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# Identification of seven haplotype-specific *SFB*s in European pear (*Pyrus communis*) and their use as molecular markers

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ABSTRACT

The European pear (*Pyrus communis*) carries the *S*-RNase-mediated gametophytic self-incompatibility (GSI) system. The *S*-haplotype is conferred by an *S*-locus, which contains the style-specific expressed *S*-RNase, and the pollen-specific expressed F-box genes (*SFB*). Both the *S-RNase* and the *SFB* genes are multi-allelic and each is characteristic of one of the *S*-haplotypes. Therefore, they are ideal markers for molecular *S*-genotyping. In this work, for the first time, seven haplotype-specific *SFB*s were isolated from European pears. Particular primers for each of these *SFB*s were generated, thus providing an additional tool for *S*-genotyping of European pear cultivars.



## 1. Introduction

The European pear (*Pyrus communis*) belongs to the Rosaceae family, which carries the *S*-RNase-mediated gametophytic self-incompatibility (GSI) fertilization system (Sassa et al., 1992). In this system, compatibility among cultivars is determined by the *S*-locus, which harbors an RNase (*S-RNase*) and an F-box gene termed *SLF* (*S*-locus F-box) or *SFB* (*S*-haplotype-specific F-box) (Entani et al., 2003; Ushijima et al., 2003). The *S*-RNase is presumed to be involved in the pistil-maternal part of the system, and the *SLF*/*SFB* in the pollen-paternal part (Kao and Tsukamoto, 2004). The F-box genes are components of the SCF complex that leads to poly-ubiquitination of the selected protein and its degradation by the 26S proteasome (Hershko and Ciechanover, 1998). However, the exact role of *SLF*/*SFB* has yet to be determined (Goldraij et al., 2006; Hua et al., 2007).

Both the *S-RNase* and the *SLF*/*SFB* genes are multi-allelic and are individually characteristic for each haplotype (Lai et al., 2002; Entani et al., 2003; Ushijima et al., 2003; Yamane et al., 2003; Goldway et al., 2007). Thus, each of the alleles can serve as a molecular marker for its respective haplotype. Eighteen European pear *S-RNase* alleles were cloned and sequenced, and more than 130 cultivars were molecularly *S*-genotyped on the basis of their *S-RNase* genes (Zuccherelli et al., 2002; Zisovich et al., 2004; Sanzol et al., 2006; Takasaki et al., 2006; Goldway et al., 2009; Sanzol and Robbins, 2008). The *SLF*/*SFB* gene has been cloned from a few members of the Rosaceae family, including almond (*Prunus dulcis*) from the sub-family Amygdaloideae (Ushijima et al., 2003), apricot (*Prunus mume*) from the sub-family Prunudeae (Entani et al., 2003), and also apple (*Malus domestica*) and Japanese pear (*Pyrus pyrifolia* Nakai) from the Maloideae (Sassa et al., 2007). Here we present, for the first time, seven European pear (*P. communis* L.) haplotype-specific *SFB* alleles, and the PCR-based identification of each of these *SFB* genes.

## 2. Materials and methods

### 2.1. Plant materials

One-month-old pear leaves were collected during the spring of 2007 and were stored at −80 °C pending use.

### 2.2. DNA extraction

DNA was extracted from the leaves according to Doyle and Doyle (1987). Briefly, 700 μl of extraction buffer (2% hexadecyltrimethylammonium bromide [CTAB], 100 mM of Tris pH 8, 20 mM of ethylenediamine tetraacetic acid [EDTA] pH 8, 1.4 M of NaCl, 1% polyvinylpyrrolidone [PVP] MW 40,000, and 1% β-mercaptoethanol) were added to 100–200 mg of leaves, which had been powdered under liquid nitrogen with a pestle and mortar. This mixture was incubated for 30 min at 65 °C, with occasional mixing. After it had cooled to room temperature, two extractions were performed with chloroform:octanol (24:1). The DNA was pelleted with ethanol and dissolved in DDW. The DNA extract was kept at −20 °C pending use.

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### 2.3. PCR amplification

The universal primers used for the PCR amplification of the *SFB* alleles were FjbFB1 (CCAAGTCTCTGATGMGRTTCAAATG) and RjbFB1 (SRGTTAGKWGTTTTGTCCATGAAC), as described by Sassa et al. (2007). FjbFB1 is from the F-box motif, about 60 nucleotides into the open reading frame (ORF), and RjbFB1 is from the hyper-variable region A (Hva), about 200 nucleotides prior to the end of the ORF. The PCR was used to extract DNA from 13 European pear cultivars (the *S*-genotype is presented according to the new numeration, Goldway et al., 2009): 'Spadona' (*S101 S103*), 'Coscia' (*S103 S104*), 'Gentile' (*S101 S106*), 'Spadonchina' (*S102 S103*), 'Dagan' (*S101 S104*), 'Etrusca' (*S103 S?*), 'Red Clapp' (*S101 S108*), 'Cascade' (*S101 S104*), 'Lawson' (*S115 S117*), 'Delbard première' (*S101 S109*), 'Forelle' (*S101 S116*), 'Rosemarie' (*S101 S116*) and 'Red Druz' (*S103 S107*). From each cultivar between 4 and 20 PCR products were cloned. Each of the clones was sequenced and aligned with the gene bank in the NCBI.

For each specific European pear *SFB* allele, a unique pair of primers was designed, based on the genomic DNA sequences; they are listed in Table 1.

The PCR tube contained about 20 ng of DNA, 5 μl of 10× Ex Taq PCR buffer (Takara Bio, Shiga Japan), 0.2 mM dNTP mixture, 20 pmol of each primer and 0.25 μl of TaKaRa Ex Taq (Takara Bio. Inc., Japan) at 5 units/μl, in 50 μl of reaction mixture.

Amplification was performed in a MyCycler PCR apparatus (Bio Rad, Hercules, CA, USA). The PCR conditions were: an initial cycle of 2 min at 94 °C, 10 cycles of 15 s at 94 °C, 30 s at the annealing temperature, which depended on the primer pair (Table 1) and 2 min at 70 °C; then 20 cycles of 15 s at 94 °C, 30 s at the appropriate annealing temperature (Table 1) and 2.5 min at 70 °C; with a final step of 7 min at 72 °C.

### 2.4. Cloning and DNA sequencing of PCR products

PCR products were cloned into pGEM-T plasmid (Promega, Madison, WI, USA). Each clone was sequenced in both directions at least twice, on an ABI PRISM 377 DNA sequencer (PE Bio Systems, USA).

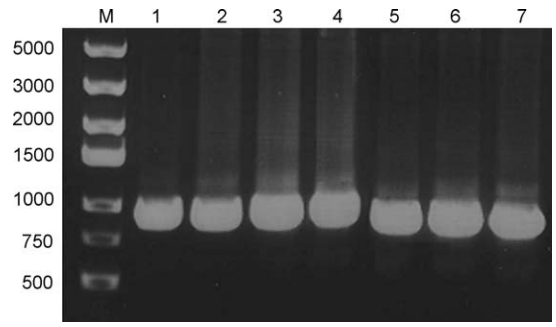


**Fig. 1.** *SFB* PCR products from a reaction performed with universal primers 'FjbFB1' and 'RjbFB1' and with DNA extracted from European pear cultivars (1% agarose gel, stained with ethidium bromide). M-size marker, GeneRuler™ Express DNA Ladder (Fermentas). Lane: (1) Spadona; (2) Coscia; (3) Gentile; (4) Spadochina; (5) Red Clapp; (6) Cascade; (7) Lawson.

### 2.5. DNA sequence analysis

The DNA sequence data were analyzed and the protein sequence was estimated with the BLAST of NCBI Clustal W software, at the EMBL BoxShade server at http://www.ebi.ac.uk/clustalw/index.html, and with the DNASTAR-Lasergene 6-SeqMan II.

## 3. Results

### 3.1. Cloning of SFBs from European pear cultivars

The PCR for amplifying European pear *SFB* alleles was performed with FjbFB1 as forward primers and RjbFB1 as reverse primer; these had first been generated by Sassa et al. (2007) for cloning apple and Japanese pear *SFB* alleles. The PCR was used to extract DNA from 13 European pear cultivars.

A single band of about 900 bp was obtained from all of the cultivars (Fig. 1). We suspected that this band contained a sequence of more than one *SFB* gene, because the cultivars are heterozygous at their *S*-locus and, moreover, the PCR primers that were applied amplified three *SFB* alleles from each *S*-locus in Japanese pear (Sassa et al., 2007).

**Table 1**
Primer pairs and the PCR conditions.

| Allele | Primers | | Annealing temp. (°C) | Comments |
|---|---|---|---|---|
| | F-sense (forward), R-anti-sense (reverse) | Sequence | | |
| *SFB* general | FjbFB1<br>RjbFB1 | CCAAGTCTCTGATGMGRTTCAAATG<br>SRGTTAGKWGTTTTGTCCATGAAC | 62 | Sassa, 2007 |
| *SFB101* | F101 fbox<br>R101 fbox | CAGGGAAAGGTACTT<br>GCAACTGTGGAGTAT | 45 | 25 cycles in stage B |
| *SFB103* | F103 fbox<br>R103 fbox | ATGCACGTTTTCCCGGACCAG<br>TCCCTTCAAGTATTGTGAACC | 62 | 25 cycles in stage B |
| *SFB104* | F104 fbox<br>R104 fbox | CAGCCGTTCTCAGATGCCGG<br>ACAGAGATTGTCTCCAATTG | 57 | 25 cycles at stage B |
| *SFB106* | F106 fbox<br>R106 fbox | GTAGGGATGACCATAAGCCCTT<br>CTCAAGTACACAGAAAAAGGATA | 60 | |
| *SFB107* | F107 fbox<br>R107 fbox | CCTCACATTTTCCCGGACCAGAA<br>AAGTAAACCGAACAAGACCAG | 60 | |
| *SFB108* | F108 fbox<br>R108 fbox | GCGAACATTTTCCCAGACCAGAG<br>AAGTACACTGAACAAGAACAT | 58–60 | Stage A—10 cycles with annealing at 60 °C and at stage B annealing at 58 °C |
| *SFB115* | F115 fbox<br>R115 fbox | CTCGTGTTTTCCCAGACAAT<br>CCGAAGACCGTTTCCAATTGG | 62 | |