Cloning of the Self-incompatibility *SFB* Gene from Chinese Apricot ‘Xiaobaixing’ and Construction of the *SFB* Expression Vectors

Hai-nan Liu, Jian-rong Feng¹, Xiao-fang Liu, Wen-hui Li, and Wen-juan Lv

Xinjiang Production and Construction Corps Key Laboratory of Special Fruits and Vegetables Cultivation Physiology and Germplasm Resources Utilization, College of Agriculture, Shihezi University, Shihezi, Xinjiang 832000, China

Ming Luo

Key Laboratory of Biotechnology and Crop Quality Improvement, Ministry of Agriculture, Biotechnology Research Center, Southwest University, Beibei, Chongqing 400715, China

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ABSTRACT. Three kinds of expression vectors of a pollen-S determinant were constructed to provide a reference for molecular breeding of self-compatible (SC) *Prunus* species. An S-haplotype-specific F-box (SFB) protein gene from the ‘Xiaobaixing’ apricot (*Prunus armeniaca*) was cloned by reverse transcription polymerase chain reaction (RT-PCR) and 3′-rapid-amplification of cDNA ends (3′-RACE). A 1136-bp sequence complementary to the 3′-end of the cDNA (GenBank accession number KP938528.2) with a 912-bp complete open reading frame (ORF) was obtained. The deduced amino acid sequence contained an F-box domain, two variable regions, and two hypervariable regions with structural characteristics similar to SFB in other Rosaceae plants. Sense, antisense, and RNA interference (RNAi) vectors for *SFB* were constructed by enzyme restriction. The target fragment was restricted using the corresponding restriction enzyme and then directionally inserted between the 35S cauliflower mosaic virus promoter and the nopaline synthase terminator (NOS-ter) of the expression vector pCAMBIA-35S-MCS-NOS-NPTII. The intron-containing hairpin RNA (ihpRNA) was obtained by fusion PCR. The constructed vectors were transferred into *Agrobacterium tumefaciens* strain LBA4404 by freezing/thawing. The RNAi vector of *SFB* was also transformed in tobacco (*Nicotiana tabacum*). The successful construction of these three expression vectors provides a basis for transforming ‘Xiaobaixing’ apricot and the breeding of SC *Prunus* cultivars.

In contrast to European apricot (*P. armeniaca*), which is generally SC, 90% of native apricot cultivars in China’s Xinjiang Province are self-incompatible (SI). The self-pollinated fruiting rate averages less than 2.0% (He et al., 2007). Therefore, it is necessary to either match cultivars for pollination or perform artificial pollination to obtain stable yield. Self-incompatibility is a major obstacle restricting the breeding and production of native apricot cultivars in Xinjiang Province.

‘Xiaobaixing’ apricot is one of the most famous apricot cultivars in southern Xinjiang Province. ‘Xiaobaixing’ is mainly produced at two locations: Kuqa (lat. 40°46′N to 42°35′N, long. 82°35′E to 84°17′E) and Luntai (lat. 41°05′N to 42°32′N, long. 83°38′E to 85°25′E). The fruit of ‘Xiaobaixing’ is oval, light yellow, and glabrous. ‘Xiaobaixing’

exhibits typical *S-RNase*-based gametophytic self-incompatibility (GSI) controlled by the S-allele. The S-allele contains at least one pollen determinant and one style determinant, which cooperatively determine self-(in)compatibility (Franceschi et al., 2011; Ikeda et al., 2005; Wünsch and Hormaza, 2004). Self-compatibility has become an important objective in *Prunus* breeding programs.

It has been reported that SFB protein is a good candidate for the pollen-S determinants in *Prunus* species (Vaughan et al., 2006). *SFB* encodes an F-box protein that has an F-box domain, two variable regions (Va and Vb), and two hypervariable regions (HVa and HVb) (Vaughan et al., 2006; Wang et al., 2013; Wu et al., 2009). The hypervariable regions play an important role in the GSI reaction (Ikeda et al., 2004; Ushijima et al., 2004). The breakdown of self-incompatibility may be caused by the absence of hypervariable regions in *SFB*, due to a type of pollen-part mutation (Halász et al., 2007). A 4-bp deletion upstream of the HVa region of *SFB*₄′ in an SC sweet cherry (*Prunus avium*) caused a frameshift that produced a transcript of a defective *SFB* lacking HVa and HVb (Ushijima et al., 2004; Zhu et al., 2004). Insertion was another reason for the absence of hypervariable regions. A 6.8-kb insertion in the middle of the *SFB*_f of SC Japanese apricot (*Prunus mume*) resulted in a defective *SFB* lacking HVa and HVb (Ushijima et al., 2004). A 7.1-kb insertion in *SFB*₃′ resulted in a premature stop codon that produced truncated dysfunctional SFB transcripts (Yamane et al., 2009), closely resembling the mode of mutation in *SFB*_f. The *SFB* mutations reported in peach (*Prunus*

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¹Corresponding author. E-mail: fengjr102@126.com.

persica) are similar to those in Japanese apricot (Tao et al., 2007). Sequence analysis of two SC apricot cultivars, Currot ($S_C S_C$) and Canino ($S_2 S_C$), revealed a 358-bp insertion in the *SFB_C* gene, whereas the *S_C-RNase* gene was unaltered (Vilanova et al., 2006). Base substitution of *SFB* also produced a premature stop codon (Marchese et al., 2007). Notably, a putative nonautonomous mutator element in *Prunus* has been reported that exerts a major indirect genome shaping force by inducing a loss-of-function mutation at the SI locus (Halász et al., 2014). The accumulation of a minimum of two nonfunctional S-haplotypes within a single individual was a reason for the breakdown of SI in tetraploid sour cherry [*Prunus cerasus* (Hauck et al., 2006)].

Most *Prunus* species become SC via mutations of *SFB* that result in a truncated protein. This suggests that manipulation of either the hypervariables or their upstream regions can be a theoretically feasible method for converting SI cultivars into SC cultivars. Understanding and using the function of *SFB* in GSI would facilitate artificial control of GSI and efficient breeding of SC *Prunus* cultivars.

Sense and antisense plant expression vectors for a given target gene are molecular and cellular tools that have useful applications in modern breeding and transgenesis (Farnum et al., 2007). Previous studies of fruit trees such as apple [*Pyrus malus* (Dandekar et al., 2004)], strawberry [*Fragaria × ananassa* (Song et al., 2009)], and papaya [*Carica papaya* (López-Gómez et al., 2009)] have primarily focused on genes related to fruit traits. In recent years, RNAi technology has led to greater accuracy and precision in the modification of plant gene expression (Jagtap et al., 2011). RNAi has been used to alter gene expression related to self-incompatibility in *Brassica* (Jung et al., 2012) and *Solanum* (Li and Chetelat, 2014; O'Brien et al., 2002). *SFB* genes have been cloned and identified for multiple apricot cultivars (Li et al., 2014; Romero et al., 2004; Wu et al., 2009), and several quantitative trait loci associated with self-incompatibility in *Prunus* have been identified (Fernández i Martí et al., 2011). These results provide a reference for the molecular breeding of SC *Prunus* species.

In this study, the *SFB* gene of 'Xiaobaixing' was cloned by RT-PCR and 3'-RACE. Restriction enzyme ligation was used to construct the sense and antisense expression vectors of *SFB*. Fusion PCR was used to construct the ihpRNA. We wanted to influence *SFB* expression by genetic transformation, thereby creating a mutant SC apricot. The successful construction of sense, antisense, and RNAi expression vectors for *SFB* will make it possible to transform SI apricot cultivars into SC ones without shuffling untargeted parts of the genome through hybridization.

Materials and Methods

Plant materials

Pollen was collected at the balloon stage of flower development from 'Xiaobaixing' grown at the China National Fruit Tree Germplasm Repository, Chinese Academy of Xinjiang Agricultural Science, Luntai, Xinjiang, China. The pollen was stored at -70°C . Genomic DNA from upland cotton (*Gossypium hirsutum*) was provided by the Biotechnology Research Center at Southwest University, Chongqing, China. Tissue culture seedlings of wild-type tobacco were used to confirm the transformation of the RNAi expression vector.

Extraction of RNA and RT-PCR for SFB

Pollen RNA was extracted using cetyltrimethylammonium bromide. The cDNA was synthesized using Moloney murine

leukemia virus reverse transcriptase (TaKaRa Biotechnology, Dalian, China). The specific PCR protocol for obtaining *SFB* was as follows: 5 min denaturation at 95°C followed by 30 cycles of amplification (each cycle: denaturation, 95°C for 40 s; annealing, 48°C for 45 s; and extension, 72°C for 60 s). The reaction was extended at 72°C for another 10 min to ensure full extension of the PCR products. The 20 μL PCR reactions were composed of 10 μL of 2 $\times$ Taq PCR Master Mix (Tiangen Biochemical Technology, Beijing, China), 2 μL of cDNA, 6 μL of sterile water, and 1 μL of each specific primer (synthesized by Sangon Biotechnology, Shanghai, China) [Saifen F1: 5'-ATGATATTTCAGACTACGTAAGA-3' (52.3°C , 10 $\mu\text{mol}\cdot\text{L}^{-1}$) and Saifen F2: 5'-CTATGGATAAATAAAAG-GACA-3' (48.8°C , 10 $\mu\text{mol}\cdot\text{L}^{-1}$) Li et al. (2014)]. The recombinant products were separated and purified. The purified cDNA fragments were then ligated to a pMD19-T vector (TaKaRa Biotechnology) and transformed into *Escherichia coli* DH5 α . The recombinants were identified using blue-white color selection and screened with primers. Positive clones were sequenced by Sangon Biotechnology. The sequencing results were BLAST searched using the GenBank database (National Center for Biotechnology Information, 2016a) and aligned with DNAMAN software (Lynnon Biosoft, San Ramon, CA). The obtained plasmids were named according to the pMD19-*SFB* scheme.

Cloning of the 3'-end of full-length cDNA

3'-RACE was performed using a 3'-Full RACE Core Set Ver. 2.0 (TaKaRa Biotechnology) following the manufacturer's instructions. Based on the sequencing results for the specific cDNA fragments, two primers (P_{f1}: 5'-TATGGAACCCATCGGTAA-GAAAGT-3' for the outer PCR and P_{f2}: 5'-ATGCCT-TAGTGGTTGAGGTTTAT-3' for the inner PCR) were designed to amplify the 3'-end fragment. Based on the spliced cDNA fragments, the following primers were designed to amplify the 3'-end of the full-length *SFB* gene: P_{g3}: 5'-ATGATATTTCAGACTACGTAAGA-3' and P_{f4}: 5'-TATACATCTGTTTAA-CAATTGAG-3'. The outer and inner PCRs began at 95°C for 5 min, followed by 30 amplification cycles of 95°C for 45 s, 48°C for 45 s, and 72°C for 120 s. Finally, the reaction was extended for another 10 min at 72°C . The 20- μL PCR reactions were composed of 10 μL of 2 $\times$ Taq PCR Master Mix, 2 μL of cDNA, 1 μL of each corresponding primer (10 $\mu\text{mol}\cdot\text{L}^{-1}$), and 6 μL of sterile water. The positive clones were sequenced and analyzed.

Construction of sense and antisense plant expression vectors

The amplified product (generated using the specific primers Saifen F1 and Saifen F2) was analyzed with ORF Finder (National Center for Biotechnology Information, 2016b) and confirmed to be the ORF of *SFB*. The cloning vector pMD19-*SFB* and the intermediate vector pBS-T (3.0 kb, ampicillin resistant) were both restricted with *Pst*I and *Kpn*I (Thermo Fisher Scientific, Beijing, China). Next, the larger fragments of pBS-T and the smaller fragments of pMD19-*SFB* were recycled and purified using a DNA gel extraction kit (Tiangen Biochemical Technology). The two purified fragments were ligated using T4 DNA ligase (TaKaRa Biotechnology) at 4°C for 12 h and then transformed into competent cells of *E. coli* DH5 α . The subsequent experiments were the same as described above. The correct positive recombinant plasmids were identified as the intermediate vector pBS-*SFB*. In comparison, the intermediate cloning vector pBS-*SFB* and the expression vector pCAMBIA [pCAMBIA-35S-MCS-NOS-NPTII,

12.8 kb, kanamycin (Km) resistant] were restricted with *SacI* and *KpnI*. The recombinants were further identified by *XbaI* and *SalI*. The positive recombinant plasmid was named pCAMBIA-S-*SFB* (Fig. 1A).

The cloning vector pMD19-*SFB* and the expression vector pCAMBIA were restricted with *KpnI* and *SalI*. The subsequent experiments were the same as described above. The positive recombinant plasmid was named pCAMBIA-A-*SFB* (Fig. 1B). The antisense expression vector was constructed without the intermediate vector pBS-T.

Construction of an RNAi plant expression vector

PRIMER DESIGN AND CLONING OF TARGET FRAGMENTS. The RNAi target was a 29-bp fragment of *SFB* located upstream of the variable region. The opposite fragments were amplified using the following two primers: 5'-AATCCCTCGTTCGGTTTCTTTGCACATGC-3' (P1) and 5'-CCACAATCCTAAGCAGCAGCAGCATGTGCAAA-GAAACCGAACGAGGGATT-3' (P2). The positive fragments were amplified using the following two primers: 5'-AGCCAAACGAGTTACCCGTCGCATGTGCAAAAGAA-ACCGAACGAGGGATT-3' (P5) and 5'-AATCCCTC-GTTCGGTTTCTTTGCACATGC-3' (P6). The opposite and positive fragments constituted the arm of the hairpin and were reverse complementary. Because no intron fragment was present in the *SFB* sequence, two primers were designed to amplify the exogenous interval fragment: 5'-AATCCCTCGTTCGGTTTCTTTGCACATGCTCGTG-CTGCTTAGGATTGTGG-3' (P3) and 5'-AATCCCT-CGTTTCGGTTTCTTTGCACATGCGACGGGTAA-CTCGTTTGGC-3' (P4). The 242-bp exogenous fragment was the loop of a hairpin from a cotton genomic DNA fragment. The adapter primers of the three fragments (primers P2, 3, 4, and 5) included "initiation" (underlined) and "overlapping" sections.

Fig. 1. Schematic representation of the expression plasmid constructs: (A) pCAMBIA-S-*SFB*, (B) pCAMBIA-A-*SFB*, and (C) pCAMBIA-RNAi-*SFB* [LB = T-DNA left border, RB = T-DNA right border, *SFB* = S-haplotype-specific F-box protein gene, S = positive fragment, A = opposite fragment, Insertion = intermediate fragment, CaMV 35S = cauliflower mosaic virus 35S promoter, NOS-ter = neomycin synthase terminator, NPTII = neomycin phosphotransferase II gene (kanamycin resistance), *GUS* = β -glucuronidase gene. *EcoRV*, *HindIII*, *KpnI*, *SalI*, *XbaI*, *SacI*, and *EcoRI* = unique restriction endonucleases at T-DNA region].

The ihpRNA for *SFB* was constructed by fusing the opposite, intermediate, and positive fragments with two PCR reactions. The protocol for the reactions was one cycle of denaturation at 95 °C for 5 min, 30 cycles of amplification (each cycle: 95 °C for 30 s, 52 °C for 45 s, and 72 °C for 1 min) and one cycle of extension at 72 °C for 10 min. The PCR amplification system was similar to that mentioned earlier. The purified opposite, intermediate, and positive fragments were concentrated for 5 min at 45 °C (447.2 g_n) and then equimolarly subjected to fusion PCR. The reaction procedure was the same as described above. The 20 μ L fusion PCR system was composed of 10 μ L of 2 $\times$ Taq PCR Master Mix and 10 μ L of an equimolar mixture. The recombinant fragments (i.e., the largest fragments) were recombined with the cloning vector pEASY-T1 (TransGen Biotech, Beijing, China) and then transformed into *E. coli* DH5 α . The recombinant plasmid was named pEASY-RNAi-*SFB*.

Construction of the RNAi expression vector

The cloning vector pEASY-RNAi-*SFB* and the expression vector pCAMBIA were both restricted with *XbaI* and *KpnI*. The ihpRNA was inserted into the CaMV35S promoter and the NOS-ter of the expression vector pCAMBIA using T4 DNA ligase. The ligated product was then transformed into *E. coli* DH5 α . The recombinants were screened with primers P1 and P6 and identified with *XbaI* and *SalI*. The positive recombinant plasmid was named pCAMBIA-RNAi-*SFB* (Fig. 1C).

Transforming the expression vectors pCAMBIA-sense-*SFB*, pCAMBIA-antisense-*SFB*, and pCAMBIA-RNAi-*SFB* into *A. tumefaciens*

The expression vectors pCAMBIA-S-*SFB*, pCAMBIA-A-*SFB*, and pCAMBIA-RNAi-*SFB* were introduced into competent cells of *A. tumefaciens* LBA4404 by freezing/thawing. The positive colonies were screened and cultured on solid yeast extract broth (YEB) containing 125 mg·mL⁻¹ streptomycin (Sm) and 50 mg·mL⁻¹ Km. A single colony was chosen and shaken in liquid YEB containing the same antibiotics. Analysis was then conducted by PCR with the corresponding specific primers.

Transformation of the RNAi expression vector

It is difficult to complete homologous verification using expression vectors for the imperfect regeneration system in apricot. We were therefore very interested to construct an RNAi expression vector for the 'Xiaobaixing' *SFB* gene. We chose to infect tobacco, one of the most frequently used model plants in gene function research (Aharoni et al., 2001; De Marchis et al., 2015). The RNAi expression vector for the apricot *SFB* gene was introduced into 1-month-old tobacco seedlings using the *A. tumefaciens*-mediated leaf disk transformation method (Horsch et al., 1985). The tobacco seeds were sterilized in 1.0% sodium hypochlorite and then shake-flask cultured in sterile water for 2 d. The sterile water was changed every 12 h. The seeds were then transferred to germination medium, which consisted of mineral salts basal (MSB) medium, 20 g·L⁻¹ sucrose, and 6.0 g·L⁻¹ ordinary agar at pH 6.5. The MSB medium contains salts and B5 vitamins. The seedling leaves were used for transformation after 1 month.

The *A. tumefaciens* strain LBA4404 containing pCAMBIA-RNAi-*SFB* was grown in YEB medium containing Sm and Km. The cultures were kept on a rotary shaker at 28 °C until they reached an OD₆₀₀ of 0.8. The *A. tumefaciens* cells were harvested by centrifugation and then resuspended in liquid Murashige and Skoog (MS) medium (pH 5.6) and 100 mmol·L⁻¹

acetosyringone. The tobacco leaves were cut into pieces (≈ 0.5 cm²) on a sterile plate, placed in liquid MS, and then shaken on a rotary shaker at 28 °C for 10 min.

After infection, the tobacco leaves were cultured on co-cultivation medium (1× MSB + 0.5 mg·L⁻¹ indole-3-acetic acid (IAA) + 2 mg·L⁻¹ 6-benzylaminopurine (6-BA) + 30 g·L⁻¹ sucrose + 6.0 g·L⁻¹ agar, pH 5.4). A piece of filter paper was placed on the surface of the medium, and then the tobacco leaves were put face down on the filter paper. The leaves were cultured for 2 d in the dark. The leaves were then transferred to selection medium (1× MSB + 0.5 mg·L⁻¹ IAA + 2 mg·L⁻¹ 6-BA + 30 g·L⁻¹ sucrose + 50 mg·L⁻¹ Km + 125 mg·L⁻¹ Sm + 200 mg·L⁻¹ Cef + 6.0 g·L⁻¹ agar, pH 5.8) and cultured for 15 d. The adventitious buds were excised and transferred to rooting medium (1× MSB + 30 g·L⁻¹ sucrose + 50 mg·L⁻¹ Km + 125 mg·L⁻¹ Sm + 200 mg·L⁻¹ Cef + 6.0 g·L⁻¹ agar, pH 5.8). Tobacco seedlings with antibiotic resistance were then selected for transgenic identification.

Histochemical staining of β -glucuronidase

β -glucuronidase (GUS) was assayed as previously described with slight modification (Jefferson et al., 1987). Leaves were detached from the tobacco seedlings in the rooting medium and then transferred into GUS staining buffer [500 mg·L⁻¹ X-Gluc, 0.1 mol·L⁻¹ K₃Fe(CN)₆, 0.1 mol·L⁻¹ K₄Fe(CN)₆, 0.01 mol·L⁻¹ Na₂EDTA, 1.0% Triton X-100 (Solarbio, Shanghai, China) (v/v), and 0.14 mol·L⁻¹ phosphate buffer saline buffer (pH 7.0)].

The leaves were then incubated for 48 h at 37 °C in the dark with continuous gentle shaking. When blue color appeared on the leaves, the leaves were washed in double-distilled water and then placed in 75% ethanol to remove chlorophyll.

Results

THE AMPLIFICATION OF THE 3'-END OF THE *SFB* GENE. A 912-bp fragment of *SFB* was obtained by PCR with Saifen F1 and Saifen F2 (Fig. 2A). The fragment was sequenced, and the result was used to amplify the 3'-end of *SFB*. A 1136-bp sequence with a 912-bp complete ORF was deduced from the sequencing (Fig. 2B). The deduced amino acid sequence of the fragment contained an F-box domain, V1, V2, HVa, and HVb. The structural characteristics were similar to *SFB* in other Rosaceae species. The deduced amino acid sequence had 91% identity with the amino acid sequence of *SFB*₂₆ (EU652887.1) in *P. armeniaca*. Bioinformatics analysis indicated that the F-box region in 'Xiaobaixing' is composed of 40 amino acids [the 9th to the 48th amino acid counted from methionine (Met; coded by the initiation codon)].

CONSTRUCTION AND IDENTIFICATION OF pCAMBIA-S-*SFB* AND pCAMBIA-A-*SFB*. The ORF was recombined with pMD19-T. The cloning vector pMD19-*SFB* was confirmed by sequencing. The restriction fragments (produced by *Pst*I and *Kpn*I) of pBS-T (3.0 kb) and pMD19-*SFB* (912 bp) (Fig. 3A) were used to construct the intermediate vector pBS-*SFB*. The fragments of interest (912 bp) were obtained (Fig. 3B) by restriction with *Xba*I and *Sal*I. The results showed that the ORF had been ligated with the pBS-T fragments. The restriction fragments (produced by *Sac*I and *Kpn*I) were obtained from pCAMBIA (12.8 kb) and pBS-*SFB* (912 bp) (Fig. 3C). PCR identification showed that the restriction fragments had recombined successfully (Fig. 3D). Restriction fragments (by *Xba*I and *Sal*I; 912 bp) containing ORF revealed that the ORF had been successfully inserted into the pCAMBIA vector (Fig. 3E).

The linearized pCAMBIA vector (12.8 kb) and ORF (912 bp) were obtained by restriction with *Kpn*I and *Sal*I (Fig. 4A and B). Two fragments (12.1 kb and 1600 bp) were obtained after restriction with *Kpn*I and *Eco*RI, indicating that the ORF had recombined with the pCAMBIA vector in reverse (Fig. 4C). These results were consistent with PCR identification (Fig. 4D), thereby confirming the successful construction of the antisense expression vector pCAMBIA-A-*SFB*.

Fig. 2. Polymerase chain reaction amplification of *S*-haplotype-specific *F*-box (*SFB*) gene from 'Xiaobaixing' apricot (lane M = DL 2000 DNA marker): (A) specific amplification of *SFB* with Saifen F1 and Saifen F2 (lane 1) and (B) amplification of the 3' end of *SFB* gene with P_{F1} and P_{F2} (lanes 1–3).

Fig. 3. Restriction enzyme and polymerase chain reaction (PCR) identification of pCAMBIA-S-*SFB* (lane M = DL 2000 DNA marker): (A) restriction pMD-*SFB* and pBS-T with *Xba*I and *Kpn*I; restriction pMD-*SFB* with *Xba*I and *Kpn*I (lane 1), restriction pBS-T with *Xba*I and *Kpn*I (lanes 2–3); (B) restriction enzyme identification of pBS-*SFB* by *Xba*I and *Sal*I (lanes 1–2); (C) restriction pCAMBIA and pBS-*SFB* with *Sac*I and *Kpn*I, restriction pCAMBIA with *Sac*I and *Kpn*I (lane 1), restriction pBS-*SFB* with *Sac*I and *Kpn*I (lanes 2–3); (D) PCR identification of pCAMBIA-S-*SFB* with Saifen F1 and F2 (lanes 1–2); and (E) restriction enzyme identification of pCAMBIA-S-*SFB* by *Xba*I and *Sal*I (lanes 1–2). *SFB* = *S*-haplotype-specific *F*-box gene.

Fig. 4. Restriction enzyme and polymerase chain reaction (PCR) identification of pCambia-A-SFB: (A) restriction pCambia with *KpnI* and *SalI* [lane M = Lambda DNA/EcoRI91I (BstEII) marker 15], restriction pCambia with *KpnI* and *SalI* (lanes 1–2); (B) restriction pMD-SFB with *KpnI* and *SalI* [lanes 1–2 (lane M = DL 2000 DNA marker)]; (C) restriction enzyme identification of pCambia-A-SFB by *HindIII* and *EcoRI* [lanes 1–2 (lane M = DL 2000 DNA marker)]; and (D) PCR identification of pCambia-A-SFB with Saifen F1 and F2 [lanes 1–2 (lane M = DL 2000 DNA marker)]. *SFB* = S-haplotype-specific F-box protein gene.

Fig. 5. Construction of pCambia-RNAi-SFB (lane M = DL 2000 DNA marker): (A) polymerase chain reaction (PCR) amplification of the positive fragment with primers P1 and P2 from S-haplotype-specific F-box protein gene (*SFB*) gene (lane 1), the opposite fragment with primers P5 and P6 from *SFB* (lane 2), and the intermediate fragment with primers P3 and P4 from *GhHSD1* (lane 3); (B) fusion PCR products of the opposite, positive, and intermediate fragments (lane 1); (C) restriction enzyme identification of pCambia-RNAi-SFB (lanes 1–3); (D) PCR identification of pCambia-RNAi-SFB (lanes 1–2); and (E) schematic representation of the intron-containing hairpin RNA (ihpRNA) sequence; the whole region represents the ihpRNA sequence, whereas the highlighted fragments indicate the arm regions, the nonhighlighted region indicates the intermediate fragment from *GhHSD1*.

Fig. 6. Polymerase chain reaction (PCR) identification of expression vectors in *Agrobacterium tumefaciens* (lane M = DL 2000 DNA marker): (A) pCambia-S-SFB in *A. tumefaciens* with Saifen F1 and F2 (lanes 1–4); (B) pCambia-A-SFB in *A. tumefaciens* with Saifen F1 and F2 (lanes 1–4); and (C) pCambia-RNAi-SFB in *A. tumefaciens* with F1 and F6 (lanes 1–4). *SFB* = S-haplotype-specific F-box protein gene.

CONSTRUCTION AND IDENTIFICATION OF pCambia-RNAi-SFB. The opposite, positive (29-bp RNAi target fragment contained in 49 bp; the others were complementary interval fragments), and intermediate (242 bp) fragments were cloned (Fig. 5A) with self-designed primers (P1 and P2, P3 and P4, and

P5 and P6, respectively). The opposite, intermediate, and positive fragments were fused together [300 bp (Fig. 5B)] by PCR and then sequenced. The results showed that the fusion was successful (Fig. 5E). Restriction by *XbaI* and *SalI* [912 bp (Fig. 5C)] and PCR identification [912 bp (Fig. 5D)] also demonstrated that the ihpRNA of *SFB* had recombined with pCambia. These results provide a new method by which fusion PCR can be used to construct ihpRNA for genes without a self-intron.

TRANSFORMING THE SENSE, ANTISENSE, AND RNAi EXPRESSION VECTORS OF SFB INTO A. TUMEFACIENS. The transformations of sense and antisense expression vectors were verified by PCR amplification with Saifen F1 and Saifen F2, and a 912-bp ORF was obtained (Fig. 6A and B). Transformations of the RNAi expression vectors were verified by PCR amplification with P1 and P6. The opposite, positive, and intermediate fragments were fused into a 300-bp target fusion fragment (Fig. 6C). PCR identification showed that the vectors pCambia-S-SFB, pCambia-A-SFB, and pCambia-RNAi-SFB had been successfully transformed into *A. tumefaciens*. The Km resistance gene, *Neomycin phosphotransferase II* (*NPTII*), and the GUS reporter gene of pCambia can be used to directly screen and identify transgenic plants.

TRANSFORMATION OF THE RNAi EXPRESSION VECTOR FOR SFB IN TOBACCO. We obtained a number of regenerated tobacco seedlings with resistance to Km and Sm after several generations of subculture.

DETECTING GUS ACTIVITY IN RNAi TRANSGENIC TOBACCO. Blue color was observed in the leaf vein and at the edges of

Fig. 7. Histochemical staining of β -glucuronidase (GUS) activity in transgenic tobacco plantlets harboring an RNAi vector. The RNAi expression vector for the apricot S-haplotype-specific F-box protein gene was introduced into tobacco by the *Agrobacterium tumefaciens*-mediated leaf disk transformation method (CK = leaves from plantlets without infection, + = intact and damaged leaves from plantlets with GUS activity). Blue color indicates that the RNAi expression vector was integrated and expressed in the tobacco genome.

wounds (Fig. 7), indicating the expression of the *GUS* gene. This meant that the RNAi expression vector had been integrated into the tobacco genome and could be expressed. We obtained a total of 16 transgenic tobacco plants.

Discussion

We cloned the 3'-end of cDNA of *SFB* from SI apricot 'Xiaobaixing' pollen using RT-PCR and 3'-RACE. We also constructed sense, antisense, and RNAi plant expression vectors for *SFB* and transferred them into *A. tumefaciens* LBA4404.

Sense and antisense expression vectors are useful tools in genetic engineering. In this study, we used sense and antisense expression vectors to learn more about *SFB*. We constructed sense and antisense expression vectors using the complete ORF of *SFB* from the cloning vector pMD-*SFB*. It is not necessary to design additional primers for cloning sense and antisense fragments. For the specific restriction sites near the CaMV35S promoter (*Hind*III) and NOS-Ter terminator (*Eco*RI) of pCambia, the ORF was directionally inserted into the expression vector without confirming the direction, thereby simplifying the construction processes used in this study. However, the restriction sites in the intermediate vector should be considered carefully to guarantee the direction of the insertion if the frequently used vector pMD19-T is used to construct the sense expression vector (i.e., the pCambia skeleton). We also constructed an RNAi expression vector for comparing the inhibition efficiency of the antisense and RNAi expression vectors for *SFB* in transgenic apricot. The combination of overexpression (via the sense expression vector) and RNAi technology could reveal the function of the target gene and its functional network (Tian et al., 2013; Uri et al., 2009), providing a theoretical basis for the molecular genetic improvement of self-incompatibility and the functional utilization of *SFB*.

Traditional enzyme restriction methods have been used for the construction of RNAi expression vectors for other fruit tree species (Freiman et al., 2012; Jiang et al., 2013; Liu et al., 2010). The efficacy of gene silencing is highest when the RNAi expression vectors contain ihpRNA (Wesley et al., 2001). However, the traditional process for constructing ihpRNA is complicated and time consuming. Fusion PCR permits the construction of ihpRNA via PCR with overlapping primers. ihpRNA is immediately generated by PCR rather than by the construction of an intermediate vector. This

technique is easier than traditional methods, and it imposes no restrictions on the target fragments. Fragments of interest can be fused, and the ihpRNA constructed by fusion PCR can be ligated with any expression vector. Several laboratories have successfully constructed ihpRNA for other crops; however, there are no reports about the use of ihpRNA in fruit tree species. Target genes containing intron sequences (i.e., using DNA as a template) are typically used for construction of ihpRNA; however, we constructed ihpRNA for *SFB* using cDNA without a self-intron. The cotton genomic DNA fragment in this study was the intron sequence of *GhHSD1* (11- β -hydroxysteroid dehydrogenase) from upland cotton. The introduction of an interval region might greatly enhance the stability of inverted repeat sequences in bacteria and could facilitate cloning (Lee and Carthew, 2003). Increasing the molar concentration of the target fragments for fusion and the molar ratio of ihpRNA to the expression vector could accelerate the construction of the RNAi expression vector. The sense, antisense, and RNAi expression vectors will provide a basis for transforming 'Xiaobaixing' and will facilitate the molecular breeding of SC apricot cultivars.

The unusual properties of woody fruit trees complicate their genetic transformation. Transgenic technology requires many mutants and a heavy genetic transformation process. Low transformation efficiency increases the duration of the process. Construction of a complete transformation system for apricot has great significance for characterizing gene function in woody fruit tree species. Our team will continue to explore and improve the regeneration system of apricot.

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