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Original Paper

AGL18-1 delays flowering time through affecting expression of flowering-related genes in *Brassica juncea*

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Abstract *Brassica juncea* is an important vegetable and condiment crop widely grown in Asia, and the yield and quality of its product organs are affected by flowering time. AGAMOUS-LIKE18-1 (AGL18-1) belongs to a member of MADS-domain transcription factors, which play vital roles in flowering time control, but the biological role of *AGL18-1* in *B. juncea* (*BjuAGL18-1*) has not been thoroughly revealed in flowering regulatory network. In this study, *BjuAGL18-1* expressed highly in inflorescence and flower, but slightly in root, stem and leaf. The sense and anti-sense transgenic lines of *BjuAGL18-1* were generated and showed that *BjuAGL18-1* functioned as a flowering inhibitor and depressed growth of lateral branching. During the vegetative phase, *BjuAGL18-1* induced another flowering repressor *AGAMOUS-LIKE15* (*BjuAGL15*) but inhibited the flowering signal integrator of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*BjuSOC1*) in *Brassica juncea*. Whereas, during the flower developmental phase, both *SOC1* and *AGAMOUS-LIKE24* (*AGL24*) were down-regulated by *BjuAGL18-1*. By contrast, *AGL15* was promoted by *BjuAGL18-1*, while *SHORT VEGETATIVE PHASE* (*SVP*) was independent of *BjuAGL18-1*. Additionally, *HISTONE DEACETYLASE 9* (*HDA9*) was highly induced by *BjuAGL18-1*. These results will provide valuable information for clarifying the molecular mechanism of *BjuAGL18-1* in mediating flowering time.

Key words: *AGL18-1*, *Brassica juncea*, flowering related genes, flowering time.

Introduction

MCM1-AGAMOUS-DEFICIENS-SRF (MADS) domain regulatory factors play essential roles in controlling the floral transition (Adamczyk et al. 2007; Borner et al. 2000; Hartmann et al. 2000; Lee et al. 2000; Yu et al. 2002). During the floral transition, MADS-domain proteins can act either as activators or repressors (Fernandez et al. 2014). In Arabidopsis, important floral activators include *SOC1* and *AGL24*. Repressors of flowering include *AGL15*, *AGL18* and *SVP*.

Transgenic plants of *AGL15* overexpression produced a lot of phenotypic changes, including reduced fertility, delayed flowering time and inhibit senescence (Fernandez et al. 2000), as with the phenotypic of *AGL18* overexpression lines (Adamczyk et al. 2007). AGL18 and AGL15 form a heterodimer to actively regulate the expression of miRNA156, which acted as a floral repressor (Serivichyaswat et al. 2015). In our previous studies, three members (*AGL18-1*, *AGL18-2* and *AGL18-3*) of the *AGL18* family, together with *SOC1* and *AGL24* were cloned from *B. juncea* (Li et al. 2018). However, only AGL18-1 protein interacted with promoters of *SOC1* and *AGL24* via yeast one-hybrid assays and Dual-Glo® luciferase assays (Li et al. 2018).

SOC1 was regarded as a pivotal flowering integrator in regulating flowering time (Immink et al. 2012; Melzer et al. 2008). Additionally, *SOC1* involved in the regulation of leaf senescence (Chen et al. 2017), floral organ aging (Tan and Swain 2007) and fruit development (Papaefthimiou et al. 2012). *SOC1* was regulated by the *FT* and *CONSTANS* (*CO*) genes (Helliwell et al. 2006), and was employed as downstream target of *CO*. *CO* regulated the expression of *SOC1* gene by binding to the site of the *SOC1* promoter region (Hepworth et al. 2002). Another crucial flowering integrator was *AGL24* (Michaels et al. 2003), which highly expressed in shoot apices, leaves, stems, inflorescence and roots (Liu et

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al. 2008; Yu et al. 2002). AGL24 protein activated the expression of *SOC1* in the flowering transition, and served as a positive regulator of *SOC1* in *Arabidopsis*. Overexpression of *AGL24* caused up-regulation of *SOC1*, and overexpression of *SOC1* also led to up-regulation of *AGL24*. It identified that the *SOC1* and *AGL24* could be regulated with each other (Michaels et al. 2003).

Interestingly, *SVP* had the highest homology with *AGL24*, but it showed the opposite biological role in the flowering process (Hartmann et al. 2000). SVP protein not only directly repressed the expression of flowering integrator *SOC1* in shoot apices and leaves (Li et al. 2008), but also regulated the expression of flowering integrator *FT* to decay flowering (Lee et al. 2007). In addition, *HDA9* was a member of histone deacetylase (HDAC) family in plants, which could delay the initiative of flowering by repressing the transcriptional factor *AGL19* in *Arabidopsis* (Kang et al. 2015; Kim et al. 2013). The MADS transcription factor *AGL18* directly interacted with the co-repressor *TOPLESS* (*TPL*) (Causier et al. 2012), which was regarded as a flowering repressor by recruiting histone deacetylase *HDA19* (Krogan et al. 2012; Liu et al. 2009). Furthermore, the FUNCTION-RELATED 1(AFR1) protein and the FUNCTION-RELATED 2(AFR2) protein which periodic regulated HDAC, were ability to form complexes with AGL18 and HDAC in flowering transition (Gu et al. 2013).

In *Arabidopsis*, *AGL18* acted as repressor of the floral transition, and redundancy with *AGL15*, Yeast two-hybrid assays reveal that AGL18 could interaction with AGL15 (Adamczyk et al. 2007). In yeast one-hybrid assays, AGL15 regulates *SOC1* via binding to promoter of *SOC1*. Therefore, both AGL15 and AGL18 were acted as *SOC1* targets (Immink et al. 2012). SOC1 directly interacts with AGL24 and forms a homodimer to integrate flowering signals. In addition, *AGL24* and *SOC1* affect expression of each other. The MADS-Domain factors AGL15 and AGL18 could along with SVP and AGL24 to inhibit expression of floral genes (Liu et al. 2008; Michaels et al. 2003; Yu et al. 2002).

However, it was still unknown how AGL18-1 functioned as a flowering regulation factor in *B. juneca*. Hence, the biological role of *AGL18-1* in floral transition was identified in transgenic plants. Subsequently, the expression patterns of flowering regulatory genes, such as *SOC1*, *AGL24*, *AGL15*, *SVP* and *HDA9*, were investigated via qRT-PCR. This study will provide valuable information for elucidating regulatory mechanisms of *AGL18-1* together with the above flowering-related genes in *B. juncea*.

Materials and methods

Transgenic plants and growth conditions

BjuAGL18-1 gene was cloned from *B. juncea* in our previous study (Li et al. 2018). Successively, the transgenic plants were generated in the background via agrobacterium-mediated method use recombinant plasmids pBI*35S::sBjuAGL18-1* and pBI*35S::aBjuAGL18-1*, which constructed by inserted the full-length sequences of *BjuAGL18-1* into the containing CaMV35S promoter plant binary vector pBI121 forwardly and reversely. Next, the above recombinants were transformed into *B. juncea* and *Nicotiana tabacum* to generate transgenic lines.

T1 transgenic plants were cultivated in the chamber under long-day conditions (16/8 h light/dark cycle at 25/20°C). The *35S::sBjuAGL18-1* transgenic lines were screened and identified via PCR with primer pairs of pBI121-F and *AGL18-1*-R (Supplementary Table S1), while the *35S::aBjuAGL18-1* lines were detected using primer pairs of pBI121-F and *AGL18-1*-F (Supplementary Table S1). Finally, 15 *35S::sAGL18-1* plants and 12 *35S::aAGL18-1* transgenic lines in *B. juncea*, 22 *35S::sAGL18-1* plants and 19 *35S::aAGL18-1* transgenic lines in *Nicotiana tabacum* were obtained.

Flowering time analysis

The number of leaves before bolting (LBB), the number of cauline leaves on lateral branches (CLLB) and the total leaf number (TL) of transgenic lines were regarded as a measure of flowering time during the bolting stage. The number of LBB was counted at the moment of the first plant bolting. The number of CLLB and TL were recorded at the tenth days after the first plant bolting. At least random 12 plants were chosen for each genotype and treatment.

gDNA and RNA extraction, cDNA Synthesis

Genomic DNA was extracted from the third and fourth attached rosette leaves of *B. juncea* using DNAsecure Plant Kit (TIANGEN, DP320-03). Total RNA was extracted from roots, stems, cauline leaves, flowering buds, sepals and petals of *B. juncea* by using of RNAprep Pure Plant Kit (TIANGEN, DP432). According to the manufacturer s recommendations, cDNA was synthesized via PrimeScript™ RT reagent kit RR047A with gDNA Eraser (TaKaRa).

Quantitative Real-Time PCR

The *ACTIN2* gene was used as an internal reference and gene expression of *BjuAGL18-1*, *BjuSOC1*, *BjuAGL24*, *BjuAGL15*, *BjuSVP* and *BjuHDA9* were detected in various tissues and developmental stages under inductive conditions. The qRT-PCR was performed in 96-well blocks using Bio-Rad CFX96 Real-Time PCR system with specific primers listed in Supplementary Table S1. The reaction system was as follows: 0.5 μl reverse primers (10 μmol/μl), 0.5 μl forward primers (10 μmol/μl), 2 μl cDNA template, 5 μl SsoFast™ EvaGreen® Supermix (Bio-Rad) and add ddH_2O to 10 μl. The reactions procedure was carried out using conditions as follows: 95°C

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for 3 min followed by 40 cycles of 95°C for 10 s, 55–65°C for 30 s. All the qRT-PCR results were presented as means±SE of three biological replicates and each sample was quantified in triplicate. The relative expression level of genes were analyzed by the $2^{-\Delta\Delta Ct}$ equation.

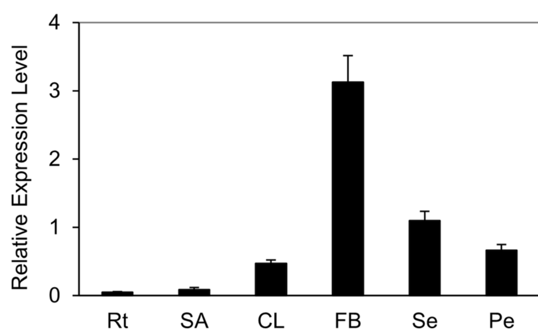

Figure 1. Expression of *BjuAGL18-1* in main tissues under LD conditions in *B. juncea*. The expression patterns of *BjuAGL18-1* in roots (Rt), shoot apex (SA), cauline leaves (CL), flowering buds (FB), sepals (Se) and petals (Pe). Total RNA was isolated from different tissues of two-month-old plants. Three technical and biological replicates were assessed. Bars represent means±SE.

Results

Expression pattern of BjuAGL18-1 in B. juncea

The qRT-PCR analysis indicated that transcript accumulation of *BjuAGL18-1* were mainly found in flowering buds, sepals and petals, but barely in roots, shoot apex and cauline leaves of *B. juncea*. The highest expression of *BjuAGL18-1* was detected in flowering buds (Figure 1). These results supported that a high transcript abundance of *BjuAGL18-1* in flower organ, which might contribute to maintaining reproduction growth.

Phenotype of 35S::sBjuAGL18-1 and 35S::aBjuAGL18-1 lines

In this study, *BjuAGL18-1* was transformed into *Nicotiana tabacum* by Agrobacterium-mediated method for identifying its biological role in the control flowering time. The results showed that two-month-old *35S::sBjuAGL18-1* lines exhibited a phenotype of delayed flowering time under inductive LD conditions. Conversely, *35S::aBjuAGL18-1* lines accelerated flowering compared with WT plants (Figure 2A). Except for flowering time, significant increase was found in the branch number between the *35S::aBjuAGL18-1* lines and the wild type or *35S::sBjuAGL18-1* lines (Figure 2B–D). Similar results also were observed in the

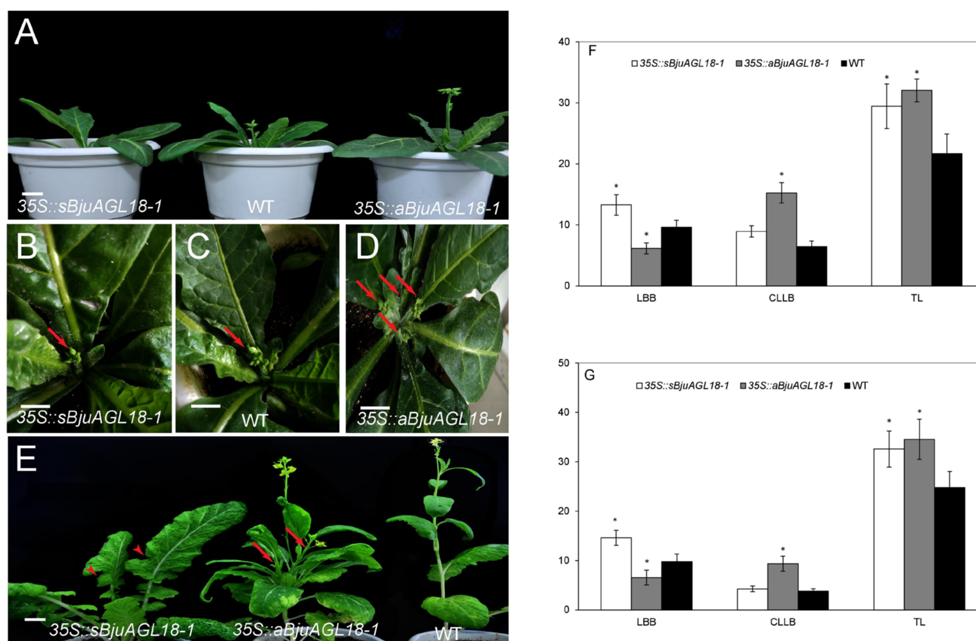

Figure 2. Phenotype of *35S::sBjuAGL18-1* and *35S::aBjuAGL18-1* in transgenic *Nicotiana tabacum* and *B. juncea*. The flowering of *35S::sBjuAGL18-1*, WT and *35S::aBjuAGL18-1* lines under LD conditions in *Nicotiana tabacum* (A). The phenotype of bolting appearance in *Nicotiana tabacum* (B–D), the arrows referred to the number of lateral branches produced when appearance of bolting. The flowering of *35S::sBjuAGL18-1*, *35S::aBjuAGL18-1* and WT lines under LD conditions in *B. juncea* (E). The arrowheads in *35S::sBjuAGL18-1* lines indicated the serrated leaves, while the arrows in *35S::aBjuAGL18-1* lines referred the lateral branches. Bars=1 cm. Effect of transgenic lines on flowering time in *B. juncea* (F) and *Nicotiana tabacum* (G). The number of leaves before bolting (LBB), the number of cauline leaves on lateral branches (CLLB) and the total leaf number (TL) were used to investigate flowering time. Data were indicated with the means±SD ($n \geq 12$ plants). Asterisks indicated statistically significant differences in means between transgenic plants and wild-type. Three technical and biological replicates were assessed. Paired *t*-tests, * $p<0.05$, ** $p<0.01$.

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transgenic *B. juncea* plants (Figure 2E). Furthermore, we investigated the number of leaves before bolting (LBB) which reflected the time of flowering, the number of cauline leaves on lateral branches (CLLB) which reflected the number of lateral branches and the total leaf number (TL) in transgenic *B. juncea* and *Nicotiana tabacum*. The LBB was the most in *35S::sBjuAGL18-1* lines and had significant difference with the wild type and *35S::aBjuAGL18-1*. The *35S::aBjuAGL18-1* lines had significantly more CLLB than the wild-type plants and *35S::sBjuAGL18-1* lines. The TL in *35S::sBjuAGL18-1* and *35S::aBjuAGL18-1* lines was dramatically higher than of wild type (Figure 2F–G). It preliminarily suggested that *BjuAGL18-1* probably served as a floral repressor in *B. juncea* and *Nicotiana tabacum*.

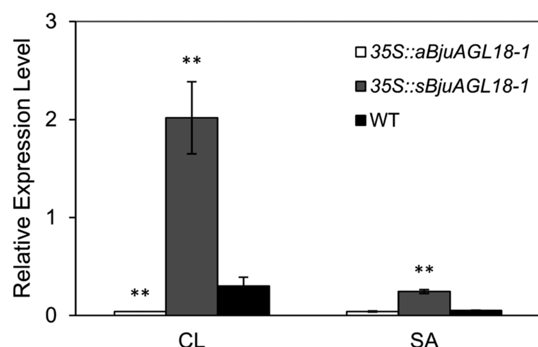

Figure 3. *BjuAGL18-1* relative expression in cauline leaves (CL) and shoot apex (SA) of transcript *B. juncea* lines and WT. Total RNA was isolated from different tissues of 40-day-old plants. Asterisks indicated statistically significant differences in means between transgenic plants and wild-type. Three technical and biological replicates were assessed. Paired *t*-tests, * $p<0.05$, ** $p<0.01$.

Expression of BjuAGL18-1 and flowering-related genes in transgenic seedlings

To further elucidate *BjuAGL18-1* function of the regulation of flowering time, flowering signal perceptive tissues of cauline leaves and shoot apex were taken to measure transcript accumulation by qRT-PCR during the vegetative stage. The relative *BjuAGL18-1* expression level in *35S::sBjuAGL18-1* lines was much higher than WT in cauline leaves and shoot apex of *B. juncea* seedlings. Conversely, we observed that *35S::aBjuAGL18-1* lines accumulated low levels of *BjuAGL18-1* transcripts (Figure 3). Compared with WT, the *35S::sBjuAGL18-1* lines had a lower abundance of *BjuSOC1* transcript in cauline leaves and shoot apex. Nevertheless, the transcript levels of *BjuSOC1* was remarkable higher in *35S::aBjuAGL18-1* lines relative to WT (Figure 4A). The transcript accumulation of *BjuAGL15* was substantially higher in *35S::sBjuAGL18-1* lines than in the WT. There was no major difference in *BjuAGL15* expression levels detected between *35S::aBjuAGL18-1* lines and WT(Figure 4C). However, no significant expression changes were detected in either *35S::sBjuAGL18-1* lines or *35S::aBjuAGL18-1* lines compared to the WT plants

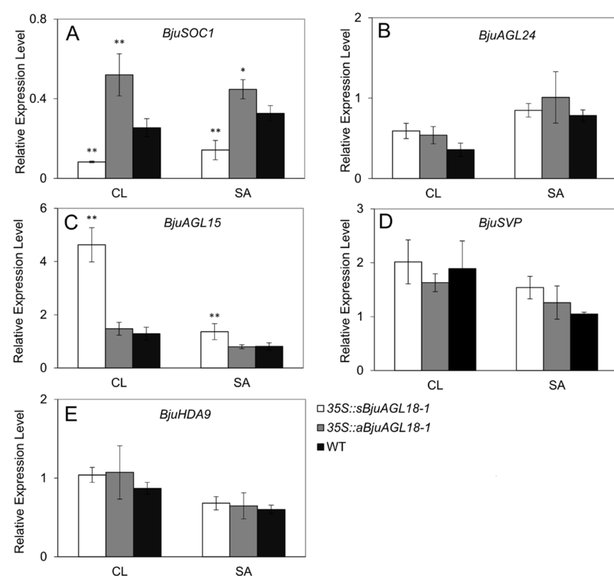

Figure 4. The transcript abundance of *BjuSOC1* (A), *BjuAGL24* (B), *BjuAGL15* (C), *BjuSVP* (D) and *BjuHDA9* (E) in different tissues of *B. juncea* seedlings grown under LD conditions. The expression was measured in cauline leaves (CL), shoot apex (SA) via qRT-PCR. The means±SE were shown. Total RNA was isolated from different tissues of 40-day-old plants. Asterisks indicated statistically significant differences in means between transgenic plants and wild-type. Three technical and biological replicates were assessed. Paired *t*-tests, * $p<0.05$, ** $p<0.01$.

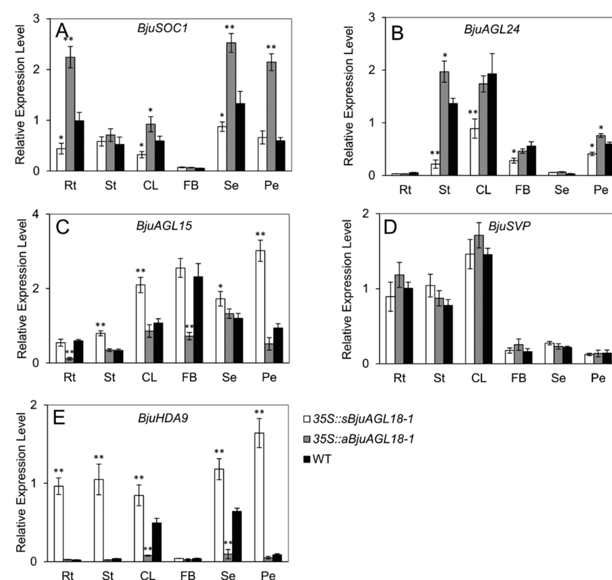

Figure 5. The transcript accumulation of *BjuSOC1* (A), *BjuAGL24* (B), *BjuAGL15* (C), *BjuSVP* (D) and *BjuHDA9* (E) in different tissues of *B. juncea* during flowering phase under LD conditions. The expression were measured in roots (Rt), stems (St), cauline leaves (CL), flowering buds (FB), sepals (Se) and petal (Pe) via qRT-PCR. The means±SE were shown. Total RNA was isolated from different tissues of two-month-old plants. Asterisks indicated statistically significant differences in means between transgenic plants and wild-type. Three technical and biological replicates were assessed. Paired *t*-tests, * $p<0.05$, ** $p<0.01$.

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in *BjuAGL24* (Figure 4B), *BjuSVP* (Figure 4D), *BjuHDA9* (Figure 4E).

BjuSOC1 and BjuAGL24 were repressed by AGL18-1 during flower developmental stage

To elucidate the function of *AGL18-1* of *B. juncea* during flower developmental phase, the transcript accumulation of *BjuSOC1* and *BjuAGL24* were assessed in different tissues of transgenic *AGL18-1* lines via qRT-PCR. *BjuSOC1* transcript abundance increased in roots, cauline leaves, sepals and petals of *35S::aBjuAGL18-1* lines. On the contrary, the expression of these tissues in *35S::sBjuAGL18-1* lines decreased except in the petals. Nevertheless, no significant changes were detected in either *35S::sBjuAGL18-1* lines or *35S::aBjuAGL18-1* lines compared to the stems and flowering buds of WT plants (Figure 5A).The abundance of the *BjuAGL24* transcript were principally detected in stems, cauline leaves, flowering buds and petals, but rarely expressed in roots and sepals regardless of transgenic lines or WT plants. *BjuAGL24* transcript accumulation at elevated levels relative to the WT in stems, cauline leaves, flowering buds and petals of *35S::sBjuAGL18-1* lines (Figure 4B). These results revealed that *BjuAGL18-1* repressed the expression of flowering-activator *BjuSOC1* and *BjuAGL24* during flower developmental phase.

BjuAGL15 was regulated by BjuAGL18-1 during flowering phase

Expression of *BjuAGL15* increased everywhere except in a portion of the roots and flowering buds in *35S::sBjuAGL18-1* lines, compared to the wild-type plants. In addition, *BjuAGL15* transcript level considerably declined in roots, and flowering buds of *35S::aBjuAGL18-1* lines. No obvious expression differences of *BjuAGL15* were detected in cauline leaves, sepals and petals relative to WT (Figure 5C). It suggested that *BjuAGL18-1* regulated the expression of inhibitor *BjuAGL15*during flower developmental stage.

BjuSVP was independent of BjuAGL18-1 during flowering phase

BjuSVP ubiquitously expressed in all organs. Higher *BjuSVP* accumulation was observed in vegetative organs, compared with floral tissues. However, there was no significant expression difference of *BjuSVP* among *35S::sBjuAGL18-1* lines, *35S::sBjuAGL18-1* lines and wild-type (Figure 5D), implying that *BjuAGL18-1* could not probably affect the expression of *BjuSVP* gene during flowering phase.

BjuHDA9 was highly induced by BjuAGL18-1 during flowering phase

The transcript level of *BjuHDA9*, a histone deacetylase, was evaluated via qRT-PCR in *B. juncea* transgenic lines. The results showed that the transcript of *BjuHDA9* was highly induced in *35S::sBjuAGL18-1* lines except in a portion of the flowering buds, There was no detectable significant expression difference of *BjuHDA9* in roots, stems, flowering buds and petal between *35S::aBjuAGL18-1* lines and wild type, except in cauline leaves and sepals (Figure 5E). It inferred that the high transcript abundance of *BjuHDA9* was probably responsible for the flower development.

Discussion

Biology role of AGL18-1 in flowering time regulation

In this study, the phenotypes of sense and anti-sense *BjuAGL18-1* transgenic lines were investigated and indicated that *BjuAGL18-1* not only delayed flowering time but also inhibited growth of lateral branching in *B. juncea* and *Nicotiana tabacum*. It suggested that *BjuAGL18-1* was a flowering time repressor in *B. juncea*, consistent with the previous reports in *Arabidopsis* (Adamczyk et al. 2007; Fernandez et al. 2014; Serivichyaswat et al. 2015). Whereas, *BjuAGL18-1* also acted as a branching regulator in *B. juncea*, which was quite different from *Arabidopsis*. Furthermore, previous studies showed that *AGL18* expressed highly in roots, inflorescences and mature flowers, but slightly in stems and leaves in *Arabidopsis* (Adamczyk et al. 2007). In contrast, the expression of *BjuAGL18-1* was barely detected in roots of *B. juncea* in this study.

Flowering activators BjuSOC1 and BjuAGL24 were down-regulated by BjuAGL18-1

Indeed, one finding from our previous studies indicated that BjuAGL18-1 protein could bind to promoters of *BjuSOC1* and *BjuAGL24* (Li et al. 2018). Here, during the vegetative phase, *BjuAGL18-1* significantly repressed the expression of *BjuSOC1*, but not *BjuAGL24*. In addition, during the flowering phase, *BjuAGL18-1* could negatively regulate the expression of flowering signal integrators of *BjuSOC1* and *BjuAGL24* in some organs, while the mRNA levels and expression patterns were different between *BjuSOC1* and *BjuAGL24* in some tissues of transgenic lines. It inferred that there might be different regulation mechanisms or redundant functions between *BjuSOC1* and *BjuAGL24* in floral transition of *B. juncea*. In *Arabidopsis*, the *AGL18* was related to flowering time via regulation of *SOC1*, and it could inhibit the expression of *SOC1*. In addition, the *SOC1*, *AGL24*, *AGL15* and *AGL18* acted partially redundantly (Fernandez et al. 2014).

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Flowering repressor AGL15 was promoted by BjuAGL18-1, and another flowering repressor SVP was independent of BjuAGL18-1

In this study, the expression level of *BjuAGL15* was dramatically higher in stems, leaves, petals, and sepals of the *35S::sBjuAGL18-1* lines than in the wild type, while there was no significant expression change in some organs of *35S::aBjuAGL18-1* lines. In previous studies, *AGL15* and *AGL18* were regarded as flowering inhibitors of the upstream of *FLOWERING LOCUS T* (*FT*) (Becker and Theissen 2003), and acted in a functional redundancy in regulating floral transition of *Arabidopsis* (Adamczyk et al. 2007). Therefore, we speculated that *BjuAGL15* and *BjuAGL18-1* probably acted redundantly as flowering inhibitors in *B. juncea*. However, as reported previously, AGL18-containing complexes could work independently with the SVP-containing complexes (Adamczyk et al. 2007). In this study, the expression pattern of *BjuSVP* in transgenic lines also demonstrated that *BjuSVP* was probably independent of *BjuAGL18-1* to affect flowering in *B. juncea*.

HDA9 was highly induced by BjuAGL18-1

In *Arabidopsis*, the expressions of *SOC1* and *AGL24* were substantially enhanced in *hda9* mutants relative to the wild-type (Kang et al. 2015). Our previous studies showed that BjuHDA9 protein interacted with promoters of *BjuSOC1* and *BjuAGL24* in *B. juncea* (Jiang et al. 2018). Here, the expression of *BjuHDA9* was significantly affected by *BjuAGL18-1* in transgenic lines during flowering phase, suggesting that histone deacetylase *BjuHDA9* was probably involved in flowering control through interacting with BjuAGL18-1 and other flowering factors in *B. juncea*. Similarly, ternary protein complexes AGL18/AFR/HDAC were capable of inhibiting expression of *FT* and then regulated the flowering (Gu et al. 2013). Hence, protein complexes AGL18-1/AFR/HDA9 or more higher-order complexes were proposed to regulate *SOC1* and *AGL24* in the regulation of flowering of *B. juncea*. Based on the above results, we proposed a regulatory network of *AGL18-1* involved in flowering control of *B. juncea* (Supplementary Figure S1).

Acknowledgements

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