

S genotyping and *S* screening utilizing *SFB* gene polymorphism in Japanese plum and sweet cherry by dot-blot analysis

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Abstract Most Rosaceae fruit trees such as Japanese plum and sweet cherry have a gametophytic self-incompatibility (GSI) system controlled by a single *S* locus containing at least two linked genes with multiple alleles, i.e., *S-RNase* as a pistil determinant and *SFB* (*S*-haplotype-specific *F*-box gene) as a candidate for the pollen *S* determinant. For identification of *S* genotypes, many methods based on polymerase chain reaction (PCR) utilizing polymorphism in length of the *S-RNase* and *SFB* gene have been developed. In this study, we developed two dot-blot analysis methods for *S*-haplotype identification utilizing allele-specific oligonucleotides based on the *SFB*-HV_a region, which has high sequence polymorphism. Dot-blotting of allele-specific oligonucleotides hybridized with digoxigenin-labeled PCR products allowed *S* genotyping of plants with nine *S* haplotypes (*S-a*, *S-b*, *S-c*, *S-e*, *S-f*, *S-h*, *S-k*, *S-7* and *S-10*) in

Japanese plum and ten *S* haplotypes (*S-1*, *S-2*, *S-3*, *S-4*, *S-4'*, *S-5*, *S-6*, *S-7*, *S-9* and *S-16*) in sweet cherry (dot-blot-*S*-genotyping). In addition, dot-blotting of PCR products of *SFB* probed with the allele-specific oligonucleotides, occasionally utilizing competitive hybridization, was successful in screening for a desirable *S* haplotype in sweet cherry (dot-blot-*S*-screening).

Keywords Allele-specific oligonucleotide · Dot-blot analysis · Japanese plum · *SFB* · Sweet cherry

Abbreviations

GSI gametophytic self-incompatibility
SFB *S*-haplotype-specific F-box protein
PCR polymerase chain reaction
RFLP restriction fragment length polymorphism
HV_a hypervariable region a

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Introduction

Rosaceae fruits trees such as *Prunus*, *Malus*, and *Pyrus* are commercially important crops cultivated worldwide. Most of Rosaceae plants have a gametophytic self-incompatibility system (GSI) controlled by a single *S* locus with multiple alleles. Since the GSI inhibits self-fertilization resulting in a decrease of fruit set and the bearing of parthenocarpic fruits of

low quality, pollination with optimum compatible pollen is essential. In commercial orchards, cross-compatible cultivars that flower simultaneously and harbor different *S* haplotypes are planted together to ensure cross-pollination. Therefore, utilization of information on *S* genotypes of cultivars is essential for fruit set and fruit quality. In breeding, new cultivars with self-compatibility have been preferred for labor efficiency. For example, self-compatible sweet cherries harboring the *S* haplotype mutated artificially by X-ray irradiation (Lewis 1949) have been developed. Since early screening of the mutated *S* haplotype enables efficient breeding of self-compatible cultivars, a reliable method by which the mutated *S* haplotype from the wild type *S* haplotype can be distinguished is necessary.

In Japanese pear, the pistil determinant of SI reaction has been identified as *S*-RNase, which is a basic glycoprotein with ribonuclease activity (Sassa et al. 1992). Similar subsequent studies have used many Rosaceae species such as almond (Tao et al. 1997; Ushijima et al. 1998), apple (Sassa et al. 1994), European pear (Zuccherelli et al. 2002), Japanese apricot (Yaegaki et al. 2001), Japanese plum (Yamane et al. 1999) and sweet cherry (Tao et al. 1999). Recently, a candidate for the pollen *S*-determinant has been characterized and designated as *SFB* (*S*-haplotype-specific *F*-box gene) in almond (Ushijima et al. 2003), sweet and sour cherry (Yamane et al. 2003a), Japanese plum (Zhang et al. 2007), apricot (Romero et al. 2004) or as *SLF* (*S*-locus *F*-box) in Japanese apricot (Entani et al. 2003). The term '*SFB*' for the pollen *S*-determinant is hereafter used in this report.

S genotypes of cultivars in Rosaceae fruit trees are commonly determined by investigating seed/fruit set or pollen tube growth after pollination. However, since the availability of flower materials is necessary for these tests, a marker-assisted selection system, which is independent of flowering age and season, is required. The recent identification of the *S*-RNase gene and the *SFB* gene have enabled characterization of the *S* haplotypes in Rosaceae fruit trees by RFLP- or PCR-based analysis using vegetative tissue as material. In sweet cherry, *S* genotyping was initially begun with zymogram analysis of stylar RNase (Bošković et al. 1997), but such analysis has been replaced by the PCR-based method using consensus primers which can detect polymorphisms of first or

second intron length of the *S*-RNase gene (Tao et al. 1999; Yamane et al. 2000). Allele-specific primers developed by Sonneveld et al. (2001, 2003) together with consensus primers have enabled the identification of *S*-1, *S*-2, *S*-3, *S*-4, *S*-5, *S*-6, *S*-7, *S*-9, *S*-10, *S*-12, *S*-13, *S*-14 and *S*-16 haplotypes in sweet cherry. In addition to these *S* haplotypes, techniques for discrimination of six (*S*-17 to *S*-22) and three (*S*-23 to *S*-25) additional *S* haplotypes, which were characterized by De Cuyper et al. (2005) and by Wünsch and Hormaza (2004), respectively, have been developed (Sonneveld et al. 2006; De Cuyper et al. 2005). Likewise, *S* genotyping has been developed for use in Japanese plum (Beppu et al. 2002, 2003; Sapir et al. 2004; Halász et al. 2007), Japanese apricot (Tao et al. 2002), sour cherry (Yamane et al. 2001), almond (Tamura et al. 2000; Ortega et al. 2005), apple (Janssens et al. 1995), Japanese pear (Ishimizu et al. 1998) and European pear (Moriya et al. 2007), resulting in a rapid and convenient technique.

On the other hand, alternative *S*-genotyping methods based on *SFB* have been developed by RFLP in sweet cherry (Yamane et al. 2003a; Ikeda et al. 2004a) and Japanese apricot (Yamane et al. 2003b). A technique based on PCR using allele-specific primers (*S*-1 to *S*-6) in sweet cherry (Ikeda et al. 2005) and one employing fragmentation analysis with consensus primers using a semi-automated sequencer (Vaughan et al. 2006) have also been developed. Ushijima et al. (2004) and Sonneveld et al. (2005) have revealed a 4-bp deletion in the region upstream of HVa of *PaSFB4* in an *S*-4 mutated haplotype, denoted *S*-4' herein, which is responsible for the loss of pollen activity for self-incompatibility. This finding led to the development of a technique for discrimination between *S*-4 and *S*-4' using a designed dCAPS primer (Ikeda et al. 2004b)

The consensus primers for *S*-RNase and *SFB* are not satisfactory for discrimination in certain alleles because of the similar size of PCR products and weak secondary bands on the gel (Sonneveld et al. 2003; Vaughan et al. 2006). Although analyses with a semi-automated sequencer for precise size fragmentation have been recently reported to be available (Ortega et al. 2005; Sonneveld et al. 2006; Vaughan et al. 2006), such techniques would not be practical and convenient for researchers or breeders. In allele-specific PCR, individual PCR has to be carried out for each *S* haplotype, and internal control might be

required for PCR to avoid the problem of false negatives as reported by Sonneveld et al. (2003). PCR-RFLP (CAPS) marker can reduce the possibility of the false negative. To our knowledge, however, a PCR-RFLP marker for *S* genotyping has been developed only in *S-1* and *S-3* to *S-6* haplotypes of sweet cherry (Yamane et al. 2000), but not in Japanese plum.

More recently, Fujimoto and Nishio (2003) have developed two methods for identification of *S* haplotypes by dot-blot analysis using *SP11* alleles in *Brassica oleracea*; one is blotting of plant genomic DNA probed with labeled *SP11*^m (mature protein region of *SP11* cDNA), and the other is dot blotting of *SP11*^m DNA fragments amplified from each allele-cDNA hybridized with the *SP11* coding region labeled by PCR using a template of plant genomic DNA. In the present study, rather than analyzing PCR products and genomic DNA blotted on the membrane, we applied dot-blot analysis to *S* genotyping in Japanese plum and sweet cherry utilizing allele-specific oligonucleotides based on the *SFB*-HVa region, which has high sequence polymorphism. As a result, our improved method allowed us to discriminate among nine *S* haplotypes (*S-a*, *S-b*, *S-c*, *S-e*, *S-f*, *S-h*, *S-k*, *S-7* and *S-10*) in Japanese plum and ten *S* haplotypes (*S-1*, *S-2*, *S-3*, *S-4*, *S-4'*, *S-5*, *S-6*, *S-7*, *S-9* and *S-16*) in sweet cherry (dot-blot-*S*-genotyping). In addition, we examined a labeled oligonucleotide as a probe for screening of a desirable *S* haplotype (dot-blot-*S*-screening) according to Shirasawa et al. (2005, 2006).

Materials and methods

Plant materials

Thirteen Japanese plum cultivars, which were managed at Nanjing Agricultural University, and thirteen sweet cherry cultivars, which were provided by Sato farm at Yamanobe-machi in Yamagata Prefecture and by the National Institute of Fruit Tree Science (Morioka, Japan), were used (Table 1).

Design of allele-specific oligonucleotides

As shown in Table 2, nine and ten allele-specific oligonucleotides for Japanese plum and sweet cherry

Table 1 Cultivars used in this study and their *S* genotypes

	Cultivar	<i>S</i> genotype
Japanese plum	Akihime	<i>S-b/S-h</i>
	Dazhili	<i>S-10/UI</i>
	Heibaoshi	<i>S-b/S-h</i>
	Huangjiazuanshi	<i>S-e/S-h</i>
	Huangpili	<i>S-f/S-7</i>
	Kaiqinman	<i>S-e/S-h</i>
	Meiguili	<i>S-c/S-e</i>
	Nvgelei	<i>S-c/S-h</i>
	Oishiwasesumomo	<i>S-c/S-d</i>
	Ozarkpremier	<i>S-a/S-f</i>
	Redgold	<i>S-h/S-k</i>
	Weikeshun	<i>S-f/S-k</i>
	Younai	<i>S-f/S-h</i>
	Sweet cherry	Benisayaka
Benishuho		<i>S-4/S-6</i>
Bigarreau de Jaboley		<i>S-6/S-9</i>
Compact Stella		<i>S-3/S-4'</i>
Early Purple		<i>S-2/S-7</i>
Napoleon		<i>S-3/S-4</i>
Rodmersham Seedling		<i>S-3/S-16</i>
Sam		<i>S-2/S-4</i>
Satonishiki		<i>S-3/S-6</i>
Seneca		<i>S-1/S-5</i>
Taishonishiki		<i>S-1/S-4</i>
Van		<i>S-1/S-3</i>
Vega		<i>S-2/S-3</i>

UI, unidentified

SFB alleles, respectively, were designed in the region covering hypervariable region a (HVa; Ikeda et al. 2004a). T_m values of all oligonucleotides were set at 70°C. In addition, PaSFBoli-4, -4' and -6 (Table 2) were used as digoxigenin-labeled probes for dot-blot-*S*-screening experiments. For competitive hybridization experiments, digoxigenin-labeled and non-labeled oligonucleotides of PaSFBoli-4new and PaSFBoli-4' new were designed (Table 2).

Dot-blot analysis with digoxigenin-labeled DNA: dot-blot-*S*-genotyping

The designed allele-specific oligonucleotide of 5 μM was dot-blotted onto a nylon membrane (Nytran,

Table 2 Designed oligonucleotide sequences

	Name	Oligonucleotide sequence (5'-3')	S haplotype	Note
Japanese plum	PsSFBoli-a	CCATTGGATGAATGGAGTGGTGC	<i>S-a</i>	dotted on membrane
	PsSFBoli-b	TTTCCTTTGGATGATTACTATTCTACA	<i>S-b</i>	dotted on membrane
	PsSFBoli-c	TATCCTCGGAGCTGTTATCGTATAA	<i>S-c</i>	dotted on membrane
	PsSFBoli-e	TTCGGATTATTATTATCGTACAATCG	<i>S-e</i>	dotted on membrane
	PsSFBoli-f	TTTATCCTTTTGATGGTTATGATAACAAT	<i>S-f</i>	dotted on membrane
	PsSFBoli-h	TTTGGATTATTATTATTATCGTACAATAG	<i>S-h</i>	dotted on membrane
	PsSFBoli-k	CCTGGGGATTATTGTTATAATGTGG	<i>S-k</i>	dotted on membrane
	PsSFBoli-7	TCCTTTGGATGAATGGAATGTTACAA	<i>S-7</i>	dotted on membrane
	PsSFBoli-10	CTTTGGATCGTTGTACACCGAACAA	<i>S-10</i>	dotted on membrane
	Sweet cherry	PaSFBoli-1	TATTTATCTTCAGATTATTGTTATCGTA	<i>S-1</i>
PaSFBoli-2		TTTATGATCCTTTGGATTATTGTCATC	<i>S-2</i>	dotted on membrane
PaSFBoli-3		TTTTTCCTTTGAATCATTGTCATCGTA	<i>S-3</i>	dotted on membrane
PaSFBoli-4		TTTTATTATCCTTCTCATTATTATGGTA	<i>S-4</i>	dotted on membrane, digoxigenin- or non-labeled probe
PaSFBoli-4'		GTGTCCTTTTATCCTTCTCATTATTAT	<i>S-4'</i>	dotted on membrane, digoxigenin- or non-labeled probe
PaSFBoli-5		TTTTCTCCTTTGGGTGATTGTGAT	<i>S-5</i>	dotted on membrane
PaSFBoli-6		TATTTATCCTGCTGGTAGTTATGGTA	<i>S-6</i>	dotted on membrane, digoxigenin- or non-labeled probe
PaSFBoli-7		TTTCCTTTTCGGTTATTGTGATCTTTTA	<i>S-7</i>	dotted on membrane
PaSFBoli-9		ATCCTTTGGGTATGATTATCGTCC	<i>S-9</i>	dotted on membrane
PaSFBoli-16		TATCCATCCTTTTGATTGTGATGGTA	<i>S-16</i>	dotted on membrane
PaSFBoli-4new	TGTCCTTTTATTTATCCTTCT	<i>S-4</i>	dotted on membrane, digoxigenin- or non-labeled probe	
PaSFBoli-4'new	TGTCCTTTTATCCTTCT	<i>S-4'</i>	dotted on membrane, digoxigenin- or non-labeled probe	

Whatman, Germany) by Multi-pin Blotter (Atto, Tokyo, Japan). The membrane was exposed to UV-light (0.12 J) for cross-linking of the oligonucleotides.

Genomic DNA was extracted from leaves (0.1 g) according to the method described by Doyle and Doyle (1987) with an extraction solution containing 2% 2-mercaptoethanol. The DNA was quantified by a spectrophotometer (Qubit Fluorometer, Invitrogen, USA). In order to amplify digoxigenin-labeled DNA,

two sets of degenerated consensus primers for a fragment of ca. 350 bp covering the HVa region of Japanese plum *SFB* and of sweet cherry *SFB* were designed (Table 3). Digoxigenin labeling of the ca. 350-bp fragments by PCR was performed in a 25- μ l reaction mixture containing 10 ng of genomic DNA, 10 pmol of each primer, 1 \times ExTaq buffer, 2.5 μ l of PCR DIG labeling mix (Roche Diagnostics, Switzerland) and 1.25 U of ExTaq DNA polymerase (TaKaRa Biomedicals, Japan). The thermal cycling

Table 3 Designed degenerated consensus primer

	Name	Oligonucleotide sequence (5'-3')	Note
Japanese plum	PsSFB-CR	AGAATTYATAGYACCAGATGCMATT	used as a forward primer
	PsSFB-R1	CAMRAATTCGATTTTCGYCATATTTTC	used as a reverse primer
Sweet cherry	PaSFB-F3	AAGARTTCATARCACCRGATG	used as a forward primer
	PaSFB-R1	ASRMKAATTCSATTTTCVYCATAT	used as a reverse primer

condition was 95°C for 30 s, 55°C for 30 s and 72°C for 3 min (45 cycles). The labeled DNA dissolved in $5 \times \text{SSC}$, 0.5% blocking reagent (Roche Diagnostics), 0.1% sodium *N*-lauroylsarcosine and 0.02% SDS was hybridized with the dot-blotted membrane at 40°C. After hybridization, the membrane was washed twice in $2 \times \text{SSC}$, 0.1% SDS at room temperature for 5 min and then twice in $1 \times \text{SSC}$, 0.1% SDS buffer at 40, 42, 45°C or 50°C for 20 min. Signals were detected using the DIG DNA Detection Kit (Roche Diagnostics) according to the manufacturer's instructions.

Dot-blot analysis with digoxigenin-labeled oligonucleotide probes: dot-blot-*S*-screening

Amplification of ca. 350-bp fragments by PCR with the same degenerated consensus primers as those described in the former section was performed in a 25- μl reaction mixture containing 10 ng of genomic DNA, 10 pmol of each primer, $1 \times \text{ExTaq}$ buffer, 200 μM dNTPs and 1.25 U *ExTaq* DNA polymerase. The thermal cycling condition was 95°C for 30 s, 55°C for 30 s and 72°C for 1 min (35 cycles). The amplified DNA was denatured in a solution of 0.4 N NaOH and 10 mM EDTA, and then dot-blotted onto a nylon membrane by Multi-pin Blotter (Atto, Japan). The digoxigenin-labeled oligonucleotide probe diluted in the same solution described in the above section was hybridized with the membrane at 60°C. The membrane was washed twice in $2 \times \text{SSC}$, 0.1% SDS at room temperature for 5 min and then twice in $0.1 \times \text{SSC}$, 0.1% SDS buffer at 60°C for 20 min. For competitive hybridization to discriminate between *SFB-4* and *SFB-4'* alleles, hybridization was carried out with a set of probe mixtures (PaSFB4-mix probe) containing 0.02 μM of the digoxigenin-labeled oligonucleotide for the *SFB-4* allele (PaSFBoli-4; Table 2) and 0.2 μM of the unlabeled counterpart oligonucleotide for the *SFB-4'* allele (PaSFBoli-4') at 60°C, or with another set of probe mixtures (PaSFB4'-mix probe) containing 0.02 μM of the digoxigenin-labeled oligonucleotide for the *SFB-4'* allele (PaSFBoli-4'new; Table 2) and 0.1 μM of the unlabeled counterpart oligonucleotide for the *SFB-4* allele (PaSFBoli-4new) at 40°C. Washing was carried out with $0.1 \times \text{SSC}$, 0.1% SDS at 60°C for the PaSFB4-mix probe or with $1 \times \text{SSC}$, 0.1% SDS at

40°C for the PaSFB4'-mix probe. Signals were detected as previously described.

Results

S-genotyping with dot-blot analysis (dot-blot-*S*-genotyping) in Japanese plum

In Japanese plum, nineteen *S*-RNase alleles have been sequenced (Beppu et al. 2002, 2003; Sapir et al. 2004), while sequences of nine *SFB* alleles (*SFB-a*, *-b*, *-c*, *-e*, *-f*, *-h*, *-7* and *-10*; Zhang et al. 2007, *SFB-k*; accession number EF490571) have been published to date. Since comparison of the HVa region of *SFB* indicated high sequence polymorphism among alleles (53–87% nucleotide identity), we designed allele-specific oligonucleotides for dot-blotting on the membrane by comparing the sequences of HVa and its adjacent regions. Using the cloned genes for *SFB-b* and *-h* alleles as templates, each digoxigenin-labeled DNA for *SFB-b* and *-h* was synthesized by PCR with consensus primers, resulting in amplification of ca. 350-bp products including the HVa region. Allele-specific signals for the *SFB-b* and *SFB-h* alleles were detected by hybridization of the allele-specific oligonucleotides blotted on the membrane with the digoxigenin-labeled DNAs (Fig. 1A). In addition, DNAs synthesized directly from genomic DNA of 'Heibaoshi' (*S-b/S-h*; Zhang et al. 2007) by PCR also detected the allele-specific signals for *SFB-b* and *-h* (Fig. 1B) consistent with the *S*-genotype of 'Heibaoshi'.

In the twelve other cultivars (Table 1), hybridization was carried out with each labeled DNA synthesized by PCR using each genomic DNA as a template. In 'Meiguili', 'Nvgelei', 'Redgold', 'Weikeshun', 'Huangjiazuanshi', 'Kaiqinman', 'Younai', 'Huangpili', 'Akihime' and 'Ozarkpremier', allele-specific signals consistent with their respective *S* genotypes were detected (Fig. 1C). Since the *SFB-d* gene has not yet been reported, only a signal for *SFB-c* was detected in 'Oishiwasesumomo' (*S-c/S-d*). Similarly, the DNA synthesized from 'Dazhili', in which one haplotype is *S-10* and the other is unidentified, hybridized only with the oligonucleotide for *SFB-10*. These experiments suggest this dot-blotting method using sequence polymorphism of the *SFB* gene is useful for identification of all the nine *S* haplotypes used here.

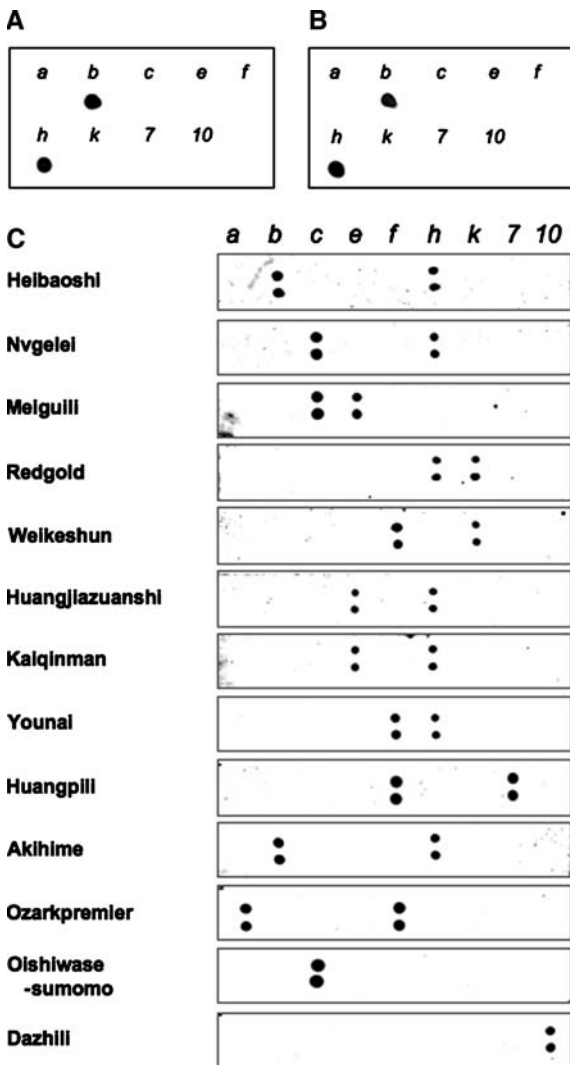


Fig. 1 Dot-blot analysis with *SFB*-allele-specific oligonucleotides in Japanese plum. Hybridization with digoxigenin-labeled DNAs synthesized from cloned *SFB-b* and *SFB-h* alleles by PCR (a), and digoxigenin-labeled DNAs synthesized from ‘Heibaoshi’ genomic DNA (b). Digoxigenin-labeled DNAs synthesized directly from genomic DNAs of 13 cultivars (c). a, b, c, e, f, h, k, 7 and 10 indicate allele-specific oligonucleotides designed from *PsSFB-a*, *-b*, *-c*, *-e*, *-f*, *-h*, *-k*, *-7* and *-10*, respectively. Two dots were blotted for each allele in (c)

Dot-blot-S-genotyping in sweet cherry

In sweet cherry, twenty-two *S* haplotypes have been identified in cultivated sweet cherry and wild cherry populations (Bošković and Tobutt 2001; Crane and Brown 1937; De Cuyper et al. 2005; Sonneveld et al. 2001, 2003), and *S*-RNase sequences of all these *S*

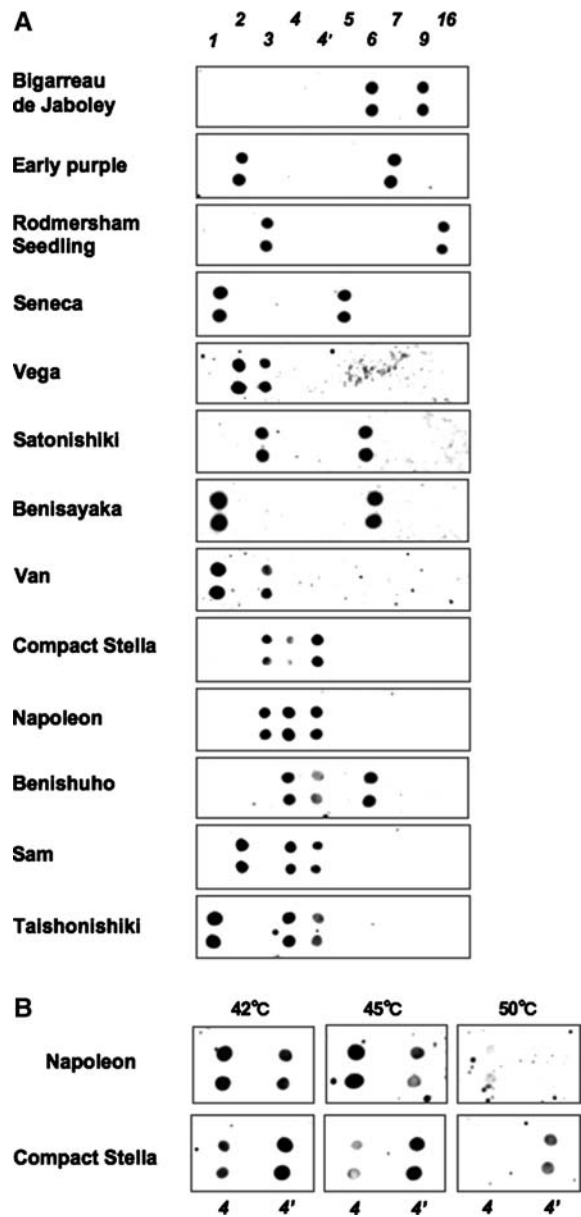


Fig. 2 Dot-blot analysis with *SFB*-allele-specific oligonucleotides in sweet cherry. (a) Hybridization with digoxigenin-labeled DNAs synthesized from genomic DNAs of 13 cultivars by PCR. (b) Discrimination between *S-4* and *S-4'* haplotypes under different temperature conditions. 1, 2, 3, 4, 4', 5, 6, 7, 9 and 16 indicate allele specific oligonucleotides designed from *PsSFB-1*, *-2*, *-3*, *-4*, *-4'*, *-5*, *-6*, *-7*, *-9* and *-16*, respectively. Two dots were blotted for each allele

haplotypes have been reported (Wünsch and Hormaza 2004; Sonneveld et al. 2001, 2003; De Cuyper et al. 2005). On the other hand, thirteen *SFB* alleles (*SFB-1*, *-2*, *-3*, *-4*, *-4'*, *-5*, *-6*, *-7*, *-9*, *-10*, *-12*, *-13* and *-16*)

among those *S* haplotypes have been characterized (Vaughan et al. 2006). Thirteen cultivars harboring ten alleles other than *SFB-10*, *-12* and *-13* were obtained as materials for this investigation (Table 1). Although the nucleotide identity of the HVa region between *SFB-4* and *-4'* is high (95%), because of only a 4-bp deletion in *SFB-4'*, comparison between the HVa sequences of the remaining alleles indicated high sequence polymorphism (55%–80% nucleotide identity). Therefore, we designed allele-specific oligonucleotides for dot-blotting on the membrane by comparing the sequences of HVa and those of adjacent regions as well as in Japanese plum (Table 2). Hybridization was carried out with each labeled DNA synthesized by PCR using consensus primers (Table 2) under the same condition as in Japanese plum. Labeled DNA synthesized from ‘Bigarreau de Jaboley’, ‘Early purple’, ‘Rodmersham seedling’, ‘Seneca’, ‘Vega’, ‘Satonishiki’, ‘Benisayaka’ and ‘Van’ detected allele-specific signals consistent with their respective *S* genotypes (Fig. 2A).

Because of the likelihood of identical sequence other than the 4-bp deletion causing cross-hybridization, signals for both *SFB-4* and *-4'* were detected in ‘Compact stella’, ‘Napoleon’, ‘Benishuho’, ‘Sam’ and ‘Taishonishiki’, together with the signals for the other *SFB* alleles. For distinct discrimination between *SFB-4* and *SFB-4'*, the temperature condition in hybridization and washing was examined using ‘Napoleon’ harboring *SFB-4* and ‘Compact Stella’ harboring *SFB-4'*. At 45°C, the signals for *SFB-4* and *SFB-4'* were preferentially detected in ‘Napoleon’ and ‘Compact Stella’, respectively (Fig. 2B). The contrast of signal intensities between these alleles was greater at 45°C than at 42°C (Fig. 2B). When the temperature was raised to 50°C, however, faint signals not allowing discrimination between these alleles were observed. Therefore, the 45°C condition for hybridization and washing is suggested to be optimum for individuals in which cross-hybridization has been observed.

S haplotype screening with digoxigenin-labeled allele-specific oligonucleotide probes (dot-blot-*S*-screening)

Although Fujimoto and Nishio (2003) and Shirasawa et al. (2005) dot-blotting genomic DNA on a

membrane, low quality of genomic DNA can cause a weak signal (Shirasawa et al. 2005). Since it is difficult to extract genomic DNA of fruit trees with high quantity and quality due to impurities such as secondary metabolites or saccharide, we utilized PCR products as a dot-blotting DNA, as also done by Fujimoto and Nishio (2003) and Shirasawa et al. (2005).

The PCR product of ca. 350 bp for each sweet cherry cultivar, which is the same region used as a probe in the above experiments, was dotted on a membrane (Fig. 3A). The membrane was hybridized with a digoxigenin-labeled *SFB-6* allele-specific oligonucleotide probe (Table 2) at 60°C. Signals were detected specifically in cultivars harboring an *S-6* haplotype (Fig. 3A). In contrast, when hybridization was carried out with the digoxigenin-labeled

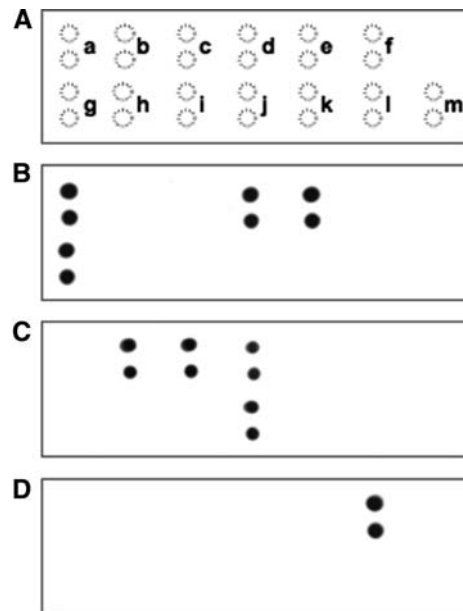


Fig. 3 Screening of *S* haplotypes with allele-specific oligonucleotide probes. (A) The position for a PCR product of each cultivar dotted on membrane. a, Satonishiki; b, Napoleon; c, Taishonishiki; d, Benishuho; e, Benisayaka; f, Compact Stella; g, Bigarreau de Jaboley; h, Early Purple; i, Vega; j, Sam; k, Van; l, Seneca; m, Rodmersham Seedling (B) Hybridization with digoxigenin-labeled PaSFBoli-6 probe at 60°C followed by washing with $0.1 \times$ SSC, 0.1% SDS at the same temperature. Competitive hybridization with the digoxigenin-labeled PaSFBoli-4 oligonucleotide mixed with the non-labeled PaSFBoli-4' oligonucleotide (1:10) as a probe (C) or with digoxigenin-labeled PaSFBoli-4'-new mixed with non-labeled PaSFBoli-4-new (1:5) as a probe (D). Two dots were blotted for a PCR product of each cultivar

SFB-4 allele-specific probe (PaSFBoli-4), signals were observed in cultivars harboring not only *SFB-4* but also *SFB-4'*, probably due to cross-hybridization (data not shown). A cross-hybridization signal was also observed in the case in which the digoxigenin-labeled *SFB-4'* allele-specific probe (PaSFBoli-4') was used. Shirasawa et al. (2006) reported a competitive hybridization method, which can detect sequence polymorphism such as SNP and insertion/deletion between one allele and a counterpart allele in rice. To improve the specific detection of *SFB-4* or *-4'*, we utilized this competitive hybridization method. The addition of unlabeled PaSFBoli-4' oligonucleotide to the hybridization buffer containing one-tenth the amount of the digoxigenin-labeled PaSFBoli-4 probe enabled specific detection of the *SFB-4* allele (Fig. 3C). However, hybridization using the reverse set of probes detected a faint signal for *SFB-4'* at 60°C (data not shown). In addition, lowering the temperature caused cross-hybridization signals (data not shown). Therefore, alternative oligonucleotides (PaSFBoli-4new and PaSFBoli-4'new; Table 2), which were shorter than the previous ones, were redesigned for the following competitive hybridization. The addition of the unlabeled PaSFBoli-4new oligonucleotide to the hybridization buffer containing one-fifth the amount of the digoxigenin-labeled PaSFBoli-4'new probe at 40°C resulted in detection of the specific signal of the *SFB-4'* allele (Fig. 3D).

Discussion

Distinct *S* genotyping by dot-blotting

In this study, the dot-blot-*S*-genotyping technique was successful in discriminating between nine-haplotypes (nine *SFB* alleles) of Japanese plum and between ten *S*-haplotypes (ten *SFB* alleles) of sweet cherry. DNA amplification of the *S-RNase* and *SFB* alleles by PCR using the consensus primers in sweet cherry and Japanese plum sometimes results in the amplification of DNAs of similar size and is accompanied by the amplification of other DNA sequences, which makes *S* genotyping complicated (Tao et al. 1999; Yamane et al. 2000; Sonneveld et al. 2003; Beppu et al. 2003; Sapir et al. 2004; Vaughan et al. 2006). In the case of allele-specific primers, successful amplification of the alleles depends on several

conditions such as the purity of template DNAs and the reaction temperature, and therefore, the reliability of *S* genotyping should be taken into consideration. False negative detection can be resolved by PCR-RFLP method. However, the method with *S-RNase* polymorphism has been developed only for *S-1* and *S-3* to *S-6* haplotypes in sweet cherry (Yamane et al. 2000), but not yet in Japanese plum. Sakamoto et al. (2000) have identified twenty-one *S* haplotypes in a large number of cabbage and broccoli by their developed PCR-RFLP method, but the *S* haplotype identification has been confirmed by nucleotide sequencing because of the difficulty of reliable identification by PCR-RFLP, especially for heterozygotes. This implies that as PCR-RFLP markers in two *Prunus* species increase, great difficulty in *S* genotyping would be expected because most cultivars or selections in both fruit species are *S* heterozygote by nature. In contrast, the method developed in the present study showed distinct signals, demonstrating satisfactory reliability of *S* genotyping. Although Ortega et al. (2005), Sonneveld et al. (2006) and Vaughan et al. (2006) reported new methods for discriminating similar sizes of PCR products by a semi-automated sequencer, the present method (dot-blot-*S*-genotyping) enables distinct identification of *S* genotypes independent of DNA fragment sizes and, furthermore, is simpler and more cost-efficient than methods using a sequencer.

In sweet cherry, we were unable to test for the *S-10*, *S-12* and *S-13* haplotypes, whose *SFB* sequences have already been published, because of the unavailability of sweet cherry materials. However, preliminary experiments exhibited no cross-hybridization between designed oligonucleotides for *S-10*, *S-12* and *S-13* and the labeled PCR products of ca. 350 bp amplified from sweet cherry cultivars used in this study. Although the experiments using cultivars harboring *S-10*, *S-12* and *S-13* haplotypes are needed, in the future, the dot-blotting method herein presented would be more practical in *S*-genotyping for any *S* haplotypes.

According to the dot-blotting method reported by Fujimoto and Nishio (2003), the genomic DNAs or *S*-determinant PCR products of target haplotypes are necessary for dot-blotting on a membrazne. Instead, we used oligonucleotides for target haplotypes, which resulted in satisfactory genotyping. Since the sequences of *SP11s* are highly variable between *S* haplotypes (Fujimoto and Nishio 2003), our method

would be applicable to the identification of *S* haplotypes in *Brassica* species.

Dot-blotting useful for *S* screening

In order to select individuals harboring a target *S* haplotype, a membrane dotted with non-labeled PCR products for each individual was hybridized with the labeled-oligonucleotide probe in sweet cherry. The labeled-oligonucleotide probe for the *S-6* haplotype enabled us to select cultivars harboring the *S-6* haplotype (Fig. 3B). However, cross-hybridization between the *SFB-4* and *SFB-4'* alleles was observed with an independent probe for *S-4* (PaSFBoli-4) or *S-4'* (PaSFBoli-4') (data not shown), possibly due to high sequence similarity. To date, Ikeda et al. (2004b) have succeeded in discrimination between *SFB4* and *SFB4'* by PCR using dCAPS primer sets that they designed, though PCR must be performed twice. Alternatively in this study, we were also successful in distinct and simple discrimination between these haplotypes utilizing the competitive hybridization method.

Although we used a cultivar population, *S* haplotypes of which have already been characterized, the dot-blot-*S*-screening method would be more practical than the method based on the PCR and would promote the screening of individuals that harbor the desirable *S* haplotype in a large-scale population.

Future application

In dot-blot-*S*-genotyping, we utilized a region of ca. 350 bp containing HVa. In our preliminary experiments, the longer sized fragments, ca. 500 bp, resulted in less intense signals, even when the temperature was below 40°C (data not shown), suggesting that the length of DNA fragments is important and that a length less than 500 bp is best for obtaining distinct signals. In *Prunus*, such as Japanese plum (Beppu et al. 2002, 2003; Sapir et al. 2004; Halász et al. 2007) and sweet cherry (Tao et al. 1999; Sonneveld et al. 2003), the first and second introns of *S-RNase* are highly variable in length. However, because the second introns of most alleles are more than 500 bp in length, they might not be suitable for dot-blot-*S*-genotyping. On the contrary,

since the length of the first intron is less than 500 bp, dot-blot-*S*-genotyping might be applicable. The availability of allele specific oligonucleotides designed from *S-RNase* along with those from *SFB* for the dot-blot-*S*-genotyping method would enable more reliable *S* genotyping. As cloning of *SFB* alleles will progress not only in Prunoideae but also in Maloideae such as apple and Japanese pear (Cheng et al. 2006; Sassa et al. 2007), the diversity of the HVa sequence will be further elucidated. To deal with the larger number of alleles and the diversity of the *SFB* gene, methodological improvements contributing not only to agronomical utilization of the *S* haplotype but also to convenient analyses of *S* haplotypes for population genetics are needed. Thus, this alternative strategy for *S* genotyping and *S* screening herein presented would seem to be promising.

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