Journal of Plant Physiology 173 (2015) 72–81

Contents lists available at ScienceDirect

Journal of Plant Physiology

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

Physiology

Characterization of expressed sequence tags from *Lilium longiflorum* in vernalized and non-vernalized bulbs

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ARTICLE INFO

Article history:
Received 22 July 2014
Received in revised form
26 September 2014
Accepted 28 September 2014
Available online 17 October 2014

Keywords:
ESTs
Flowering
Meristem
Lilium longiflorum
Subtraction libraries
Vernalization

ABSTRACT

In *Lilium longiflorum*, vernalization is both an obligatory requirement and the major factor affecting flowering time, however, little is known about the molecular regulation of this mechanism in *Lilium* and other flowering bulbs. Exposure of *L. longiflorum* bulbs to 9 weeks at 4 °C greatly promoted stem elongation within the bulb, floral transition and flowering. Subtraction libraries of vernalized (V) and non-vernalized (NV) bulb meristems were constructed. 671 and 479 genes were sequenced, from which 72 and 82 proteins were inferred for the NV–V and the V–NV libraries, respectively. Much lower transcription levels and putative gene functions were recorded in the NV–V libraries compared the V–NV libraries. However, a large number of genes annotated to transposable elements (TEs), represented more than 20% of the sequenced cDNA were expressed in the NV–V libraries, as opposed to less than 2% in the V–NV libraries. The expression profile of several genes potentially involved in the vernalization pathway was assessed. Expression of *LlSOC1*, the lily homologue of *SUPPRESSOR OF OVER-EXPRESSION OF CO1* (*SOC1*), an important flowering gene in several plant species, found in the V–NV library, was highly up-regulated during bulb meristem cold exposure. The subtraction libraries provided a fast tool for relevant gene isolation.

Introduction

Cold periods strongly affect various aspects of the life cycle of numerous geophytes. In *Lilium longiflorum*, a prominent ornamental crop (Grassotti and Gimelli, 2011), cold exposure is required to fill the vernalization requirements to allow flowering, while in other geophytes, cold exposure is required to break dormancy (Kamenetsky et al., 2012).

L. longiflorum has an obligatory requirement to vernalization, which is also the major factor affecting flowering time and quality (Dole and Wilkins, 1994; Miller, 1993). The plant is receptive to the vernalization signal while in a broad spectrum of physiological stages, including the bulb and shoot elongation phases until flower initiation (Miller, 1993; Roh and Willkins, 1977b).

The duration of lily bulbs cold storage (2–10 °C) quantitatively reduces flowering time, stem length, leaf and flower counts, up to a saturation point (Dole and Wilkins, 1994, 1999; Holcomb and Berghage, 2001; Miller, 1993; Roh and Willkins, 1977a, 1977b, 1977c). In the literature, optimal cold treatments refer to the duration of bulb vernalization that ensures the production of the highest quality plants in the shortest period. Both morphological and biochemical markers have been investigated to determine whether bulb cold treatment was sufficient to induce flowering (Dole, 2003), but suitable markers still need to be established that will accurately indicate the amount of vernalization needed to attain optimal flower quality and flowering time and avoid problems related devernalization of cold-treated bulbs, causing the delay of flowering (Miller, 1993).

Although the physiological aspects of vernalization have been studied, leading to effective manipulation in lily flowering, needed for success in the floriculture industry, the molecular regulation of this major mechanism remains almost unknown. Identification of molecular components of lily vernalization will bring about new breeding means as well as interesting data on the conservation of this mechanism across plant species. Moreover, this could lead to the isolation of vernalization markers for best flower production.

Most of our understanding about the molecular basis of vernalization derives from studies performed in *Arabidopsis* and, more

Abbreviations: EST, expressed sequence tags; qPCR, quantitative real-time polymerase chain reaction; TE, transposable elements; V, vernalized; NV, non-vernalized.

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http://dx.doi.org/10.1016/j.jplph.2014.09.015
0176-1617/

recently, in cereals (Amasino, 2010; Distelfeld et al., 2009; Kim et al., 2009).

In *Arabidopsis*, *FLOWERING LOCUS C* (*FLC*), a MADS-box gene encoding a potent repressor of flowering, is active in meristems in autumn. While FLC represses genes that direct meristems to form flowers, it relies on *FRIGIDA* (*FRI*) to elevate its autumnal expression to a level that prevents flowering. During winter, vernalization causes the acquisition of meristem competence to flower by repressing *FLC* expression. Once it has been repressed by vernalization, *FLC* remains off for the rest of the plant's life cycle after the return of warm conditions, *i.e.*, the repression is epigenetic in the sense that it is mitotically stable in the absence of the inducing signal (cold exposure). The mechanism of epigenetic repression of *FLC* involves histone modifications that convert *FLC* into a heterochromatin-like state. A key player in the vernalization-mediated silencing of *FLC* is *VERNALIZATION INSENSITIVE 3 (VIN3)*. Probably a component of a chromatin-remodeling complex, *VIN3* is required for all *FLC* chromatin modifications associated with vernalization-mediated silencing. The induction of *VIN3* expression is the only known output of the system that measures cold, and only a complete winter season of cold will induce *VIN3* expression (Sung and Amasino, 2004).

In winter cereals requiring vernalization, a system similar to that in *Arabidopsis* exists. Specifically, a flowering repressor prevents flowering prior to cold exposure and the expression of this repressor is turned off by cold. In wheat, the repressor is a type of zinc-finger protein VERNALIZATION 2 (VRN2). One of the genes repressed by VRN2 is *VRN1*, which encodes a MADS-box protein that promotes flowering. In fact, the *VRN1* gene from cereals plays a crucial and dual role in flowering, first by inducing the expression of the cereal *FT* homologue, through the vernalization pathway, and secondly, acting as a floral meristem identity gene (Li and Dubcovsky, 2008; Yan et al., 2006).

Several homologues to vernalization and flowering-related genes, such as *VRN1*, have been detected in expressed sequence tags (EST) libraries from cold-exposed axillary buds of the orchid species *Dendrobium nobile* (Liang et al., 2012). However, homologues of the floral repressors *FLC* and *VRN2* were not detected in this study – either due to limitations of the technique or to the absence of these genes from the orchid genome, hinting that a different mechanism of flowering repression would operate in this species.

Previous studies performed on diverse plant species have therefore demonstrated that, although the general mechanism of vernalization is conserved among distant species, the sequence of the main regulating genes, the "repressors", is not. Different genes, regulating vernalization, are therefore expected to be isolated from geophytes, as many of them, and lily in particular, have strong vernalization requirements (Kamenetsky et al., 2012). *L. longiflorum* was chosen as a model bulb plant to study the molecular events regulating vernalization for the following reasons: (1) the availability of extensive physiological characterization; (2) the obligatory vernalization requirement and the quantitative response to the vernalization period, which indicates that flowering time may be manipulated; (3) the similarities of the *Arabidopsis* and lily flowering processes; (3) the relative rapidity of lily flowering compared to other important flowering bulbs (such as tulip); (4) the possibility of genetically transforming lily plants; (5) the major importance of lily cut flowers and potted plant production worldwide.

Significant progress in the understanding of lily vernalization is expected from the molecular studies planned in this project. Because exposing the bulbs to low temperatures dramatically accelerates flowering, our hypothesis was that the cold induces the differential expression of genes involved in the vernalization pathway, which can be isolated by a system revealing differential gene expression. To this end, we constructed subtraction libraries using cDNAs from the meristems of bulbs either exposed or not exposed to cold.

In this study, we first investigated the physiological response of *L. longiflorum* cv. White Heaven to vernalization and demonstrated the quantitative effect of cold exposure on bolting and flowering. We then produced and sequenced subtraction libraries of meristems cDNA from bulbs exposed to 25 °C or 4 °C for 9 weeks. These libraries revealed a series of differentially expressed genes, including a group of transposable elements (TEs), only expressed in bulbs without cold exposure.

Materials and methods

Plant material, meristem characterization and growth conditions

Lilium longiflorum bulbs (cultivar White Heaven) were purchased from a nursery in August. Bulbs were sanitized and stored in moist medium mixture of peat and vermiculite (1:1, v/v) at 25 ° or 4 ° for 9 weeks. Every week, shoot apical meristems were excised from the bulbs, immediately frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Stem elongation within the bulb during cold treatment was measured every week in 15 bulbs (replicates). After planting, meristems were isolated from the plants, their developmental stage was characterized under a stereo microscope (Stemi 2000C, Zeiss, Germany) and they were immediately stored in liquid nitrogen for further RNA extraction.

For the flowering experiment, lily bulbs were stored for 0, 1, 2, 3, 4, 5, 6, 7, 8 or 9 weeks, at 4 °C and otherwise were kept at 25 °C. All bulbs were planted on 26.10.10 in a greenhouse, under natural day length and monthly average temperatures fluctuating between 19.1 °C (January) and 32.1 °C (May). During growth, plants were sampled at intervals for meristem observation under a Stemi microscope. The floral transition stage was assessed when first flower primordia were formed in the meristem. Flowering was determined when the first flower of the inflorescence in each plant reached anthesis. For each cold treatment, mean values of the time from planting to flower transition and time from planting to flowering were based on 20 plants (replicates).

Subtraction libraries

Total RNA was isolated from 27 meristems from bulbs kept at 25 °C (non-vernalized, NV) and 13 meristems from bulbs kept at 4 °C (vernalized, V) for 9 weeks, using Tri reagent (Sigma–Aldrich), following the manufacturers protocol. Any contaminating DNA was removed by DNase digestion (DNA-free kit, Ambion). The RNA concentration was estimated spectrally (Nano Drop ND-1000; Nano Drop Technologies). mRNA was purified from total RNA using the PolyATtract mRNA isolation system (Promega, Madison, WI) according to the manufacturer's instructions.

The PCR-Select cDNA Subtraction kit (Clontech) was used to isolate genes of differentially expressed genes during cold exposure, following manufacturer's protocol. Basically, 5 μg mRNA from meristems of NV and V bulbs was first converted into cDNA. These two cDNA populations were used both as "tester" (population containing specific transcripts) and "driver" (reference cDNA) for the production of different libraries, namely "NV–V" and "V–NV". This method has been successfully used in lily to isolate differentially expressed genes in pollen (Wang et al., 2004).

Sequences analyses and functional annotation

Gene fragments generated from the libraries were cloned into the pGEM-T vector (Promega) and several hundreds of clones from each library were sequenced and built into contigs at the Genome Center at Washington University St. Louis, MO, USA (outsourcing).