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Characterization of a novel self-incompatibility allele in *Malus* and *S*-genotyping of select crabapple cultivars

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ABSTRACT

Apples (*Malus* ×*domestica* Borkh.) are one of the largest economically significant fruit crop worldwide. Due to the self-incompatibility of most *Malus* cultivars, the apple industry relies on insect pollinators to aid in cross-pollination to improve seed and fruit set. The introduction of crabapples as pollinizers has become a popular practice to promote cross-pollination in commercial apple orchards. Genetic compatibility with a desired apple cultivar is a critical feature of effective pollinizers, but the *S*-genotypes of many crabapple cultivars used for cross-pollination have not yet been reported. In this study, seven crabapple cultivars were genotyped, and the genomic DNA of one novel *S*-allele in 'Mt. Blanc' and three variants of previously discovered alleles in 'Manchurian', 'Snowdrift' and 'Indian Summer' were characterized. Genomic DNA sequences were submitted to National Center for Biotechnology Information (NCBI) GenBank, and PCR-based detection methods were developed. The methods and results of this study aim to enrich *S*-genotyping methodologies and inform pollinizer-cultivar compatibility in commercial orchards.

1. Introduction

Sterility and sexual incompatibility are the two main causes of poor fruit set in apple (Janick et al., 1996). Whereas sterility can occur as a result of the developmental processes associated with the pollen, embryo-sac, embryo, or endosperm, incompatibility occurs when the pollen is unable to grow down the style and fertilize the ovary (Janick et al., 1996; Ramírez and Davenport, 2013). Unlike sterility, sexual incompatibility is a mechanisms that has evolved to prevent self-fertilization, and functions to limit the detrimental effects associated with inbreeding (Ramírez and Davenport, 2013).

Many rosaceous fruit trees, including wild and domestic apples (*Malus* spp.), exhibit a gametophytic self-incompatibility (GSI) mechanism in which sexual reproduction is regulated by a multi-allelic *S* locus (Kobel et al., 1939; de Nettancourt, 1977; Sassa, 2016). In the GSI system, pollen tube growth is inhibited in styles containing matching self-incompatibility alleles (*S*-alleles) (Dennis, 2003). Depending on their combination of *S*-alleles, apple cultivars can be fully incompatible, semi-compatible, or fully compatible (Schneider et al., 2005). Full incompatibility occurs when the *S*-alleles between parents are identical; semi-compatibility occurs when one *S*-allele differs; and full compatibility occurs when both *S* alleles differ (Schneider et al., 2005). Although commercial apples are vegetatively propagated, consistent crop production relies upon insect pollinators to transfer compatible pollen between cultivars (Mayer and Lunden, 1988). To promote cross-pollination and improve fruit set, orchards must include at least two different, cross-compatible cultivars; alternatively, pollinizer varieties that are genetically distinct from crop cultivars can be used. In an open pollination trial, Schneider et al., (2005) reported lower seed and fruit set rates in semi-compatible crosses in comparison to fully-compatible crosses. Thus, ideal orchard design should integrate fully-compatible pollinizers for optimal seed and fruit set (Schneider et al., 2005).

The *S* locus in apple harbors genes encoding an extracellular ribonuclease (S-RNase) in the pistil (Sassa et al., 1996) and a collection of F-box proteins, known as *S*-locus F-box brothers (SFBB), in the pollen (Cheng et al., 2006; Sassa et al., 2007). The proteins encoded at the *S*-locus play their role in a specific pollen-pistil recognition mechanism that results in the selective rejection of 'self' pollen tubes. The precise mechanism of self-recognition and inhibition of pollen tube growth in apple has long remained unclear; however, recent work supports a model in which the pollen SFBB are incorporated into a SKP1-Cullin1-F-box-RBx1 (SCF) -type complex that serves as an E3 ubiquitin ligase to mediate ubiquitination and degradation of non-self S-RNases (Minamikawa et al., 2010; Yuan et al., 2014; Sassa, 2016). In this 'non-

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self' recognition system, the SFBB proteins encoded within a pollen tube are able to inactivate all S-RNases except those matching the pollen's own *S* allele. In this case, 'self' S-RNases remain active and exert cytotoxic activity leading to pollen tube death. Thus, pollen tube growth is inhibited in the style and fertilization is prevented if the pollen *S*-haplotype is identical to either of the seed parent *S*-haplotypes.

Most apple cultivars are diploid and contain two sets of chromosomes; however, even triploid cultivars exhibit self-incompatibility, and fruit production is improved by cross-pollination with a compatible pollen source (Janick et al., 1996). Triploid cultivars, which include 'Bramley's seedling' (Scott, 1972), 'Mutsu', 'Jonagold', and 'Hokuto' (Sassa et al., 1994), among others, are generally unfavorable for use as a pollen source due to the reported low viability of triploid pollen (Scott, 1972). Crabapples are favored as pollinizers because they can be interplanted between trees of a commercial variety, eliminating the need to maintain less profitable cultivars used primarily for cross-pollination purposes (Crassweller et al., 1980) and increasing the homogeneity inside the orchard. The 'Manchurian' crabapple has been widely planted as a source of cross-compatible pollen in apple orchards, but due to its apparent susceptibility to some economically significant diseases, including speck rot and Sphaeropsis rot (Sikdar et al., 2018; Xiao et al., 2005; Xiao et al., 2014), the apple industry is seeking alternative crabapple varieties to serve as pollinizers (Hansen 2014).

Understanding genetic compatibility among cultivars has been a topic of research for several decades. Kobel et al. (1939) reported eleven distinct incompatibility alleles in apple on the basis of phenotypic incompatibility. Later work sought to supplement early phenotyping studies with *S*-genotyping by characterization of stylar S-RNases (Sassa et al., 1994; Bošković and Tobutt, 1999) and *S* locus nucleic acid sequences (Broothaerts et al., 1995; Janssens et al., 1995; Sakurai et al., 1997; Broothaerts, 2003; Kim et al., 2009; Larsen et al., 2016). These molecular-based methods have proven useful in genotyping self-incompatibility alleles more accurately and efficiently than traditional crossing experiments, which are laborious, time-consuming, and can be influenced by environmental factors (Van Nerum et al., 2001). In recent years, the increase in deposition of *S* locus sequences to central databases (e.g. NCBI GenBank), combined with inconsistencies in naming conventions and some erroneous *S*-genotypes reported in the literature, has led to some confusion in the *S*-allele nomenclature and reported *S*-genotypes in apple (Broothaerts et al., 2004). Some of this confusion was resolved by genomic sequencing (Matsumoto et al., 2003a) and pollination studies (Matsumoto et al., 2006), and a fully reorganized list of *S*-alleles and GenBank accessions was subsequently published by Kim et al. (2016).

Although previously published studies provide effective detection methods for many *S*-alleles common in *Malus* × *domestica*, there are currently few genotyping methods available for *S*-alleles discovered in wild apples and crabapples of mixed ancestry. Dreesen et al. (2010) reported 16 novel S-RNase sequences in *Malus sylvestris* and Li et al. (2012) identified five previously undiscovered *S*-alleles in wild *Malus* species, but neither study reported efficient molecular methods for detecting these alleles. Larsen et al. (2016) developed a screening method for detecting 39 *S*-alleles in *Malus*, including wild and domestic apples; however, this method requires costly equipment and can produce ambiguous results due to its reliance on subtle restriction fragment length differences. Furthermore, the authors identified 25 *S*-alleles in a collection of 432 *Malus* accessions but were unable to experimentally verify the efficacy of this method for many *S*-alleles. With the growing trend of utilizing crabapples as pollinizers in commercial apple production, the importance of defining self-incompatibility alleles in crabapples is becoming increasingly evident. In the present work, previously reported *S*-genotyping methods were used in conjunction with genomic DNA sequencing to identify *S*-genotypes in seven crabapple varieties. One novel *S*-allele and three variants of previously reported *S*-alleles were characterized, and PCR-based methods were developed for rapid detection of these alleles.

2. Materials and methods

2.1. Plant material

Seven crabapple cultivars, *Malus* 'Adirondack', *Malus* 'Evereste', *Malus* 'Frettingham', *Malus* 'Indian Summer', *Malus baccata* 'Manchurian', *Malus* 'Mt. Blanc', and *Malus* 'Snowdrift' were acquired from commercial nurseries between 2014 and 2016.

2.2. Extraction of genomic DNA

Young leaves were collected from a representative tree of each variety evaluated in this study. Genomic DNA was extracted from frozen leaves with the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) using the manufacturer's recommended protocol. Nucleic acid concentration was measured spectrophotometrically using the NanoDrop™ 2000 (Thermo Scientific, Waltham, MA).

2.3. Molecular screening for S-haplotypes

Allele-specific PCR was used in conjunction with PCR-RFLP methods to screen *Malus* varieties for *S*-haplotypes (Table 1). The method reported by Broothaerts (2003) was used to screen for S1, S2, S3, S5, S7, S9, S10 and S24 as described by the author, with the exceptions of increasing $MgCl_2$ concentration to 2.5 mM and decreasing the buffer concentration to 0.8× for some primer combinations. The allele-specific PCR detection method for S41 (De Franceschi et al., 2016) was used as described, with the exception of using 0.3 μM of each primer and 0.6 U *Taq* polymerase with 1 × PCR buffer. The primers developed by Long et al. (2010) were used to screen for S46 (FJ008672), S54 (FJ008671), and S55 (FJ008673) in a modified protocol using 0.8 U *Taq* polymerase with 0.8 × PCR buffer, 2.0 mM $MgCl_2$, and 0.25 μM each forward and reverse primers. Additionally, the presence of S46 was further investigated using the consensus primer PycomC1F (Sanzol and Robbins, 2008) with MdS46SpR (Long et al., 2010) and digesting the 555 bp amplicon with *Swa*I, yielding two fragments sized 289 bp and 266 bp. The CAPS (cleaved amplified polymorphic sequences) method described by Kim et al. (2009) was used to screen for S1, S2, S3, S4, S5, S7, S9, S10, S11, S16, S20, S21,

Table 1
List of crabapple cultivars included in this study and their respective *S*-genotypes and methods adopted.

Cultivar	*S* genotype	Method
Adirondack	S30 S45b S46	S30: CAPS (Kim et al. (2009)) S45b: current work S46: Allele-specific PCR (Long et al. (2010)), current work
Evereste	S20 S26	S20, S26: CAPS (Kim et al. (2009); see Supplementary Fig. 1)
Frettingham	S24 S25	S24: CAPS (Kim et al. (2009)), Allele-specific PCR (Broothaerts (2003)) S25: PCR-RFLP (Kitahara and Matsumoto (2002))
Indian Summer	S26 S50b	S26: CAPS (Kim et al. (2009)) S50b: current work
Manchurian	S5 S39b	S5: Allele-specific PCR (Broothaerts (2003); Matsumoto et al. (2010)) S39b: current work
Mt. Blanc	S3 S58	S3: Allele-specific PCR (Broothaerts (2003)) S58: current work
Snowdrift	S25 S45b	S25: PCR-RFLP (Kitahara and Matsumoto (2002)) S45b: current work