time through the regulation of expression of the *Flowering Locus T* gene (*FT*), which encodes a systemic signaling molecule that is synthesized in the leaves but moves to the shoot apex to induce flowering, making it a key component of the long-sought florigen (Song et al., 2015). Transcription of *FT* in the leaf phloem companion cells is activated by the CONSTANS protein (CO), the key component of the photoperiodic pathway in the leaves. An intricate network of factors shapes CO expression at the transcriptional and post-translational levels to confine *FT* transcription to long-day conditions (Johansson and Staiger, 2015). The circadian clock and light signaling converge to regulate the expression and activity of the CO transcription factor (Golembeski and Imaizumi, 2015). This model is termed the external coincidence model because it describes the coincidence of a fluctuating internal signal with a periodic external signal. *CO* gene expression is regulated by the circadian clock, which is also regulated by light through phytochromes (PHYs) and cryptochromes (CRYs), which synchronize it to the solar day (Devlin, 2002; Somers et al., 1998). The *CO* gene has a unique daily expression pattern that has a global minimum in the morning and a maximum at night (Suárez-López et al., 2001).

In addition, the stability of the CO protein changes under a variety of external light conditions, with light quality being an important factor through which CO protein activity is regulated. Generally, CO is stabilized under blue and far-red light and destabilized under red light and darkness (Valverde et al., 2004). Both cryptochromes 1 and 2 (CRY1 and CRY2), which are activated by blue light, and phytochrome A (PHYA), which is activated by far-red light, stabilize the CO protein through a mechanism that disrupts the function of the COP1-SPA complex (Shim et al., 2016). PHYA and CRY1/CRY2 activity in the late afternoon increases CO protein abundance. In the morning, phytochrome B (PHYB) is activated by red light and acts antagonistically to PHYA and destabilizes the CO protein (Golembeski and Imaizumi, 2015) through HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 1 (HOS1) E3 ubiquitin ligase, which restricts the accumulation of CO protein by degrading it (Shim et al., 2016).

The overlap between photoreceptor signaling and developmental pathways in plants such as flowering time, fruiting and tuberization, fruit ripening, and nutritional quality suggests that photoreceptor genes may be involved in determining the quantitative traits that can affect productivity, which are therefore of agronomic value. Thus, information about photoreceptor genes and their expression could become the focus of future breeding programs for enhancing the agronomic value of crops (Kharshiing and Sinha, 2015). In this sense, studies on phytochrome expression patterns have been developed in woody plants such as *Citrus* (Dornelas et al., 2007) and *Vitis* (Kühn et al., 2009; Pérez et al., 2009, 2011). In kiwifruit, molecular analysis of male plants displaying different flowering periods allowed us to determine two regions associated with flowering: one corresponded to a partial sequence of the *PHYB* gene (AJ606884), and the other region contained a 7-nucleotide-long element involved in the repression of the *PHYA* gene (Novo et al., 2010). These results indicate the possible involvement of phytochromes, and hence the photoperiodic pathway, in flowering regulation in this species, an issue which was unclear in previous work (Snelgar et al., 2007).

As described above, the contributions of photoreceptors to photoperiodic flowering in *Arabidopsis* have long been known (Horvath 2009; Mockler et al., 2003; Tóth et al., 2001), but this topic is generally much more difficult and time-consuming to address in woody plants due to their long life cycles. Here, we present an initial approach to studying the role that photoreceptors play in kiwifruit development. In the present work, we identified gene sequences in *Actinidia chinensis* var. *deliciosa* 'Hayward' that belong to three photoreceptors (AcPHYA, AcPHYB and AcCRY2) to study their involvement in kiwifruit flowering regulation. We monitored the annual and daily expression profile of the *AcPHYA*, *AcPHYB* and *AcCRY2* genes in leaves of field-grown kiwifruit plants and determined their spatial expression patterns using *in situ* hybridization analysis. In addition, we also analyzed the expression pattern of the kiwifruit *AcFT* and *AcCO*-like genes to gain a better understanding of the relationship among these genes in the framework of the regulation of flowering in this species.

2. Materials and methods

2.1. Plant material

Kiwifruit leaves were collected from mature female plants of kiwifruit (*Actinidia chinensis* var. *deliciosa* (A. Chev.) A. Chev. 'Hayward') growing in a commercial orchard located in northwestern Spain (Galicia) and operated by Kiwi Atlántico S.A. Mature leaves from at least five individual plants were collected in the morning (10:00) and in the late afternoon (20:00) monthly from April to December (on 23rd, with the exception of April 28th and December 17th). In addition, leaves were collected approximately every 2 h for a 24-h period on April 28th (LD, daylength 12 h 45 min), June 23rd (LD, daylength 14 h 15 min), and December 17th (SD, daylength 9 h 00 min). Critical daylength was considered 12 h (corresponding to approximately 50% budbreak in our environmental conditions, as described for grapevine by Kühn et al., 2009). Samples were prepared in six random pools each containing 10 leaf discs from at least five plants; three pools from these were selected for independent RNA extraction (biological replicates). Plant material collected was either stored in RNALater Storage Solution (Sigma, St. Louis, MO, USA) at 4 °C for 24 h and then stored at −20 °C, or fixed in FAE solution (3.7% formaldehyde, 5% glacial acetic acid, 50% absolute ethanol) for further analysis.

2.2. Total RNA isolation, quantification and cDNA synthesis

Ten frozen kiwifruit leaf discs (approximately 60 mg) were ground to a fine powder in liquid nitrogen using a Mikro-Dismembrator-S (Sartorius AG, Goettingen, Germany). RNA was extracted using the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's instructions with some modifications. PVP (1%) was added to the RNA lysis buffer and extracts were incubated with DNase for 60 min and with nuclease-free water for 1–2 min prior to centrifugation.

The RNA concentration and the 260/280 nm ratio of the samples were determined using a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). RNA integrity was determined by 2% agarose gel electrophoresis and stained with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology Inc., Suwon, Gyeonggi, South Korea) and confirmed using an Agilent 2100 bioanalyzer (Agilent, Mississauga, ON, Canada). First-strand cDNA was synthesized from 0.5 μg of total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions.

2.3. Primer design

Different primer pairs were designed for different PCR techniques: conventional PCR, RACE PCR and qPCR (Table 1). Primer design and optimization were performed using the Gene Runner