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Identification of acting domains mediating the protein interactions between flowering repressors *flowering locus C* (*FLC*) and *short vegetative phase* (*SVP*) from birch (*Betula platyphylla* Suk.)

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Abstract

Flowering, transition from vegetative to reproductive phase in plants, is regulated by both endogenous and environmental signals. *Flowering locus C* (*FLC*) and *short vegetative phase* (*SVP*) are the key genes in delaying flowering. For study on the mechanism of interaction between SVP and FLC in birch, the test was performed by yeast two-hybrid. The target genes (*BpFLC* and *BpSVP*) and truncated genes of *BpFLC1~6* and *BpSVP1~6* were cloned from subcloning vectors, digested with *Nde* I and *Eco*R I, then ligased into yeast expression vector pGBKT7 and pGADT7. The test of transformed strains in different conditions showed there were no self-activated abilities and no toxic effects in yeast bait vectors of pGBKT7-*BpFLC* and pGADT7-*BpSVP*. The process of yeast two-hybrid indicated there was a direct interaction between BpFLC and BpSVP. Further study showed Y2HGold [BD-*BpFLC2~5* × AD-*BpSVP*] could grow on selective agar plates TDO, QTO/A, or QDO/X/A with blue stains indicating that BpFLC2~5 truncated forms and BpSVP protein could act with each other to form heterodimers. In addition, the result of yeast two-hybrid also showed that BpFLC and BpSVP2~5 truncated forms could combine with each other to activate downstream reporter genes. The interactions between BpFLC3 and BpSVP3 strongly showed that the K domain of BpFLC and the K domain of BpSVP were the key structure domains and mediated the protein interactions between BpFLC and BpSVP. These results greatly increase our understanding of the mechanisms extending the vegetative phase and enhance genetic studies of birch traits and other woody plants.

Keywords: *Betula platyphylla;* BpFLC; BpSVP; yeast two-hybrid; truncated form; protein interaction.
Abbreviations: AbA_aureobasidin A; AD_activation domain; Ade_adenine; BD_binding domain; BiFC_bimolecular fluorescence complementation; Co-IP_co-immunoprecipitation; CTAB_hexadecyl trimethyl ammonium bromide; DDO_minimal media double dropouts; FD_flowering locus D; FLC_flowering locus C; FT_flowering locus T; His_histidine; LB_lysogeny broth; Leu_leucine; ORF_open reading frame; QDO_minimal media quadruple dropouts; QTO/A/X_minimal media quadruple dropouts with adding aureobasidin A and X-alpha-D-galactopyranoside; QTO/A_minimal media quadruple dropouts with adding aureobasidin A; RT-PCR_reverse transcriptase polymerase chain reaction; SD_synthetic dropout medium; SOC1_suppressor of overexpression of constans1; SVP_short vegetative phase; TDO_minimal media triple dropouts; Trp_tryptophan; X-α-Gal_X-alpha-D-galactopyranoside.

Introduction

Flowering is a highly complicated and complex physiological, biochemical and morphogenetic process that is controlled under a certain genetic background, in which plants are induced by environmental conditions, controlled by signal transduction and flowering determination genes, and restricted by various metabolic pathways, resulting in differentiation of flower buds, thereby leading to organogenesis. Previous studies show multiple genetic pathways are involved in the floral transition of *Arabidopsis*, such as photoperiod pathway, vernalization pathway, autonomous pathway and gibberellin pathway (Blázquez et al., 2000；Mouradov and Weigel 2002；Simpson and Dean 2002；Boss et al., 2004). *Flowering locus C* (*FLC*) and *short vegetative phase* (*SVP*) are two core regulatory genes of flowering signal integrator and the interactions between these two proteins can regulate flowering signal integrator. As an MADS-box transcription factor, *FLC* is mainly expressed in shoot tips and root tips (Sheldon et al., 2002). In vernalization pathway, *FLC* plays a key role. *FLC* mutants and wild-type varieties of *Arabidopsis* exhibit strong vernalization requirement. After vernalization treatment, the expression level of *FLC* in *Arabidopsis* is reduced; however, overexpression of *FLC* will delay the flowering of transgenic *Arabidopsis* (Michaels and Amasino 1999; Sheldon et al., 2002). As a negative regulatory protein of flowering, SVP contains conserved MADS-box, which can be expressed throughout the vegetative growth stage of plants but hardly expressed in inflorescence meristem (Hartmann et al., 2000; Michaels and Amasino 2000; Liu et al., 2007). Inducing the expression of flowering pathway integrators *flowering locus T* (*FT*) and suppressor of *overexpression of constans 1* (*SOC1*) can promote flowering (Hsu et al., 2011; Moon et al., 2003). FLC and SVP can not only inhibit the transcription of *SOC1* gene in shoot apical meristem, but also regulate the expression of *FT* gene in young leaves, thus preventing the transfer of FT protein and other flowering signals from leaves to meristem (Helliwell et al., 2006; Lee et al., 2007; Li et al., 2008; Giakountis and Coupland 2008). Moreover, FLC

protein affects the expression of *flowering locus D* (*FD*) and weakens the response of meristem to flowering signals (Abe et al., 2005). *Betula platyphylla* Suk., also known as birch, is the main tree species in Northeast China, with strong resistance to barren and cold, rapid growth and other characteristics. Birch provides raw materials for furniture manufacturing and papermaking, which is also a natural health medicine due to abundnat valuable nutrients in the bark, juice and seed oil (Zhang et al., 2002; Qu et al., 2013., Huang et al., 2014). In order to extend the vegetative growth stage of birch and obtain high-quality birch timber, it is essential to investigate the late-flowering mechanism in birch. This study aimed to clone flowering repressor genes *BpFLC* and *BpSVP* from *B. platyphylla* and construct yeast expression vector to establish yeast two-hybrid system harboring *FLC* and *SVP*, which provided the theoretical and technical basis for in-depth analysis of the interactions of domains and interaction sites between FLC and SVP in *B. platyphylla*.

Results

Cloning and bioinformatics analysis of BpFLC and BpSVP from B. platyphylla

BpFLC and *BpSVP* genes were successfully cloned from cDNA of young leaves of *B. platyphylla*. Specifically, ORF of *BpFLC* gene (Genebank NO.: KC020112) was 627 bp, encoding 208 amino acids, and the predicted molecular weight and isoelectric point was 23.85 kDa and 9.04, respectively; ORF of *BpSVP* gene (Genebank NO.: KP693588) was 678 bp, encoding 225 amino acids, and the predicted molecular weight and isoelectric point was 25.25 kDa and 6.68, respectively. According to results of homology analysis, BpFLC protein shared the highest similarity (76 %) with TcFLC from *Theobroma cacao* (Fig. 1). BpSVP protein shared the highest similarity (92 %) with CcSVP from *Carya cathayensis* Sarg.; SVP protein was more conservative than FLC protein (Fig. 2). Domain analysis (http://pfam.sanger.ac.uk/) showed BpFLC and BpSVP both belonged to MIKC proteins (Fig. 3). MADS-box of BpFLC and BpSVP was composed of 51 (9-59 aa, PF00319; E-value= 4.9e-28) amino acids; K-box of BpFLC and BpSVP was composed of 100 (74-173aa, PF01486; E-value=1.5e-15) and 104 (69-172 aa, PF01486; E-value=1.5e-15) amino acids, respectively. MADS-box of these two proteins was more conservative than K-box. Signal peptide prediction (http://www.cbs.dtu.dk/services/SignalP/) indicated that N-terminus of BpFLC and BpSVP contained no signal peptide, suggesting that these two proteins were not extracellular secretory proteins or membrane proteins. Therefore, yeast expression plasmids were constructed with complete coding frames of *BpFLC* and *BpSVP*, which could detect the interactions between proteins in yeast two-hybrid system.

Construction and verification of yeast expression vector

PCR products of *BpFLC*, *BpFLC1~6*, *BpSVP* and *BpSVP1~6*, yeast empty vectors pGBKT7 and pGADT7 were double-digested with *Nde* I and *Eco*R I, ligated using T_4 ligase and transformed into *E. coli* to construct recombinant vectors pGBKT7-*BpFLC*, pGBKT7-*BpSVP*, pGBKT7-*BpFLC1~6*, pGADT7-*BpFLC*, pGADT7-*BpSVP* and pGADT7-*BpSVP1~6*. To be specific, these six truncated *BpFLC1~6* genes (*BpFLC1*, *BpFLC2*, *BpFLC3*, *BpFLC4*, *BpFLC5* and *BpFLC6*) were 219, 519, 300, 450, 408 and 108 bp, encoding 73, 173, 100, 149, 135 and 35 amino acids (MI domain, MIK domain, K domain, IKC domain, KC domain and C domain of full-length MIKC-type protein BpFLC, respectively) (Fig. 3A, Supplementary Table 2). As the same with *BpFLC*, six truncated *BpSVP1-6* genes (*BpSVP1*, *BpSVP2*, *BpSVP3*, *BpSVP4*, *BpSVP5* and *BpSVP6*) were 204, 516, 312, 501, 474 and 162 bp, encoding 68, 172, 104, 166, 157 and 53 amino acids (MI domain, MIK domain, K domain, IKC domain, KC domain and C domain of full-length MIKC-type protein BpSVP, respectively) (Fig. 3B, Supplementary Table 2). Recombinant plasmids were extracted for PCR verification (Fig. 4A and 4C) using universal primers (Supplementary Table 1), restriction digestion (Fig. 4B and 4D) and sequencing. According to sequencing results, *BpFLC*, *BpSVP* and their truncated genes were cloned into the yeast expression vector without base mutation, indicating that yeast bait expression vectors were constructed successfully.

Self-activation and toxicity test of yeast bait vectors

At 3 d post-transformation, observation results of binding domain (BD) showed that Y2HGold [BD, BD-BpFLC or BD-BpSVP] could grow on SD/-Trp media, and the colonies were white with good growth; however, no colony was observed on DDO, TDO/X and QTO/A/X plates, which indicated that yeast bait expression vectors were not self-activated (Fig. 5A). Positive colonies of Y2HGold [BD, BD-BpFLC or BD-BpSVP] were selected to measure the absorbance at 600 nm. Results showed that OD_{600} of positive colonies Y2HGold [BD-BpFLC and BD-BpSVP] and colonies Y2HGold [BD] increased first and then tended to stabilize with the extension of incubation time, but the growth conditions exhibited no significant variation with consistent overall growth trend, which indicated that Y2HGold [BD-BpFLC] and Y2HGold [BD-BpSVP] had no toxic effects on yeast (Fig. 5B).

Verification of protein interactions between BpFLC and BpSVP

Recombinant vectors pGBKT7-*BpFLC* × pGADT7-*BpSVP* and pGBKT7-*BpSVP* × pGADT7-*BpFLC* were co-transformed into yeast strain Y2HGold meanwhile with activation domain (AD) empty vectors as control. Results showed that all these four combinations could grow normally on DDO plates (Fig. 6A, Supplementary Table 3). Further experiments showed that negative control and empty vector controls could not grow on TDO, QTO/A and QTO/A/X plates, suggesting that these vectors could not activate downstream reporter genes. However, positive control and Y2HGold [BD-BpFLC × AD-BpSVP] could grown on TDO and QTO/A plates, suggesting that reporter genes *HIS3*, *AUR1-C* and *ADE2* were activated in these three yeast lines. In addition, Y2HGold [BD-BpFLC × AD-BpSVP] could also grow on QDO/A/X plates and generate blue colonies (Fig. 6A, Supplementary Table 3), which indicated that BpFLC could bind with BpSVP to activate expression of four reporter genes: *HIS3*, *AUR1-C*, *ADE2* and *MEL1*. Two genes transformed into opposite vectors could also activate expression of these four reporter genes, indicating a certain interactions between BpFLC and BpSVP.

Screening of BpFLC domains acting on BpSVP

Recombinant vectors pGBKT7-*BpFLC1-6* × pGADT7-*BpSVP* were co-transformed into yeast strain Y2HGold meanwhile with empty vectors as control. The results showed BpFLC2, BpFLC3, BpFLC4 and BpFLC5 could be fused with BpSVP protein and grow on TDO and QDO/A solid

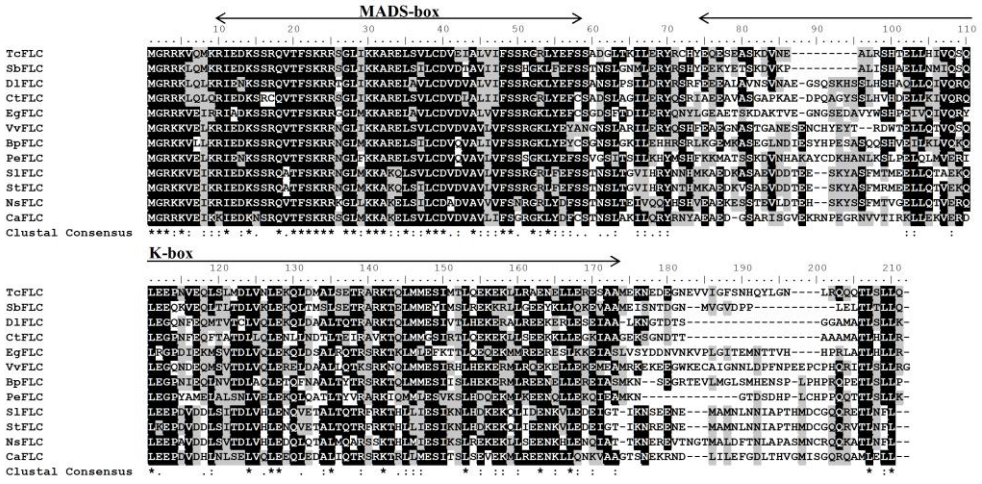

Fig 1. Analysis of homologous sequences of BpFLC protein. The alignment was generated using Clustal W and Bioedit program. The conservative MADS-box and K-box are indicated by line with arrows above the sequences. TcFLC (*Theobroma cacao*, XP_007043954); DlFLC (*Dimocarpus longan*, AHZ89709); VvFLC (*Vitis vinifera*, NP_001268057); EgFLC (*Eucalyptus grandis*, XP_010025294); PeFLC (*Populus euphratica*, XP_011020779); SlFLC, (*Solanum lycopersicum*, XP_004239235); CtFLC, (*Citrus trifoliata*, ACB72864); StFLC (*Solanum tuberosum*, XP_006352169); CaFLC, (*Coffea arabica*, ADU56823); NsFLC (*Nicotiana sylvestris*, XP_009795052); SbFLC (*Shorea beccariana*, BAN89459). Identical and conserved residues are shaded in black and dark grey, respectively.

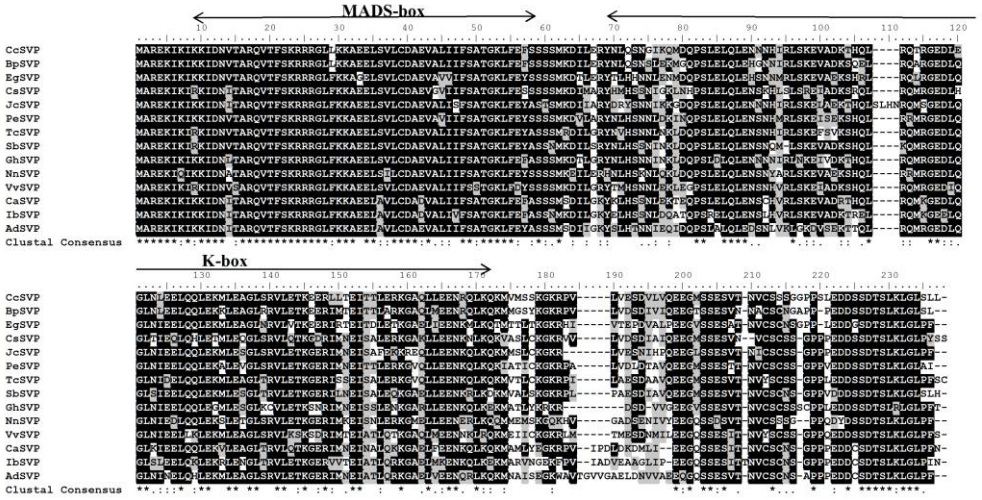

Fig 2. Analysis of homologous sequences of BpSVP protein. The alignment was generated using Clustal W and Bioedit program. The conservative MADS-box and K-box are indicated by line with arrows above the sequences. CcSVP (*Carya cathayensis*, AIA58526); PeSVP (*Populus euphratica*, XP_011016930); TcSVP (*Theobroma cacao*, XP_007018219); EgSVP (*Eucalyptus grandis*, XP_010060099); SbSVP (*Shorea beccariana*, BAN89455); VvSVP (*Vitis vinifera*, XP_002285687); CsSVP (*Citrus sinensis*, XP_006472470); GhSVP (*Gossypium hirsutum*, AGW23339); NnSVP (*Nelumbo nucifera*, XP_010254525); CaSVP (*Coffea arabica*, ADU56833); JcSVP (*Jatropha curcas*, KDP41475); AdSVP (*Actinidia deliciosa*, AFA37965); IbSVP (*Ipomoea batatas*, AAK27151). Identical and conserved residues are shaded in black and dark grey, respectively.

Fig 3. Domain structure of BpFLC (A) and BpSVP (B) in *B. platyphylla*. MADS domain (MADS), Intervening domain (I), Keratin-like domain (K) and C-terminal domain (C) represented structure domains of proteins. NH2 and COOH represented the amino and carboxyl, respectively.

medium, and QDO/A/X with blue colonies (Fig. 6B, Supplementary Table 3), and the colony growth was similar to Y2HGold [BD-BpFLC × AD-BpSVP], which indicated that the expression of four reporter genes (*AUR1-C*, *HIS3*, *ADE2* and *MEL1*) were activated in yeast. Moreover, Y2HGold [BD-BpFLC1 × AD-BpSVP], Y2HGold [BD-BpFLC6 × AD-BpSVP], empty vector controls and negative control only could grow on DDO media, which indicated that two vectors were transformed successfully into yeast. However, they could not grow on TDO, QDO/A and QDO/A/X solid medium, which indicated that they could not be fused with each other to activate downstream reporter genes.

Screening of BpSVP domains acting on BpFLC

As above, recombinant vectors pGBKT7-*BpFLC* × pGADT7-*BpSVP1-6* were co-transformed into yeast strain Y2HGold meanwhile with empty vectors as control. The results is similar to above, which showed BpSVP2, BpSVP3, BpSVP4 and BpSVP5 could be fused with BpFLC protein and grow on TDO and QDO/A, and QDO/A/X with blue colonies (Fig. 6C, Supplementary Table 3), which indicated that they activated the expression of four reporter genes (*AUR1-C*, *HIS3*, *ADE2* and *MEL1*). Moreover, Y2HGold [BD-BpFLC × AD-BpSVP1], Y2HGold [BD-BpFLC × AD-BpSVP6], empty vector controls and negative control only could grow on DDO media, but not grow on TDO, QDO/A and QDO/A/X solid medium, which indicated that they could not be fused with each other to activate downstream reporter genes.

Identification of the interactions between BpFLC3 and BpSVP3

Recombinant vectors pGBKT7-*BpFLC3* × pGADT7-*BpSVP3* were co-transformed into yeast strain Y2HGold meanwhile with empty vectors as control. The result showed BpFLC3 could be combined with BpSVP3 protein and grow on TDO and QDO/A solid medium, and QDO/A/X with blue colonies (Fig. 6D, Supplementary Table 3), and the colony growth was similar to that on positive control, which indicated that Y2HGold [BD-BpFLC3 × AD-BpSVP3] activated the expression of four reporter genes (*AUR1-C*, *HIS3*, *ADE2* and *MEL1*) in yeast, indicating a certain interactions between the K box of BpFLC and the K box of BpSVP.

Discussions

In the life cycle of plants, the transition from vegetative growth to reproductive growth is critical, and "flowering" is a switch of such transition. Physiological and genetic analyses show that flowering is a result of a variety of environmental factors and endogenous factors. Multiple flowering regulatory pathways co-regulate flowering integrators and promote the transformation from apical meristem to reproductive organs (Araki 2001; Bernier and Périlleux 2005). In this study, *BpFLC* and *BpSVP* cloned from *B. platyphylla* both encode MIKC-type proteins, belonging to flowering repressor genes. According to researches of model plant *Arabidopsis*, FLC plays a crucial role in vernalization pathway, while SVP is involved in the regulation of autonomous pathway, thermosensitive pathway and gibberellin pathway (Sung and Amasino, 2004; Hepworth et al. , 2002; Michaels, 2009). MADS-box of BpFLC and BpSVP is highly conserved, but K-box is poorly conserved. Bioinformatics prediction shows that both BpFLC and BpSVP contain three consecutive hydrophilic α helixes. Yang et al.(2003) found that the former two α helixes of K-box might play a decisive role in the formation of dimer structure, but specific sites of the identified target sequence is still unclear. Both *BpFLC* and *BpSVP* genes encode MIKC-type proteins and contain MADS domain, I domain, K domain and C domain (Fig. 3). In previous studies, *AtFLC* and *AtSVP* genes of *Arabidopsis* were ligated into the yeast expression vector for two-hybrid verification in AH109, results showed that there were certain interactions between AtFLC and AtSVP (Fujiwara et al., 2008; Balanzà et al., 2014). Tang et al. (2011, 2012) results confirmed the interactions between *BjSVP* and *BjFLC* from *Brassica juncea* with yeast two-hybrid system, further performed by *in vitro* protein expression and SDS-PAGE. In this study, yeast two-hybrid system of BpFLC and BpSVP was established, which provides a reliable technology platform for screening domains and interaction sites of BpFLC and BpSVP. In order to investigate the specific domains of the interactions between BpFLC and BpSVP proteins, six truncated *BpFLC* genes (Fig. 4A and 4C) and six truncated *BpSVP* genes were constructed (Fig. 4B, 4D). Specifically, K domain and C domain were truncated from BpFLC1 and BpSVP1; C domain was truncated from BpFLC2 and BpSVP2; only K domain was retained in BpFLC3 and BpSVP3; M domain was truncated from BpFLC4 and BpSVP4; only K domain and C domain were retained in BpFLC5 and BpSVP5; only C domain was retained in BpFLC6 and BpSVP6 (Supplementary Table 2). According to these truncated proteins, the decisive role of M, I, K and C domains can be determined. In this study, BpFLC2 (MIK domain), BpFLC3 (K domain), BpFLC4 (IKC domain) and BpFLC5 (KC domain) all contain K domain. Results show that they all exhibit interactions with BpSVP protein and can activate four reporter genes (*AUR1-C*, *HIS3*, *ADE2* and *MEL1*) in yeast, with blue colonies on QDO/X/A plates (Fig. 6B), which indicates that K domain may play a key role in the verification of interactions. At early incubation stage, the growth and color of colonies on QDO/X/A plates are not the same (data not shown), suggesting that other domains (M, I or C domains) of BpFLC protein may affect (enhance or weaken) the influence of K domain on BpSVP protein. Similarly, K domain of BpSVP also plays a key role in the interactions with BpFLC (Fig. 6C), which may be affected by other domains (M, I or C domains). According to verification results, there are certain interactions between BpFLC3 (K domain of BpFLC) and BpSVP3 (K domain of BpSVP) in Y2HGold [BD-BpFLC3 × AD-BpSVP3] (Fig. 6D), which is similar to the screening results of *BjSVP* and *BjFLC* domains of *Brassica juncea* (Tang et al., 2011, 2012). Therefore, it can be inferred that other domains (M, I or C domains) may affect the interactions between K domains, which requires further verification. Despite high efficiency and wide application, yeast two-hybrid screening technology has inherent technical defects. For instance, as a lower eukaryote, yeast varies in protein translation, folding and processing compared with higher organisms; furthermore, yeast two-hybrid system can only detect binding between two proteins but cannot detect interaction between multiple proteins; false positives caused by self-activation of proteins and other false negative results such as toxicity and secretion also limit the application of yeast two-hybrid technology (Fields, 2005; Bickle et al., 2006). To overcome these technical defects, various techniques based on other principles, such as *in vivo* co-immunoprecipitation (Co-IP) technology and bimolecular fluorescence complementation (BiFC) assay, can be adopted for protein interaction analysis,

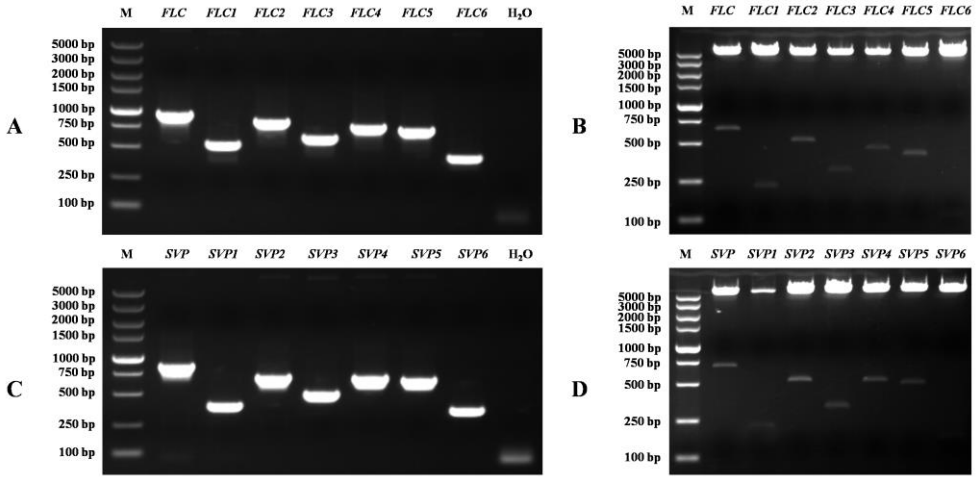

Fig 4. The verification of yeast expression plasmids. (A) Electrophoretogram of PCR products of recombinant vector pGBKT7-*BpFLC1~6* with BD universal primers. (B) Restriction enzymes detection of recombinant vector pGBKT7-*BpFLC1~6* with *Nde* I and *Eco*R I. (C) The PCR verification of recombinant vector pGADT7-*BpSVP1~6* with AD universal primers. (D) Restriction enzymes detection of recombinant vector pGADT7-*BpSVP1~6* with *Nde* I and *Eco*R I. M: DNA marker DL5000; *FLC*: the recombinant vector pGBKT7-*BpFLC*; *FLC1~6*: the recombinant vector pGBKT7-*BpFLC1~6*; *SVP*: the recombinant vector pGADT7-*BpSVP*; *SVP1~6*: the recombinant vector pGADT7-*BpSVP1~6*; H_2O: Negative control with ddH_2O as template.

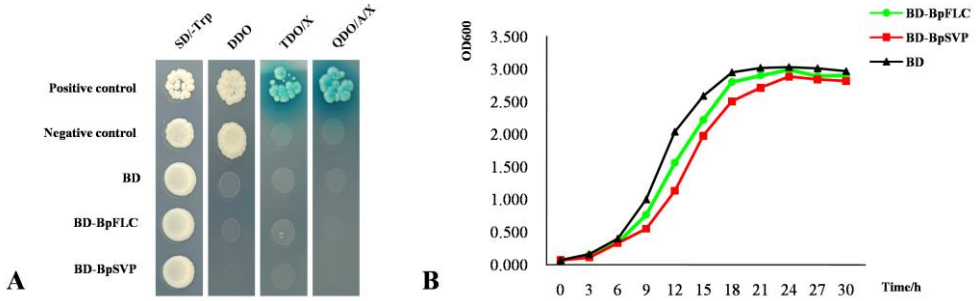

Fig 5. Verification of self-activation and toxic effects of yeast bait vectors.
(A) The recombinant yeast harbouring pGBKT7-*BpFLC* (BD-BpFLC), pGBKT7-*BpSVP* (BD-BpSVP) or empty vector pGBKT7 (BD, binding domain) were transferred to media with different dropouts to verify their self-activated ability. (B) The recombinant yeast harbouring pGBKT7-*BpFLC* (BD-BpFLC), pGBKT7-*BpSVP* (BD-BpSVP) or empty vector pGBKT7 (BD) were inoculated to liquid SD/-Trp media to measure the value of OD600. Positive control: Y2HGold [pGBKT7-53 × pGADT7-T]; Negative control: Y2HGold [pGBKT7-Lam × pGADT7-T]; BD: Y2HGold [pGBKT7]; BD-BpFLC: Y2HGold [pGBKT7-*BpFLC*]; BD-BpSVP: Y2HGold [pGBKT7-*BpSVP*]. SD/-Trp: synthetic dropout medium without tryptophan. DDO: minimal media double dropouts; TDO/X: minimal media triple dropouts with adding X-alpha-D-galactopyranoside; QTO/A/X: minimal media quadruple dropouts with adding aureobasidin A and X-alpha-D-galactopyranoside.

thus revealing the actual interactions between proteins *in vivo* and further confirming the existence of interactions between BpFLC and BpSVP.

Materials and methods

Plant material and growth conditions

The *B. platyphylla* was obtained from the campus of Northeast Forestry University in Heilongjiang province, China. For cloning the *BpFLC* and *BpSVP* gene, young leaves were sampled, immediately frozen in liquid nitrogen, and stored at -80 °C before isolating total RNA.

Isolation of BpFLC and BpSVP

To clone the *BpFLC* and *BpSVP* gene in birch, total RNA was isolated with improved CTAB method (Qu et al., 2013) from *B. platyphylla* young leaves. The purity and quality of RNA was checked by NanoDrop 2000c (Thermo-Scientific, USA). A 1 μg aliquot containing total RNA was treated with DNase I (Invitrogen, Carlsbad, CA, USA), and was reverse-transcribed using the PrimeScript RT reagent kit (TaKaRa). The full length cDNA for the ORF of *BpFLC* and *BpSVP* were cloned from cDNA using RT-PCR with *ExTaq*^{HS} enzyme (TaKaRa). The primers used to amplify *BpFLC* and *BpSVP* are listed in Supplementary Table 1. The PCR cycle profile consisted of an initial denaturation at 94 °C for 4 min; 35 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; and a final extension at 72 °C for 7 min. The PCR product was cloned into pMD18-T vector (TaKaRa), and the positive clones were picked out, then the recombinants were identified by sequencing. The deduced BpFLC and BpSVP protein was characterized using Expasy tools (http://www.expasy.org/tools). Sequence alignments were performed using the Bioedit program (7.0.4.1 version).

Construction of yeast bait expression vector

To construct the *BpFLC*, *BpSVP*, or their truncated forms (*BpFLC1~6* and *BpSVP1~6*) in yeast bait expression vector, the PCR profile was executed as above with the primers in

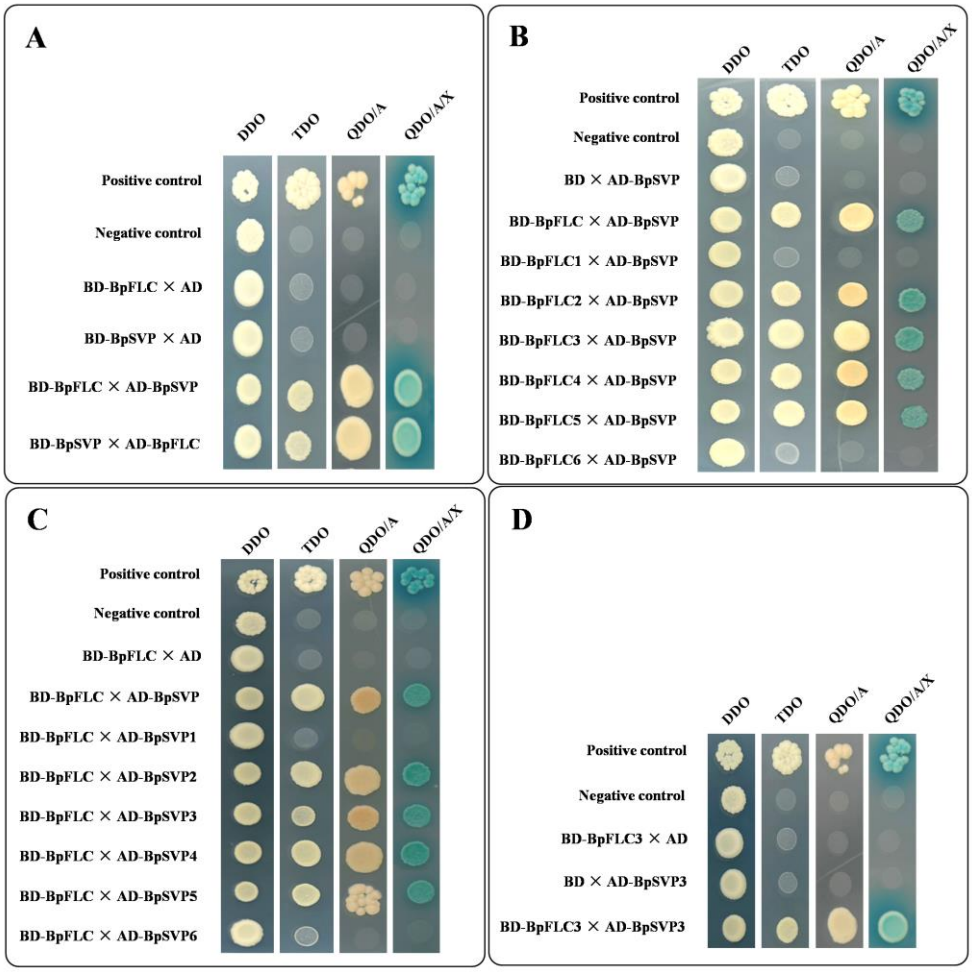

Fig 6. Yeast two-hybrid assays between BpFLC and BpSVP. (A) The BpFLC as a BD fusion was tested for interaction with BpSVP expressed as AD (activation domain) fusions or with the empty AD vector as a control. A strong interaction between BpFLC and BpSVP was detected, with the blue color indicating β-galactosidase activity. Two genes transformed into opposite vectors could also activate expression of these four reporter genes. (B) Analysis of the interactions between BpFLC1~6 truncated forms and BpSVP in yeast. The truncated BpFLC1-6 as BD fusions were respectively tested for interaction with BpSVP expressed as AD fusion and with the full length BpFLC expressed as BD fusion as a positive control. The empty BD vector was tested for interaction with AD-BpSVP as a control. (C) Analysis of the interactions between BpFLC and BpSVP1~6 truncated forms in yeast. The BpFLC as a BD fusion was respectively tested for interaction with truncated BpSVP1-6 expressed as AD fusions and with the full length BpSVP expressed as AD fusion as a positive control. The empty AD vector was tested for interaction with BD-BpFLC as a control. (D) Identification of the interactions between BpFLC3 and BpSVP3 in yeast. The BpFLC3 (K box of BpFLC) as a BD fusion was tested for interaction with BpSVP3 (K box of BpSVP) expressed as a AD fusion or with the relevant empty vector as a control. Positive control: Y2HGold [pGBKT7-*53* × pGADT7-*T*]; Negative control: Y2HGold [pGBKT7-*Lam* × pGADT7-*T*]; BD: Y2HGold [pGBKT7]; BD-BpFLC × AD: Y2HGold [pGBKT7-*BpFLC* × pGADT7]; BD-BpSVP × AD: Y2HGold [pGBKT7-*BpSVP* × pGADT7]; BD-BpFLC × AD-BpSVP: Y2HGold [pGBKT7-*BpFLC* × pGADT7-*BpSVP*]; BD-BpSVP × AD-BpFLC: Y2HGold [pGBKT7-*BpSVP* × pGADT7-*BpFLC*]; BD × AD-BpSVP: Y2HGold [pGBKT7 × pGADT7-*BpSVP*]; BD-BpFLC1-6 × AD-BpSVP: Y2HGold [pGBKT7-*BpFLC1-6* × pGADT7-*BpSVP*]; BD-BpFLC × AD-BpSVP1-6: Y2HGold [pGBKT7-*BpFLC* × pGADT7-*BpSVP1-6*]; BD-BpFLC3 × AD-BpSVP3: Y2HGold [pGBKT7-*BpFLC3* × pGADT7-*BpSVP3*]; DDO: minimal media double dropouts; TDO: minimal media triple dropouts; QTO/A: minimal media quadruple dropouts with adding aureobasidin A; QTO/A/X: minimal media quadruple dropouts with adding aureobasidin A and X-alpha-D-galactopyranoside.

Supplementary Table 1. PCR products were purified using PCR Purification Kit (TaKaRa). The target fragments (*BpFLC*, *BpFLC1~6*, *BpSVP* and *BpSVP1~6*) and vector (pGBKT7 and pGADT7) were digested using restriction enzymes *Nde* I and *Eco*R I. The total digestion profile was containing 1 μL of *Nde* I, 1 μL of *Eco*R I, 2 μL of 10× H buffer and 1 μg of nucleotide, ddH_2O was added to a final volume of 20 μL. The mixture was incubated in a water bath at 37 ℃ for 2.5 h. Subsequently, digestion products were detected by 1 % agarose gel electrophoresis and recovered using Gel Extraction Kit (TaKaRa) for ligation.

The total ligation profile was 10 μL, containing 1 μL of 10 × T_4 DNA ligase buffer, 1 μL of T_4 DNA ligase, 6 μL of target fragment and 2 μL of vector. The mixture was incubated at 16 ℃ overnight, transformed into *E. coli* DH5α competent cells, screened on LB (lysogeny broth) plates containing kanamycin (50 μg mL^{-1}, for pGBKT7 vector) or ampicillin (50 μg mL^{-1}, for pGADT7 vector), and incubated at 37 ℃ for 14~16 h. Single colonies were selected and detected by PCR. Plasmids were extracted for PCR identification and restriction analysis. Finally, positive colonies were identified by sequencing.

Preparation and transformation of yeast Y2HGold competent cells

In accordance with introductions of Gold Yeast Two-Hybrid System (Clontech) and the method modified by our laboratory (An et al., 2011), recombinant plasmids pGBKT7-*BpFLC* and pGBKT7-*BpSVP* were separately transformed into Y2HGold competent cells with PEG/LiAc method and coated on SD/-Trp media. Subsequently, pGBKT7-*BpFLC*/pGADT7-*BpSVP*, pGBKT7-*BpSVP*/pGADT7-*BpFLC*, pGBKT7-*BpFLC*/pGADT7-*BpSVP1~6* and pGBKT7-*BpFLC1~6*/pGADT7-*BpSVP* were co-transformed into Y2HGold competent cells, as the same time with empty vector as a control. After transformation, the mixture was coated on DDO media and incubated at 30 ℃ for 3~4 d to observe the transformation result.

Self-activation and toxicity test of yeast bait vectors

On SD/-Trp media, three single colonies Y2HGold (BD-BpFLC and BD-BpSVP) with the diameter larger than 2 mm was selected randomly, resuspended using 50 μL of sterile water to detect the target genes by PCR amplification. Then transferred to SD/-Trp, DDO, TDO/X and QDO/A/X medium respectively, and incubated at 30 ℃ for 3~5 d. Subsequently, growth and color changes of colonies on these plates were observed to detect whether the bait protein was self-activated. Three single colonies Y2HGold (BD, BD-BpFLC and BD-BpSVP) with the diameter larger than 2 mm were selected and identified by PCR. Then inoculated to 5 mL of SD/-Trp liquid media, incubated at 30 ℃ with shaking at 230~250 r min^{-1} for 12 h, diluted to 1: 100, inoculated to 100 ml of SD/-Trp liquid media, and incubated continuously under the same condition. Subsequently, 3 mL of broth was collected at 0, 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 h respectively, with fresh SD/-Trp liquid media as a blank control. The absorbance was measured at 600 nm using a visible spectrophotometer to record the growth of yeast and analyze the toxic effect of bait protein on yeast cells.

Verification of protein interaction by two-hybrid system

On DDO media, three single colonies with the diameter larger than 2 mm were selected and resuspended using 50 μL of sterile water to detect the target genes by PCR amplification. In addition, 2 μL of resuspended liquid was transferred to DDO, TDO, QDO/A, QDO/A/X media respectively, and incubated at 30 ℃ for 3~5 d to observe growth and color changes of colonies on these plates.

Conclusion

FLC and SVP are two core regulatory proteins of flowering signal integrator. There was no self-activation and no toxic effects in yeast bait vectors of pGBKT7-*BpFLC* and pGADT7-*BpSVP*. The results of yeast two-hybrid showed there was a direct interaction between BpFLC and BpSVP. Further study of truncated genes strongly showed that the K domain of BpFLC and the K domain of BpSVP were the key structure domains and mediated the protein interactions between BpFLC and BpSVP.

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