Molecular Markers for the Self-compatible S^4 -haplotype, a Pollen-part Mutant in Sweet Cherry (*Prunus avium* L.)

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ABSTRACT. S^4 is a pollen-part mutant in sweet cherry (*Prunus avium* L.) that is extensively used to develop self-compatible cultivars. The S^4 -haplotype is known to have a functional stylar component and a nonfunctional pollen component. The pollen component in sweet cherry necessary for the specificity of the pollen reaction is believed to be an S -haplotype specific F-box protein gene, called *SFB*. This study describes two molecular markers that distinguish between *SFB*⁺ and *SFB*⁴ by taking advantage of a four base pair deletion in the mutant allele. The resulting polymerase chain reaction (PCR) products can either be separated directly on a polyacrylamide gel or they can be subjected to restriction enzyme digestion and the different sized products can be visualized on an agarose gel. The latter technique utilizes restriction sites created in the PCR products from the *SFB*⁴ allele, but not the *SFB*⁺ allele. Because the primer sets created differential restriction sites, these primer sets were termed dCAPS (derived cleaved amplified polymorphism sequence) markers. These molecular assays can be used to verify self-compatibility conferred by the S^4 -haplotype.

Sweet cherry is an out-crossing species where pollination success is controlled by a gametophytic self-incompatibility (GSI) system. Pollen rejection results if there is a match between the pollen and stylar specificity alleles for genes at the complex S -locus, termed S -haplotype (McCubbin and Kao, 2000). The stylar specificity gene in *Prunus* is known to be a ribonuclease termed S -RNase (Bošković and Tobutt, 1996; Tao et al., 1997, 1999; Ushijima et al., 1998). Recently, a candidate gene controlling pollen specificity, called *SFB* for S -haplotype specific F-box protein gene, has been identified in several *Prunus* species such as almond (*P. dulcis*) (Ushijima et al., 2003), sweet and sour cherries (*P. avium* and *P. cerasus*) (Ikeda et al., 2004; Yamane et al., 2003a), and Japanese apricot (*P. mume*) (Entani et al., 2003; Yamane et al., 2003b).

In self-incompatible tree species such as sweet cherry, a self-incompatible production cultivar must be planted with a pollinator cultivar to provide the compatible pollen required for fruit set. In addition, supplemental bee populations are generally required to transfer the pollen and ensure good fruit set. Because of the production inefficiencies associated with planting pollinator cultivars, the development of self-compatible sweet cherry cultivars is a common breeding goal (Brown et al., 1997).

The self-compatible mutant used in sweet cherry breeding programs worldwide is an X-ray induced mutation developed at the John Innes Institute in the United Kingdom (Lewis and Crowe, 1954a, 1954b). Of the artificially produced self-compatible S -haplotypes, S^4 , where S^4 indicates the loss of pollen activity in S^4 [pollen-part mutation (PPM)], has been most extensively used in breeding programs worldwide. The S^4 -haplotype originated from 'J12420' (S^4S^4), a seedling from the cross between 'Emperor

Francis' (S^3S^4) and X-ray irradiated pollen of 'Napoleon' (S^3S^4). Although the S -haplotype of 'J12420' had been believed to be S^3S^4 , a recent study has demonstrated that it is S^4S^4 (Bošković et al., 2000).

Molecular markers for S -locus genotyping in sweet cherry can be used to determine pollination group and to detect self-compatible seedlings in breeding populations. This assay is independent of age and season because it uses DNA extracted from vegetative tissues. Since the identification of a series of polymorphic S -RNases in sweet cherry, S -RNase genotyping has been used to determine the S -alleles in individual selections (Bošković and Tobutt, 1996; Bošković et al., 2000; Choi et al., 2002; Hauck et al., 2001; Iezzoni et al., in press; Sonneveld et al., 2001; Tao et al., 1999; Wiersma et al., 2001). However, since the S^4 -haplotype contains a functional S^4 -RNase, S -RNase genotyping can not distinguish between the S^4 PPM and the normal S^4 .

Recently, extensive comparisons of the *SFB* sequences of the S^4 - and S^4 -haplotypes identified a four base pair (bp) deletion in S^4 that confers the PPM (Ushijima et al., 2004). This paper reports the development of two different polymerase chain reaction (PCR) primer sets that can be used to distinguish between the *SFB*⁺ and *SFB*⁴ alleles based on the four base pair deletion in *SFB*⁴. The resulting PCR products can either be separated directly on a polyacrylamide gel or they can be subjected to restriction enzyme digestion and the different sized products can be visualized on an agarose gel. The latter technique utilizes restriction sites created in the PCR products from the *SFB*⁴ allele but not the *SFB*⁺ allele. Because the primer sets created differential restriction sites, these primer sets were termed dCAPS (derived cleaved amplified polymorphism sequence) markers (Michaels and Amasino, 1998; Neff et al., 1998). These markers will be very useful for cherry breeders when discrimination between S^4 and S^4 is necessary.

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Materials and Methods

PLANT MATERIAL AND DNA ISOLATION. Twenty sweet cherry cultivars, representing *S*¹ to *S*⁶ including the *S*⁴ PPM were used in this study (Table 1). Young leaves were collected, frozen in liquid nitrogen, lyophilized and stored at -20 °C until use. Lyophilized leaves were ground to a powder using a mortar and pestle, and homogenized in homogenization buffer [0.5 M sucrose, 10 mM Na₂EDTA, 80 mM KCl, and 0.5% (v/v) 2-mercaptoethanol in 10 mM Tris-HCl (pH 9.0)]. The homogenate was centrifuged (1800 g_n, at 4 °C, for 15 min) to collect the pellet. The pellet was re-suspended in the homogenization buffer and centrifuged (1800 g_n, at 4 °C, for 20 min). After repeated re-suspensions and centrifugations, genomic DNA was isolated from the pellet by Nucleon PhytoPure for plant DNA extraction kit (Amersham Biosciences, Uppsala, Sweden), and further purified by phenol/chloroform extraction.

PCR FOR THE SPECIFIC AMPLIFICATION OF *SFB*⁴ AND *SFB*^{4'}. Genomic DNA was used for PCR with the *SFB*⁴ and *SFB*^{4'} gene specific primer set SFB4F0 and SFB4R0 (Ushijima et al., 2004, Table 2 and Fig. 1). The PCR mixture contained 1× *ExTaq* buffer, 200 μM of each dNTP, 400 nM of each primer, 20 ng of template DNA, and 0.5 U of TaKaRa *ExTaq* polymerase (TaKaRa Shuzo Co., Kyoto, Japan) in a 25 μL reaction volume. PCR reactions for the amplification of *SFB*⁴ and *SFB*^{4'} were run with a program of 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min 30 s, with an initial denaturing at 94 °C for 2.5 min and a final extension of 72 °C for 7 min. As a reference, PCR with the *S-RNase* allele-specific primers, Pru-C2 and Pru-C4R, was also conducted as previously described (Tao et al., 1999). PCR products were separated on a 1% agarose gel (Type I-A agarose; Sigma, St. Louis) in 1× TAE buffer and stained with ethidium bromide.

NESTED PCR. Nested PCR was performed separately with two primers sets using the products from the *SFB*⁴ and *SFB*^{4'} gene-specific PCR as the DNA template. The primer set EVS4F and emS4R (Table 2) created an *EcoRV* restriction site specifically in the PCR products from *SFB*^{4'} (Fig. 1). Likewise, the primer pair Mbs4F (Table 2) and emS4R created an *MboI* restriction site specifically in *SFB*^{4'} (Fig. 1). These primer sets were designed to yield PCR products of 89 and 85 bp for *SFB*⁴ and *SFB*^{4'}, respectively.

The PCR mixture contained 1× Taq buffer, 200 μM of each dNTP, 400 nM of each primer, 1 μL of the 100 times diluted PCR mixture that was obtained with the *SFB*⁴ and *SFB*^{4'} gene-specific

primers, and 0.5 U of TaKaRa *Taq* polymerase (TaKaRa Shuzo Co., Kyoto, Japan) in a 25 μL reaction volume. PCR reactions were run with a program of 30 cycles of 94 °C for 15 s, 50 °C for 15 s and 72 °C for 15 s, with an initial denaturing at 94 °C for 2.5 min and a final extension of 72 °C for 7 min. PCR products were separated on a 3% agarose gel (Type I-A agarose, Sigma) in 0.5× TBE buffer and stained with ethidium bromide.

DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS. PCR products obtained with the EVS4F and emS4R primer set and those obtained with the Mbs4F and emS4R primer set were used for denaturing polyacrylamide gel electrophoresis. The PCR products were separated on a denaturing polyacrylamide gel that contained 6% acrylamide, 0.25% methylene bisacrylamide, and 7.5 M urea in 1× TBE buffer. The gel was run at 80 W for 2.5 h and stained with Silver Sequence Staining Reagents (Promega, Madison, Wis.).

DIGESTION WITH RESTRICTION ENZYME. PCR products obtained with the EVS4F and emS4R primer pairs and those obtained with the Mbs4F and emS4R primer pairs were digested with *EcoRV* and *MboI*, respectively. Five microliters of the PCR mixtures from the nested PCR was digested with 3 U of respective restriction enzymes at 37 °C for 2 h. Digests were separated on a 3% agarose gel (Type I-A agarose, Sigma) in 0.5× TBE buffer and stained with ethidium bromide.

Results and Discussion

We designed two dCAPS primer sets that can be used to visualize the four base pair deletion in *SFB*^{4'}, which is the only difference between the *SFB*⁴ and *SFB*^{4'}. These two primer sets were designed to amplify a ≈90 bp of DNA segment to produce unique restriction sites in *SFB*^{4'} but not in *SFB*⁴. The two primer sets included a common reverse primer termed emS4R (Fig. 1). Two forward primers, EVS4F and MBS4F, were designed to selectively produce *S*^{4'} products with *EcoRV* and *MboI* restriction sites, respectively (Fig. 1, Table 2).

Initial amplification from genomic DNA with the two dCAPS primer sets did not yield sufficient amplification product (data not shown). Thus we first amplified *SFB*⁴ and *SFB*^{4'} with the SFB4F0 and SFB4R0 primer set (Ushijima et al., 2004) to increase allele specificity of dCAPS markers and then used the PCR product as a template for the nested PCR with the dCAPS primer set. Since the SFB4F0 and SFB4R0 primer set is designed from the sequence of 5' and 3' UTR region of *SFB*⁴ (Fig. 1), respectively,

Table 1. Twenty sweet cherry cultivars used in this study.

Genotype	Phenotype ^z	Cultivars	Reference for <i>S</i> -genotype
<i>S</i> ⁴ <i>S</i> ^{4'}	SC	J12420	Boskovic et al., 2000
<i>S</i> ¹ <i>S</i> ⁴	SI	Rainier	Tao et al., 1999
<i>S</i> ¹ <i>S</i> ^{4'}	SC	Celeste, Lapins, Skeena	Wiersma et al., 2001; Sonneveld et al., 2001
<i>S</i> ³ <i>S</i> ⁴	SI	Bing, Emperor Francis, Napoleon	Tao et al., 1999; Wiersma et al., 2001
<i>S</i> ³ <i>S</i> ^{4'}	SC	Newstar, Sandra Rose, Sonata, Stella, Sweetheart	Wiersma et al., 2001; Sonneveld et al., 2001
<i>S</i> ¹ <i>S</i> ²	SI	Early Rivers	Tao et al., 1999; Wiersma et al., 2001
<i>S</i> ¹ <i>S</i> ³	SI	Van	Tao et al., 1999; Wiersma et al., 2001
<i>S</i> ¹ <i>S</i> ⁶	SI	Rockport Bigarreau	Yamane et al., 2000
<i>S</i> ² <i>S</i> ³	SI	Velvet	Tao et al., 1999; Wiersma et al., 2001
<i>S</i> ² <i>S</i> ⁶	SI	NY 54	Iezzoni et al., 2003
<i>S</i> ³ <i>S</i> ⁵	SI	Hedelfingen	Wiersma et al., 2001; Hauck et al., 2001
<i>S</i> ³ <i>S</i> ⁶	SI	Satonishiki	Tao et al., 1999

^zSI = self-incompatible; SC = self-compatible.

A. *EcoRV* dCAPS

B. *MboI* dCAPS

Fig. 3. PCR fragments generated by nested PCR with the primer sets *EcoRV* dCAPS and *MboI* dCAPS. (A) Uncleaved fragments on agarose gels, (B) Uncleaved fragments separated on denaturing polyacrylamide gels, and (C) restriction enzyme cleaved fragments separated on agarose gels. Lanes: 1, 'JI2420' (S^4S^4); 2, 'Celeste' (S^4S^4); 3, 'Lapins' (S^4S^4); 4, 'Skeena' (S^4S^4); 5, 'Newstar' (S^3S^4); 6, 'Sandra Rose' (S^3S^4); 7, 'Sonata' (S^3S^4); 8, 'Stella' (S^3S^4); 9, 'Sweetheart' (S^3S^4); 10, 'Rainier' (S^4S^4); 11, 'Bing' (S^3S^4); 12, 'Emperor Francis' (S^3S^4); and 13, 'Napoleon' (S^3S^4). SI = self-incompatible and SC = self-compatible.

restriction enzyme digestion (Fig. 3). *EcoRV* digestion of the PCR products obtained with the EVS4F and emS4R primer set yielded 65- and 20-bp fragments for *SFB'* (*EcoRV* dCAPS). This 65-bp fragment was distinguished from the 89 bp fragment from *SFB'* using agarose gel electrophoresis. Similarly, *MboI* digestion of the PCR products obtained with the MbS4F and emS4R primer set yielded 64- and 21-bp fragments for *SFB'* (*MboI* dCAPS). The 64-bp fragment was distinguished from the 89-bp fragment from *SFB'* by agarose gel electrophoresis.

The *MboI* dCAPS marker was preferred over the *EcoRV* dCAPS marker because both the *SFB'*- and *SFB'*-derived fragments from 'JI2420' could be observed more clearly in the *MboI* dCAPS than *EcoRV* dCAPS. With the *EcoRV* dCAPS primer set, the 89 bp band derived from *SFB'* was faint and sometimes difficult to resolve for 'JI2420'. This result is similar to that observed when the uncleaved products were separated on a denaturing polyacrylamide gel.

In our study we devised two different approaches for taking advantage of a four base pair deletion to distinguish between *SFB'* and *SFB'*. The nested PCR products from the dCAPS primer sets can either be separated directly on a denaturing polyacrylamide gel or they can be subjected to restriction enzyme digestion and visualized on an agarose gel. Both gel systems were equally accurate; therefore, the choice of which gel system to use to differentiate these two alleles will depend upon available equipment and resources.

Because the sweet cherry *S-RNase* primers are designed to take advantage of the different intron lengths among the *S*-alleles,

resolving different *S*-alleles and identifying the *S*-genotypes of selections is easiest with *S-RNase* primer pairs. The markers described in this study could then be used to distinguish among the S^4S^4 , S^4S^4 , and S^4S^4 genotypes in selections shown to have the S^4 -*RNase* in order to confirm self-compatibility.

These markers are well suited for early identification of self-compatible seedlings in a breeding program prior to field planting. However, implementation of early detection and elimination of self-incompatible seedlings should take into consideration the effect of this selection would have on linked traits. This is a particular concern for the *S*-locus region which has been demonstrated in two independent studies in peach (*P. persica* L.) to contain QTL for fruit size and quality (Dirlewanger et al., 1999; Yamamoto et al., 2001). A sweet cherry fruit quality QTL study is under way with the objective of identifying important horticultural traits linked to the *S*-haplotype and those alleles that produce positive effects. The long-term goal is to develop a set of molecular markers that can be used to "design" the desired allelic complements for the *S*-haplotype and the *S*-haplotype region in sweet cherry. The development of molecular markers to distinguish between the S^4 - and S^4 -haplotypes is a significant achievement toward this objective.

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