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The protein J3 regulates flowering through directly interacting with the promoter of *SOC1* in *Brassica juncea*

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ABSTRACT

DNA J HOMOLOG 3 (J3) is a special transcriptional regulator in flowering time control, but the molecular mechanism of J3 in regulating flowering time has not been thoroughly revealed in *B. juncea* which is one important oilseed and vegetable crop. In this study, *J3* gene was cloned from *B. juncea* (*BjuJ3*). Phylogenetic relationship analysis showed that the BjuJ3 had high amino acid sequence similarity (>93%) with other *Brassica* plants. The *BjuJ3*-transgenic tobacco plants exhibited early flowering, suggesting that *BjuJ3* was an activator of flowering time. The qRT-PCR analysis found that *BjuJ3* could be ubiquitously induced by the long-day and vernalization treatments in all the tissues of *B. juncea*. Yeast two-hybrid assays and GST pull-down experiments revealed that BjuJ3 could not directly interact with BjuSOC1, BjuSVP and BjuAGL24. Whereas, yeast one-hybrid and Dual-Glo® Luciferase assays found that BjuJ3 could not interact with *BjuAGL24* promoter but could specifically bind to *BjuSOC1-1* which is one of truncated fragments of *BjuSOC1* promoter. Our research will provide valuable information for unraveling regulatory mechanisms of flowering time in *B. juncea*.

1. Introduction

The floral transition is a significant developmental stage from vegetative to reproductive in flowering plants [1]. Flowering is regulated mainly by four complex genetic pathways including long-day photoperiod, low-temperature vernalization, autonomous and gibberellin (GA)-dependent pathways [2–5]. These pathways ultimately focus on two major integrations of flowering signals, i.e. *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*), and thus promote the formation of floral meristems [6,7].

In *Arabidopsis*, many studies have demonstrated that lots of regulators mediated the floral transition, among which a group of MADS-box transcription factors such as *FLOWERING LOCUS C* (*FLC*), *SHORT VEGETATIVE PHASE* (*SVP*), *SOC1* and *AGAMOUS-LIKE 24* (*AGL24*) play crucial roles in controlling flowering [1,8]. *SOC1* and *AGL24* directly regulate each other to promote flowering [9–11]. By contrast, other two MADS-box proteins, SVP and FLC, suppress the expression of *SOC1* [9,12,13].

Brassica juncea (AABB, 2n = 36) is one important oilseed and vegetable crop in the world [14]. The yield and quality of the product organs are affected greatly by flowering time. Thus it is of great importance to focus on the regulation mechanism of flowering time triggered by environmental signals and endogenous stimuli. It was reported that a novel flowering regulator factor, *DNAJ HOMOLOG 3* (*J3*), could promote flowering in *Arabidopsis* partly through up-regulating the expression of *SOC1* in a way that J3 interacts directly with SVP in the nucleus and attenuates *SVP* binding to the regulation sequences of *SOC1* [15–17]. However, it is unknown that whether and how J3 interacts with SOC1 in *B. juncea* in flowering time control.

In this study, the biological function of *J3* in *B. juncea* (*BjuJ3*) was investigated by examing expression patterns of *BjuJ3* in different organs of *B. juncea* under long-day and vernalization treatments and by transformation study of *BjuJ3* in tobacco. More importantly, the protein-protein and protein-DNA interactions of BjuJ3 with BjuSVP, BjuSOC1 and BjuAGL24 were investigated with multiple

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methods, and a molecular model of BjuJ3 in mediating the flowering signals was herein proposed. This study will broaden our understanding on the complex regulation network of flower time in *B. juncea*.

2. Materials and methods

2.1. Plant materials and growth conditions

Five-leaf-old seedlings of a green mustard 'QJ' (a homozygous line of *B. juncea*) were treated separately in RXZ-type chambers for 30 days under two conditions, i.e. a long daylight photoperiod (16 h light/8 h dark at 22 °C) and a low temperature vernalization (12 h light/12 h dark at 4 °C), and then grown in a normal condition (12 h light/12 h dark at 22 °C). The plants grown under the normal condition throughout the whole study were taken as the control.

2.2. Cloning of BjuJ3

The sequences of cruciferous *J3s* in GenBank database were used to perform homologous alignment with BLASTp. Based on the conserved regions of *J3s* in cruciferous plants, the primers were designed for cloning *BjuJ3* (Table S1). Total RNA was extracted in *B. juncea* using RNAprep Pure Plant Kit (TIANGEN) and then reversely transcribed according to manufacturer's instructions. The *BjuJ3* coding sequence was amplified by PCR using J3-F and J3-R primers (Table S1). The target bands were isolated using agarose gel electrophoresis, inserted into the vector pEASY-T1 and then sequenced by Invitrogen.

2.3. Plant transformation

BjuJ3 was sub-cloned with primers of PBI121-J3-F and PBI121-J3-R (Table S1) and then ligated into pBI121 plasmid for tobacco transformation. The kana of 30 mg/mL was added into MS medium for screening positive transgenetic plants. The DNA was extracted from the leaves of transgenic plants using DNAsecure Plant Kit (TIANGEN), and PCR was performed with primer pairs of J3-F and J3-R (Table S1) to identify the positive plants with transformed *BjuJ3* gene.

2.4. Quantitative real-time PCR

The qRT-PCR of *BjuJ3* was performed with primers of RT-J3-F and RT-J3-R and the *TUBULIN* (*TUB*) was acted as the internal reference with the primers of TUB-F andTUB-R (Table S1). The qRT-PCR mixtures contained 1 μL primers, 2 μL cDNA, 5 μL SsoFast™EvaGreen®Supermix (Bio-Rad) and distilled water to a final of 10 μL. The reaction conditions were as follows: 95 °C for 3 min (one cycle); 95 °C for 10 s, 59 °C for 30 s and 72 °C for 30 s (39 cycles). The above assays were carried out in BIO-RAD C1000/CFX96 fluorescence quantitative PCR unit using Livak Method ($2^{\Delta\Delta Ct}$) for three times.

2.5. Yeast two-hybrid assay

The yeast recombinants of *BjuSVP*, *BjuAGL24* and *BjuSOC1* had been constructed in our previous studies (data not shown). In this study, *BjuJ3* was sub-cloned with primers of pGBKT7-J3-F and pGBKT7-J3-R (Table S1), and then was ligated into pGADT7 or pGBKT7 to construct recombinant plasmids. Using Matchmaker™ Gold Yeast Two-Hybrid System, the recombinants were respectively transformed into Y187 and Y2H (Clontech) for detecting the protein interactions of BjuJ3 with BjuSVP, BjuAGL24 and BjuSOC1 in yeast, respectively. Toxicity and self-activation detection of BjuJ3 had been performed and found that the recombinant plasmids didn't have self-activating effect and did not show toxicity to yeast cell.

2.6. GST pull-down assay

The *BjuJ3* was sub-cloned with primers of pET32a(+)-J3-F and pET32a(+)-J3-R (Table S1), and then was cloned into pET32a (+) vector, while the *BjuSVP*, *BjuSOC1* and *BjuAGL24* were respectively sub-cloned with the corresponding primers (Table S1) and then were recombined into pGEX-4T-1 vector. Subsequently, the labelled fusion proteins of BjuJ3-HIS, BjuSVP-GST, BjuSOC1-GST and BjuAGL24-GST were induced in the *E.coli* BL21 (DE3) with 1.0 mM IPTG for 4 h at 37 °C, respectively. BjuJ3-HIS protein was purified by BeaverBeads™ IDA-Nickel Kit-10(Beaver). BjuSVP-GST, BjuSOC1-GST and BjuAGL24-GST were purified by BeaverBeads™ GSH (Beaver). The magnetic beads which adsorbed BjuJ3 protein respectively incubated with other three proteins (BjuSVP, BjuSOC1 and BjuAGL24) to detect protein-protein interactions *in vitro* by SDS-PAGE.

2.7. Yeast one-hybrid assay

The *BjuJ3* gene was ligated into the pGADT7 vector to construct the recombinant plasmid of pGADT7-*Bju*J3. The promoters of *SOC1* and *AGL24* in *B. juncea* were inserted individually into the pAbAi vector and formed plasmids of *pAbAi-BjuSOC1* and *pAbAi-BjuAGL24* which were transformed into Y1HGold for screening the optimal resistance concentrations of AbA. Then the pGADT7-BjuJ3 was respectively transformed into Y1H (*pAbAi-BjuSOC1*) and Y1H (*pAbAi-BjuAGL24*) for examing the interactions between BjuJ3 protein and promoters of *BjuSOC1* and *BjuAGL24* on media lacking Leu (SD/-Leu) supplemented with the optimal AbA of 100 ng/mL and 350 ng/mL, respectively [18]. To identify the motif or sequence region of protein-DNA interactions, *SOC1* promoter of *B. juncea* was truncated into three fragments (*SOC1-1*, *SOC1-2* and *SOC1-3*) with primers of SOC1-1-F/SOC1-1-R, SOC1-2-F/SOC1-2-R and SOC1-3-F/SOC1-3-R (Table S1) based on the character of CArG-box in *SOC1* promoter [19]. Similarly, *AGL24* promoter of *B. juncea* was also respectively trucated as *AGL24-1*, *AGL24-2* and *AGL24-3* with primers of AGL24-1-F/AGL24-1-R,AGL24-2-F/AGL24-2-R and AGL24-3-F/AGL24-3-R [19].

2.8. Dual-Glo® luciferase assay

The interactions of BjuJ3 protein with promoters of *BjuSOC1* and *BjuAGL24* were verified using the Dual-Glo® Luciferase Assay system (Promega, Madison, USA). The *BjuJ3* was subcloned with primers of pGreen II 62-SK-J3-F and pGreen II 62-SK-J3-R (Table S1), and then was recombined into pGreenII 62-SK and named as 62SK-J3. Meanwhile, the promoters of *BjuSOC1* and *BjuAGL24* were ligated into pGreenII 0800-LUC and named as *LUC-BjuSOC1* and *LUC-BjuAGL24*, respectively. All the above recombinants were transformed into agrobacterium (GV3101). The agrobacterium bacterial fluid of 62SK-J3 was respectively mixed with *LUC-BjuAGL24* and *LUC-BjuSOC1*, and then co-infiltrated the leaves of *Nicotianabenthamian* for measuring the activities of fluorescein enzyme via GLOMAX® multifunctional instrument (Promega, USA). In addition, the truncated fragments of promoters of *BjuSOC1* and *BjuAGL24* were also used to detect the interactions with J3 protein via Dual-Glo® Luciferase Assay system in this study.

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