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Investigation of the *Phaseolus vulgaris* circadian clock and the repressive role of the PvTOC1 factor by a newly established *in vitro* system

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ABSTRACT

The circadian clock is crucial for the synchronization of an organism's physiology and metabolism with the geophysical time. In plants, previous work on the common bean (*Phaseolus vulgaris*) has identified various differing aspects of clock function compared to the widely studied *Arabidopsis thaliana* clock. However, transformation of legumes for the study of the circadian clock regulatory mechanisms is extremely laborious. In the present work, we describe an easy-to-follow and rapid method of preparing bean leaf protoplasts with high transformation potential and a functional circadian clock. In this system, we show that application of trichostatin A differentially changes the expression levels of several clock genes. More importantly, we investigate the effect of the clock protein PvTOC1 (Phaseolus vulgaris TIMING OF CAB EXPRESSION 1) on the activity of bean circadian promoters. We present new evidence on the function of PvTOC1 as a repressor of the promoter activity of its own gene, mediated by its conserved CCT (CONSTANS, CO-LIKE and TOC1) domain. Using our protoplast system we were able to uncover functions of the bean circadian clock and to identify an additional target of the PvTOC1clock transcription factor, not previously reported.

1. Introduction

The majority of living organisms possess an endogenous mechanism, the circadian clock, which allows them to synchronize their physiological functions with the daily environmental fluctuations (e.g. photoperiod). It coordinates physiology, development, stress responses and utilization of resources. This way it contributes to the enhanced fitness of plants, conferring an evolutionary advantage (Sanchez and Kay, 2016; Yerushalmi and Green, 2009).

The molecular system of the clock in the model plant, *Arabidopsis thaliana*, involves rhythmically expressed transcription factors, organized in feedback loops. The best characterized loop consists of two MYB transcription factors, LATE ELONGATED HYPOCOTYL (LHY) and CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and a pseudo-response regulator, TIMING OF CAB EXPRESSION 1 (TOC1). LHY/CCA1 are expressed at higher levels at dawn and repress *TOC1* expression by binding to its promoter (Alabadi, 2001). The protein TOC1 is one of the five clock-associated members of PSEUDO-RESPONSE REGULATORS (PRRs). PRR5, 7 and 9 have been characterized to be transcriptional repressors and to suppress *LHY* and *CCA1* expression by rhythmic association with their promoters (Nakamichi et al., 2010). Initially, work with TOC1 (PRR1)-overexpressing *A. thaliana* plants showed an altered, lowered, rhythmic mRNA profile of *CCA1* and *LHY* (Makino et al., 2002), while two TOC1 alleles, *toc1-1* and *toc1-2*, resulted in reduced *CCA1/LHY* levels (Alabadi, 2001). Later findings elucidated the biochemical function of TOC1 as a transcription factor that binds to the promoters of *CCA1*and *LHY* and as a general repressor of oscillator gene expression of the morning and evening loops (Gendron et al., 2012; Huang et al., 2012). LHY also negatively regulates a set of proteins referred to as the evening complex (EC), comprised of LUX (LUX ARRHYTHMO) and EARLY FLOWERING 3 (ELF3) and ELF4 (Adams et al., 2015). Recently, a new loop was described, involving the REVEILLE (RVE) group of genes. RVE8 is a morning-phased factor with positive transcriptional activity for evening-phased genes such as TOC1, LUX, and ELF4 (Hsu et al., 2013). All these factors are involved in multiple feedback loops forming a complex network which gives the pace for the rhythmic expression of a plethora of output genes, such as the widely studied photosynthesis-related genes, LIGHT HARVESTING PROTEIN COMPLEX (LHC) (Millar and Kay, 1996).

Abbreviations: Pv, *Phaseolus vulgaris*; TOC1, TIMING OF CAB EXPRESSION 1; LHY, LATE ELONGATED HYPOCOTYL; PRRs, PSEUDO-RESPONSE REGULATORS; ELF4, EARLY FLOWERING 4; RVE8, REVEILLE 8; LHCB2, LIGHT HARVESTING PROTEIN COMPLEX OF PSII 2; GFP, GREEN FLUORESCENT PROTEIN; L12:D12, photoperiod of 12 h light and 12 h dark; TSA, trichostatin A; HDAC, histone deacetylase; LUC, firefly LUCIFERASE; CCT, CONSTANS CO-LIKE and TOC1; PR, PSEUDORECEIVER; EV, empty vector; PL, promoterless

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Legumes constitute the third largest family among angiosperms and the second largest class of crops. The legume, common bean (*Phaseolus vulgaris* L.), is a major source of protein and thus fundamental for the nutrition of more than 500 million people in developing countries (Graham and Vance, 2003). In recent years, advances have been made regarding *P. vulgaris* genetics, as its full genome sequence has become available (Schmutz et al., 2014). Furthermore, the circadian clock of the common bean has been shown to possess distinct properties from that of *A. thaliana*, regarding the light-induced resynchronization mechanism of *PvLHY*, *PvTOC1* and *PvELF4* (Galeou and Prombona, 2012; Kaldis et al., 2003). Due to the growing scientific interest in bean biology, the need to develop an *in vitro* system for studying the circadian clock of *P. vulgaris* has become essential.

The highly demanding and laborious nature of transgenic bean production and the lack of available mutants prompted us to develop a bean protoplast system as an attractive alternative for elucidating aspects of circadian clock function. Utilizing this system we were able to show oscillations in gene expression and reporter activity, along with histone deacetylase (HDAC) activity involvement in the expression of bean clock genes. Moreover, our results lead to the novel finding that PvTOC1 functions, apart from repressing the morning oscillator gene, *PvLHY*, as a transcriptional repressor of its own gene promoter activity.

2. Materials and methods

2.1. Plant material and growth conditions

Bean seeds (*P. vulgaris* L., cv. Red Kidney) were germinated in the dark for seven days on perlite, at 22 °C and 80% relative humidity. Seedlings were switched to a photoperiod of 12 h day (light intensity: 35 $\mu Em^{-2}s^{-1}$) and 12 h night (light-dark cycle L12:D12).

2.2. Cloning

Promoter fragments of *PvTOC1*, *PvELF4* and *PvLHY* genes (1653 bp, 1445 bp and 1905 bp upstream of the open reading frame (ORF) of each gene, respectively) were PCR amplified from bean genomic DNA (supplementary Table S1) and inserted into the *Pst*I/*Bam*HI sites of pBI221LUC (Iwata and Koizumi, 2005), replacing the 35S promoter and creating the −1653*PvTOC1*:*LUC*, −1449*PvELF4*:*LUC* and −1905*PvLHY*:*LUC* constructs (or T-1653, E-1449 and L-1905). The same method was implemented for the constructs L-1375, L-1008, L-452, T-1108, T-558 and E-940. E-381 was created by digesting the E-940 construct at *Pst*I/*Pme*I sites.

All the intermediate size promoter fragment constructs were generated via site directed mutagenesis (Q5-SDM, E0554S, NEB) using T-1108 and E-940 as templates (supplementary Table S1). The promoterless pBI221LUC vector (PLpBI) was assembled by *Hind*III/*Xba*I excision of the 35S promoter.

PvTOC1 ORF was PCR amplified (supplementary Table S1) from cDNA and the *PvTOC1* overexpression vector (PvTOC1ox) was created by replacing the *EGFP* ORF with the *PvTOC1* ORF in the *Nco*I/*Hind*III sites of pSAT6-EGFP-C_1 (Tzfira et al., 2005). The HA epitope coding sequence was added to its 5′-end using the Q5-SDM kit. This vector was used in Q5-SDM reactions to create deletions of the CONSTANS, CONSTANS-LIKE, and TOC1 domain (CCT, amino acids 478–561) and PSEUDORECEIVER domain (PR, amino acids 1–157), respectively (constructs PvTOC1ΔCCT and PvTOC1ΔPR). The empty vector of pSAT6-EGFP-C_1 (EV) was created by removing the *EGFP* ORF with *Nco*I/*Hind*III digestion. All constructs were verified via sequencing.

2.3. Bean leaf protoplast isolation

Primary leaves of 12–15 days-old bean seedlings were peeled with a razor blade, removing as much as possible of the lower epidermis. Protoplast isolation was performed in the digestion solution (10 mL solution/200 mg leaves, 1% cellulase Onozuka R10, 0.3% macerozyme R10, 0.5 M mannitol, 5 mM Mes:Tris pH 5.7, 20 mM KCl, 10 mM $CaCl_2$, 0.1% bovine serum albumin-BSA) at 30 °C for 2 h. The protoplast suspension was filtered through nylon gauze (79 μm) and centrifuged (80*g*, 5 min, 4 °C). The pellet was washed twice with 10 mL wash solution (0.5 M mannitol, 5 mM Mes:Tris pH 7.0, 20 mM KCl, 10 mM $CaCl_2$, 0.1% BSA) and was resuspended in preservation solution (0.5 M mannitol, 5 mM Mes:Tris pH 7.0, 5 mM $CaCl_2$). For the transformation experiments, protoplasts (2×10^6 cells/mL) were incubated at 4 °C, on ice, for 30 min. For the rhythm experiments, protoplasts (1×10^6 cells/mL/sample) were returned to the preexisting photoperiod in 6-well plates, coated with 0.1% BSA. Trichostatin A (TSA, Sigma) was added at the indicated time points, at a concentration of 20 μM. The time the lights went on at the day of the protoplasts' isolation is considered as zero hours.

2.4. Protoplast transformation

Approximately 4×10^5 protoplasts in 0.2 mL of preservation solution were mixed with 20–30 μg plasmid DNA. An equal volume of freshly-prepared polyethylene glycol (PEG) solution (40% (w/v) PEG, MW 4000, 0.4 M mannitol, 100 mM $Ca(NO_3)_2$) was added and incubated at room temperature for 5 min. Three mL of preservation solution were added slowly, mixed and centrifuged (100*g*, 10 min, 4 °C). The protoplasts were resuspended in 1 mL of preservation solution and incubated in 6-well plates, coated with 0.1% BSA, at 22 °C in the dark for 16–20 h. Alternatively, for rhythmic gene expression analysis in post-transformation samples, protoplasts were returned to the pre-existing photoperiod.

For the real-time monitoring of promoter activity, post-transformation protoplast samples were resuspended in preservation solution containing 50 mM final $CaCl_2$ concentration, supplemented with 100 μM D-luciferin (BIOSYNTH), 50 μM ampicillin (Sigma-Aldrich) and 5% FBS (Fetal Bovine Serum, Merck Millipore) at a final volume of 0.8 mL. Each sample was split in 4 parts (100000 cells/200 μL/well) in 96-well plates (Greiner 96 flat bottom white polystyrol) and was transferred at ZT 7 to constant red light (10 $\mu Em^{-2}s^{-1}$) at 22 °C.

2.5. Luminescence assay

Firefly luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, # E1910). *Renilla reniformis* luciferase luminescence served as a transformation control (10 ng 35S:*Ren* plasmid per sample). In the reporter-effector experiments, 15 μg of each reporter plasmid were combined with 15 μg of either the effector plasmid or the empty vector (EV). Reporter experiments were performed with 20 μg DNA. Luminescence was accessed in a microplate (Greiner 96 flat bottom white polystyrol) using an Infinite M200 microplate reader (Tecan Group Ltd). Each sample was run in duplicate and each experiment was performed three times.

Alternatively, for the real-time promoter activity experiments, firefly luciferase luminescence was measured *in vivo*, every two to five hours in protoplasts transformed with 20 μg of promoter-reporter constructs. Measurements were performed in an Infinite M200 microplate reader (Tecan Group Ltd), with an integration time of 1000 ms. Each experiment was performed twice.

2.6. RNA extraction and RT-PCR

Total RNA was isolated using the RNA II kit (Macherey-Nagel). For quantification of relative RNA levels by the SYBR Green real-time PCR technology, total RNA (1 μg) was reverse transcribed using random hexamers and Superscript III reverse transcriptase (Invitrogen) following the manufacturer's protocol. cDNAs were diluted 10 times and 4 μL of each sample were PCR-amplified on a Stratagene thermal cycler (MX-3000P) using the KAPA SYBR® FAST Universal qPCR kit (KAPA