Genetic Studies on a Pollen-Part Self-Compatible Mutant in Japanese Pear

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Summary

Self-incompatibility (SI) is a widespread mechanism in flowering plants that prevents self-fertilization and promotes outcrossing. Though advantageous from this standpoint, SI is disadvantageous for agriculture. Most of the important fruit crops in subtribe Pyrinae (formerly Maloideae) of the Rosaceae, such as *Malus* and *Pyrus*, exhibit *S*-RNase (*S*-ribonuclease)–based SI controlled by a multigene complex, the *S*-locus. The genes at the *S*-locus in Pyrinae encode an *S*-RNase as a stylar factor and multiple F-box proteins called *S* locus F-box brothers (SFBBs) as pollen factors, and segregate as a single unit referred to as the *S* haplotype. Elucidation of the mechanism of SI is important for the regulation of fruit setting in agricultural production. A stylar-part self-compatible mutant (SPM) of Japanese pear (*Pyrus pyrifolia*), 'Osa-Nijisseiki', has contributed to our understanding of the function of *S*-RNase and to breeding of stylar-part self-compatible cultivars. Until the present studies, however, no pollen-part self-compatible mutants (PPMs) with an altered *S* haplotype had been discovered in the Pyrinae.

This thesis describes the discovery of a novel, artificially obtained diploid PPM of Japanese pear, the analysis of the genetic and phenotypic basis of self-compatibility (SC) in this mutant, and the evaluation of its potential for use as breeding material. The thesis comprises four series of experiments, described in Chapters 2 to 5.

In the experiments described in Chapter 2, a self-compatible mutant selection (designated 415-1) of Japanese pear was generated by a cross using pollen from a 'Kosui' tree chronically exposed to low-dose-rate γ-irradiation. Cleaved amplified polymorphic sequence (CAPS) analysis of the *S-RNase* genes revealed that the *S* haplotype of 415-1 was S_4S_5. Self- and cross-pollination tests revealed that the styles of this selection

maintained the *S*-allele-specific rejection of S_4 and S_5 pollen, whereas the pollen was not rejected by the styles of self-incompatible cultivars containing the same *S* haplotypes. Thus, this mutant was determined to be a PPM.

In the experiments described in Chapter 3, flow cytometry analysis and segregation analyses of *S* haplotypes and simple sequence repeats (SSRs) in the same linkage group as the *S* haplotype were performed to investigate the genetic control of this PPM. Flow cytometry analysis revealed that 415-1 was diploid. Segregation analyses of *S* haplotypes and SSR markers revealed a duplication of the S_5 haplotype and demonstrated that the accurate *S* haplotype of 415-1 is $S_4S_5S_5$. These results indicated that the pollen-part SC of 415-1 is not caused by a mutation of a pollen *S* factor, but by a segmental duplication encompassing the *S* haplotype. Further, the results suggested that 415-1 could produce *S*-heteroallelic pollen grains capable of breaking down SI through the function of two different *S* factors in a single pollen grain. This result provided further support for the proposal that the SI/SC systems in Pyrinae are regulated by the collaborative non-self recognition system postulated to function in the Solanaceae.

In the experiments described in Chapter 4, the segregation of *S* haplotypes and SSR genotypes in pollen generated from 415-1 was directly demonstrated by using polymerase chain reaction (PCR) analysis of single pollen grains to evaluate the frequency of *S*-heteroallelic pollen. The frequency of *S*-heteroallelic pollen from 415-1 was 16%, approximately equal to the frequency of outcross progeny of 415-1 with a duplicated *S* haplotype (14%). Moreover, about half of the pollen grains from 415-1 were aborted or smaller than those from ‘Kosui’. Together, these results indicated that the decrease in frequency of pollen with the duplicated *S* haplotype occurred during pollen formation, but that the probability of fertilization by mature *S*-heteroallelic pollen was equal to that

of single-allelic pollen. To our knowledge, this is the first time in the study of *S*-RNase–based self-incompatible plants that *S*-heteroallelic pollen with the potential to cause breakdown of SI was detected in a diploid PPM with a duplicated *S* haplotype.

In the experiments described in Chapter 5, the allelic composition and dosage of the *Pyrus pyrifolia S locus F-box brothers gamma* (*PpSFBB*$^{-\gamma}$) gene in the progeny of 415-1 were determined by TaqMan allelic discrimination assays designed to evaluate the correlation between SC and inheritance of the duplicated *S* haplotype. This assay revealed that 90% of the progeny of 'Syuugyoku' (S_4S_5) × 415-1 ($S_4S_5S_5$) had a duplicated *S* haplotype. Furthermore, 85% of the progeny with a duplicated *S* haplotype were self-compatible. From these results, the SC of 415-1 was confirmed to be transmitted to the progeny. However, some of the progeny with a duplicated *S* haplotype were self-sterile. The reasons are undetermined but might include detrimental mutations caused by γ-irradiation, inbreeding depression, or a combination of the two.

In conclusion, the self-compatible selection 415-1 is the first diploid PPM of Japanese pear, and will be useful for breeding of self-compatible cultivars. Furthermore, 415-1 will be valuable for elucidating the mechanisms of regulation and function of SI, especially that of collaborative non-self recognition of self *S*-RNases caused by a duplicated *S* haplotype.

Abbreviations

CAPS: cleaved amplified polymorphic sequence

CI: competitive interaction

LG: linkage group

NIAS: National Institute of Agrobiological Sciences

PCR: polymerase chain reaction

PPM: pollen-part self-compatible mutant

PpSFBB: *Pyrus pyrifolia S locus F-box brothers*

SC: self-compatibility

SFB: *S* haplotype-specific F-box

SFBB: *S* locus F-box brothers

SI: self-incompatibility

SLF: *S*-locus F-box

SNP: single-nucleotide polymorphism

SPM: stylar-part self-compatible mutant

S-RNase: *S*-ribonuclease

SSR: simple sequence repeat

Chapter 1

General introduction

1−1 Self-incompatibility in agricultural crops

Self-incompatibility (SI) is a reproductive strategy that prevents inbreeding and maintains genetic diversity in flowering plants (de Nettancourt 2001). Since the beginning of agriculture, artificial selection has produced changes in the genetic composition of crops. The ease and effectiveness of selection depended on the type of reproduction pattern, including the presence or absence of SI, in the plants being domesticated (Buckler et al. 2001; Haudry et al. 2007; Mariette et al. 2010). Early in the domestication of annual crops such as wheat and rice, plants with excellent phenotypes were selected and propagated by seeds. To breed desirable characteristics in sexually reproducing crops, self-compatibility (SC) is advantageous because self-pollination enables recessive but agronomically important genes to become and remain fixed (i.e., homozygous). Most of the major crop species are self-compatible, probably because annual crops were more successfully selected from self-compatible progenitors than from self-incompatible ones (Glémin and Bataillon 2009).

Meanwhile, during the domestication of perennial crops, especially fruit trees and others having a long juvenile phase, propagation procedures shifted from reproduction by seed to vegetative propagation. Once a self-incompatible plant with preferable traits has been selected, it is usually clonally propagated because many genes in self-incompatible plants are heterozygous and segregate in sexually derived progeny (Zohary 2004; McKey et al. 2011). Therefore, SI is not considered to have been an obstacle to domestication of perennial crops (Miller and Gross 2011). However, SI is disadvantageous for stable and

satisfactory yield in fruit production because cross-pollination with compatible pollen would produce normal-shaped fruit (Pinillos and Cuevas 2008). To address this limitation, self-compatible mutants from several self-incompatible fruit species, such as almonds (*Prunus dulcis*, Rosaceae) and apricots (*Prunus armeniaca*), were selected after domestication. Consequently, the prevalence of self-compatibility tends to be higher in current cultivars than in wild populations (Godini 2002; Maghuly et al. 2005). On the other hand, almost all cultivars of the species belonging to subtribe Pyrinae (formerly subfamily Maloideae, Rosaceae) such as apple (*Malus*) and pear (*Pyrus*), which are widely cultivated in the temperate regions, are still self-incompatible.

1–2 Agricultural and breeding practices for a self-compatible mutant of Japanese pear

Japanese pear (*Pyrus pyrifolia*), one of the most important fruit crops cultivated in the temperate zone of East Asia, exhibits *S*-ribonuclease (*S*-RNase)–based SI (which will be described in section 1–3). In orchards for commercial production of this species in Japan, artificial pollination is conventionally performed using compatible pollen collected from other cultivars to improve fruit quality and quantity (Sakamoto et al. 2009) because insect pollinators are inactive during cold and cloudy weather, which often occurs during the flowering period (Saito et al. 2012). For other self-incompatible fruit crops such as apple and sweet cherry (*Prunus avium*), a plantation of different *S*-haplotyped cultivars is recommended, and pollination by flower-visiting insects is required (Granger 1997; Ramírez and Davenport 2013). As a result of searches for self-compatible mutants of Japanese pears, a bud sport of ‘Nijisseiki’ (S_2S_4) called ‘Osa-Nijisseiki’ was identified to be self-compatible (Furuta et al. 1980). This cultivar has been used in agricultural

production, as breeding material, and as experimental material for studying the genes controlling SI (Norioka et al. 1996). Phenotypic and molecular studies revealed that 'Osa-Nijisseiki' is a stylar-part self-compatible mutant (SPM); that is, it exhibits stylar-specific dysfunction of SI (Sato 1993). Later, this stylar-part SC was characterized as a deletion of the *S_4-RNase* gene (Sassa et al. 1997). Several self-compatible cultivars have been bred using 'Osa-Nijisseiki' as a parent (Tanabe et al. 2001; Kitagawa et al. 2014). However, since 'Osa-Nijisseiki' has been the only genetic material available for breeding of self-compatible cultivars in Japanese pear, SPM cultivars originated from this cultivar often resemble each other in characteristics such as eating quality and harvest time. Moreover, it is likely that inbreeding depression will occur in the progeny of crosses between cultivars originating from 'Osa-Nijisseiki' or 'Nijisseiki' in the future (Sato et al. 2008). Accordingly, new self-compatible mutants with different genetic backgrounds are needed for use as breeding material.

1–3 *S*-RNase–based self-incompatibility

Many species of Rosaceae, as well as of Solanaceae and Plantaginaceae, exhibit *S*-RNase–based SI that is gametophytically controlled by a multigene complex, the *S*-locus. The genes at this locus encode *S*-RNase as a stylar factor (McClure et al. 1989; Sassa et al. 1996; Xue et al. 1996), and F-box proteins called *S*-locus F-box (SLF) in an *Antirrhinum* interspecific hybrid (Lai et al. 2002), Japanese apricot (*Prunus mume*; Entani et al. 2003), and *Petunia inflata* (Sijacic et al. 2004), *S*-haplotype-specific F-box (SFB) in *Prunus dulcis* (Ushijima et al. 2003), or *S* locus F-box brothers (SFBBs) in *Pyrus pyrifolia* (Sassa et al. 2007) as pollen factors for self/non-self recognition. At meiosis, these two genes at the *S*-locus segregate as a single unit, which is referred to as the *S*

haplotype. When a pollen *S* allele matches either one of the *S* alleles of the pistil, *S*-RNase secreted by the pistil tissue degrades the ribosomal RNA in the pollen tube produced by that pollen grain, inhibiting pollen tube elongation and preventing fertilization (Fig. 1.1).

The *S*-haplotype–specific recognition system was initially investigated through genetic and molecular characterization of self-compatible mutants. This analysis revealed that *S-RNase* genes were indispensable for a stylar self-incompatible response in all members of the Rosaceae studied to date, because this response was always lost upon deletion or silencing of an *S-RNase* gene. In contrast, the pollen factors in the Rosaceae are currently classified into two different types. In *Prunus*, there is a single functional SLF/SFB that is predicted to recognize and protect self *S*-RNase from degradation, because almost all pollen-part self-compatible mutants (PPMs) analyzed had lost SLF/SFB (Ushijima et al. 2004; Sonneveld et al. 2005). In the Pyrinae and Solanaceae, there are multiple *S*-related F-box proteins that are predicted to collaborate in recognizing and degrading non-self *S*-RNase (Fig. 1.2) (Kubo et al. 2010; Kakui et al. 2011; Saito et al. 2012); for example, 'Osa-Nijisseiki', which lacks both S_4*-RNase* and one of the *Pyrus pyrifolia S locus F-box brother* (*PpSFBB*) genes (Okada et al. 2008, 2011), maintains pollen-part SI and shows cross-incompatibility with a style harboring a non-S_4 (e.g., S_1) haplotype.

Furthermore, genetic and molecular evidence shows that non-*S*-locus factors with pollen- or style-specific expression may be necessary for *S*-RNase–based SI, because self-compatible mutants containing mutations unlinked to the *S*-locus have been discovered in sweet cherry (Wünsch and Hormaza 2004) and apricot (Vilanova et al. 2006), both in the Rosaceae, and in *Nicotiana* (McClure et al. 1999; Goldraij et al. 2006) and *Petunia* (Tsukamoto et al. 2003), both in the Solanaceae. These mutations were

predicted to modify the SI response by interacting with *S*-RNase or *S*-related F-box proteins. In fact, pollen-specific proteins interacting with SLFs/SFBBs have been found in recent years (Chen et al. 2010). In general, F-box proteins are indispensable in the ubiquitin-mediated protein degradation pathway, which acts in cell cycle regulation and stress-responsive gene expression in eukaryotes (Skowyra et al. 1997; Kipreos and Pagano 2000). The F-box protein functions as a component of an E3 ubiquitin ligase complex [consisting of Skp1 (S-phase kinase-associated protein 1), Cullin1 (CUL1), F-box, and Rbx1 (RING-box protein 1)], which ubiquitinates targeted proteins and degrades them by the 26S proteasome (Ciechanover 1998; Deshaies 1999). Thus, it has been hypothesized that the cross-compatible response occurs by non-self *S*-RNase degradation mediated by an E3 ubiquitin ligase complex containing an F-box protein (SLF/SFB/SFBB) (Lai et al. 2002; Takayama and Isogai 2005) (Fig. 1.3). Indeed, Skp1-like proteins were reported to interact with both SLF/SFB/SFBB and CUL1-like proteins in *Antirrhinum* (Plantaginaceae; Huang et al. 2006), sweet cherry (Matsumoto et al. 2012), *Petunia hybrida* (Solanaceae; Zhao et al. 2010), and Chinese pear (*Pyrus bretschneideri*) (Pyrinae; Xu et al. 2013). Meanwhile, *S*-RNase binding protein1 (SBP1), which contains a RING-finger domain characteristic of E3 ubiquitin ligases, was shown to interact with *S*-RNase in *Petunia inflata* (Sims and Ordanic 2001), *Petunia hybrida* (Hua and Kao 2006), and *Solanum chacoense* (O'Brien et al. 2004), all in the Solanaceae. Recently, an SBP1 homolog of apple was also shown to interact with *S*-RNase (Minamikawa et al. 2013; Yuan et al. 2014).

Until now, the molecular mechanism of the *S*-RNase–based gametophytic SI response in the Rosaceae has not been fully explored. The different mechanisms by which SC can arise from SI and the different molecules that modify pollen- and stylar-*S*-function

in different species raise the possibility of different patterns of self/non-self recognition and inactivation of *S*-RNase in self-incompatible responses. Therefore, it is necessary to obtain various self-compatible mutants from different self-incompatible species and to study the mutations involved in SC and their original functions in the self-incompatible progenitor.

1–4 Overview of this study

This thesis describes a novel, artificially obtained PPM of Japanese pear and the analysis of the genetic and phenotypic basis of SC in this mutant. First, a PPM was generated by pollination using pollen from a chronically γ-irradiated pear tree (Chapter 2). Moreover, this mutant was shown by flow cytometric analysis and genetic analysis of the progeny to be diploid but carrying a segmental chromosomal duplication encompassing an *S* haplotype (Chapter 3). Further, the rate of *S*-heteroallelic pollen generated from the PPM was demonstrated directly using polymerase chain reaction (PCR) analysis of single pollen grains. In this analysis, the segregation ratio of *S* haplotype and simple sequence repeat (SSR) markers in the pollen from the mutant was approximately the same as that observed in outcross progeny (Chapter 4). Finally, the relationship between the segregation of the duplicated *S* haplotype (revealed by TaqMan allelic discrimination analysis using quantitative PCR) and the inheritance of pollen-part SC (determined by self-pollination) was examined (Chapter 5). These findings provide new insight into the molecular mechanisms involved in self- and non-self-recognition of SC in Pyrinae. They also yield a novel genetic resource for breeding pollen-part self-compatible cultivars and provide markers for early selection in breeding of pollen-part self-compatible cultivars.

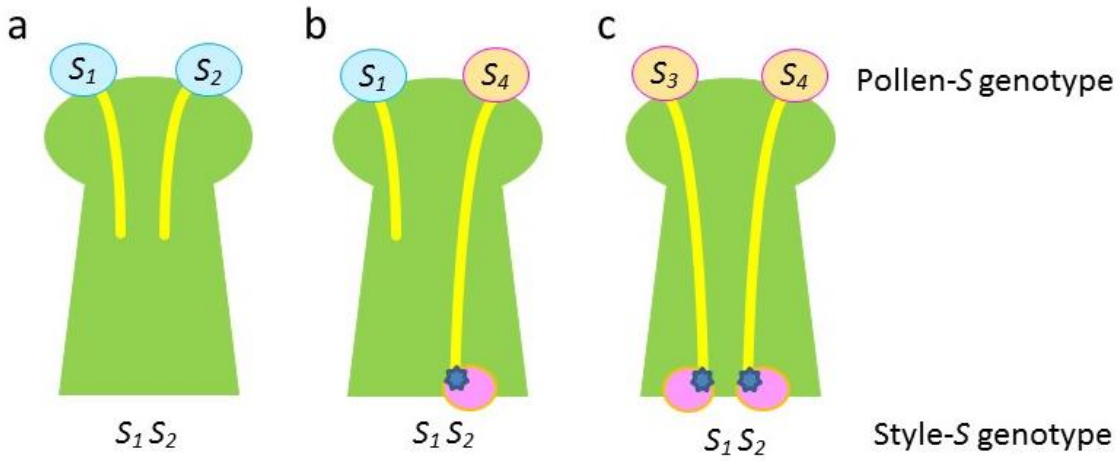

Fig. 1.1 Fertilization patterns of pollen with different pollen *S* genotypes in the gametophytic self-incompatibility system. (a) After self-pollination, pollen tube growth from pollen grains with both pollen *S* genotypes is arrested because the same two *S* genotypes (S_1 and S_2) are also present in the style. (b) After cross-pollination with an S_1S_4 plant, pollen tube growth of S_1 pollen is arrested, as in (a), but pollen tube growth of S_4 pollen continues and leads to fertilization because it carries an allele different from those in the style. (c) After cross-pollination with an S_3S_4 plant, pollen tubes from pollen of both pollen *S* genotypes continue to elongate and lead to fertilization because neither allele in the pollen matches those in the style.

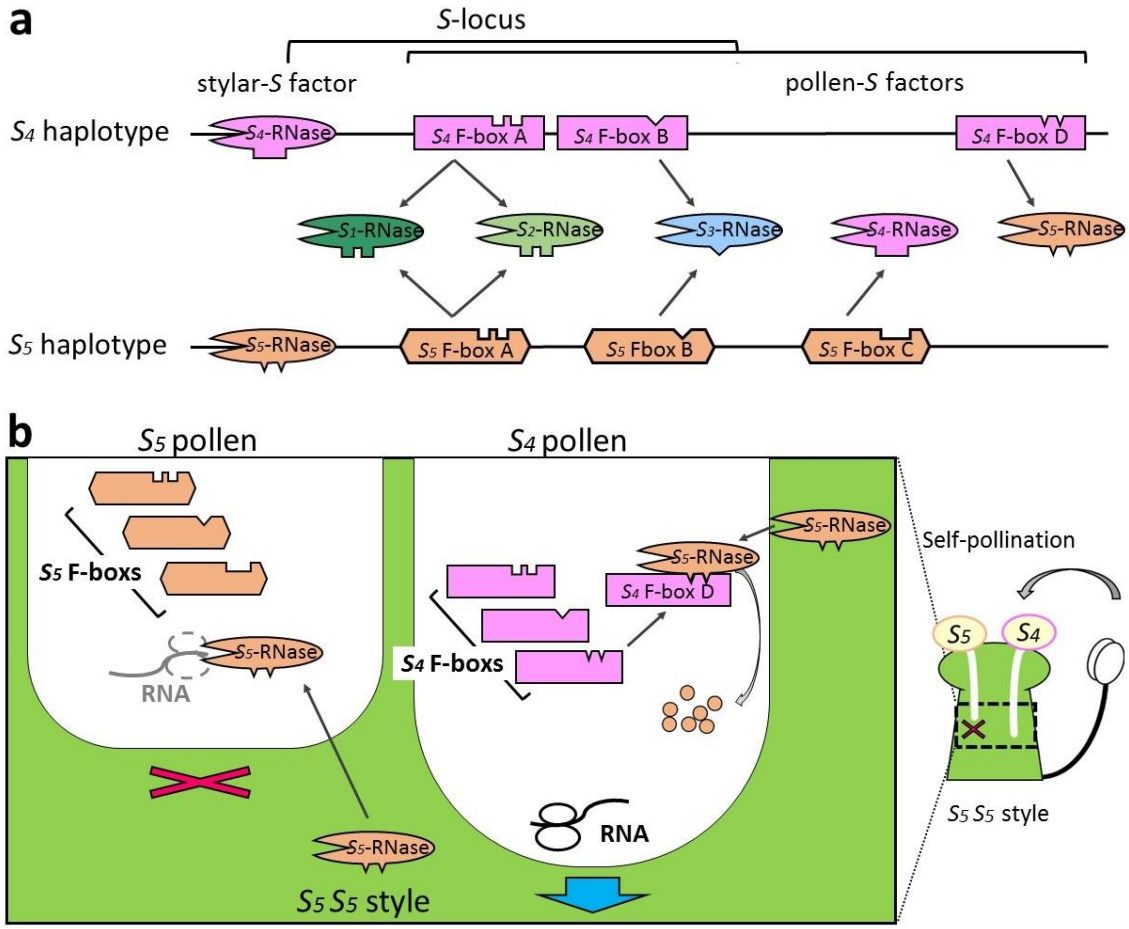

Fig. 1.2 Illustration of the collaborative non-self recognition system in *S*-RNase–based self-incompatibility proposed in the Solanaceae and Pyrinae (Kubo et al. 2010). (a) Each *S*-locus typically encodes an *S*-RNase and multiple *S*-locus F-box proteins (SLFs/SFBBs). Each F-box protein recognizes one or more non-self *S*-RNases, but none can recognize self *S*-RNase. (b) Rejection of S_5 pollen and acceptance of S_4 pollen by an S_5S_5 style. There is no F-box protein in the S_5 pollen tube that can recognize and degrade the S_5-RNase produced in the style, so the S_5 pollen tube stops elongating because S_5-RNase digests rRNA. On the other hand, one of the F-box proteins (S_4-F-boxes) in the S_4 pollen tube can recognize and degrade S_5-RNase, so the S_4 pollen tube continues to elongate.

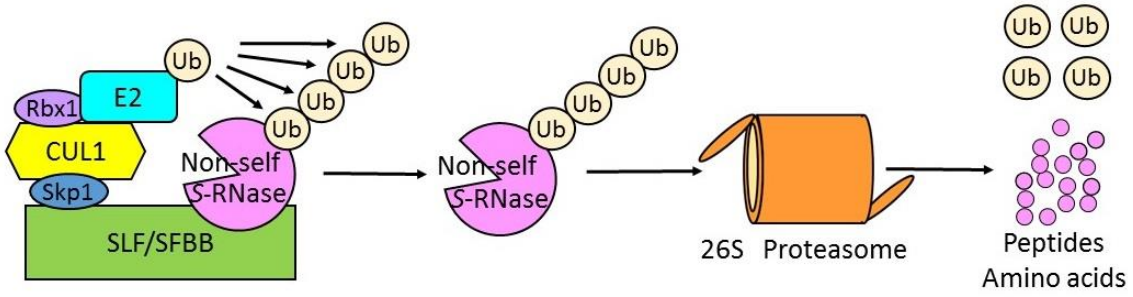

Fig. 1.3 The ubiquitin-mediated protein degradation pathway in the cross-compatible response hypothesized in the *S*-RNase–based self-incompatibility system. Ub; ubiquitin, E2; ubiquitin-conjugating enzyme. The *S*-locus F-box protein (SLF/SFBB) binds to non-self *S*-RNase through an *S* haplotype recognition domain and forms an E3 ubiquitin ligase complex (consisting of the F-box, Skp1, CUL1, and Rbx1). This complex interacts with E2 and transfers ubiquitin to the non-self *S*-RNase. The ubiquitinated non-self *S*-RNase is degraded into amino acids or peptides by the 26S proteasome.

Chapter 2

Generation of a new pollen-part self-compatible mutant of Japanese pear by crossing a self-incompatible cultivar with pollen from a gamma-irradiated tree

2–1 Introduction

Until the studies described in this thesis, there was only one self-compatible mutant in Japanese pear, ‘Osa-Nijisseiki’. It is an SPM cultivar (Sato 1993) that has been cultivated as a substitute for the original (self-incompatible) cultivar ‘Nijisseiki’ as a means of saving the labor required for artificial pollination. The mutant has also been used as breeding material and as experimental material for studying the genes controlling SI (Norioka et al. 1996). However, the pollen of ‘Osa-Nijisseiki’ (and other SPM materials bred from it) still maintains SI, so it cannot be used to pollinate existing self-incompatible cultivars having the same *S* genotypes. For production of pear fruits without artificial pollination using SPM cultivars, all of the trees in an orchard eventually need to be replaced by self-compatible ones, so many SPM cultivars with differing horticultural characteristics are necessary. On the other hand, PPMs, which have lost the pollen function of SI, do not have this limitation. A few spontaneous PPMs have been discovered in self-incompatible species in *Prunus* (Rosaceae), such as Japanese apricot (mume) and sweet cherry (Ushijima et al. 2004; Marchese et al. 2007). In recent commercial production of sweet cherry, PPM cultivars have been planted in mixtures with self-incompatible cultivars. This practice promotes stable fruit production because the PPM cultivars are able to fertilize not only themselves but also all of the self-incompatible cultivars (Granger 1997). A PPM of Japanese pear would contribute to the identification

of pollen functions in SI in Pyrinae as well as to breeding of PPM cultivars. However, all hitherto-known PPMs of Japanese pear have been polyploids (Tahira et al. 2010). Because of their ploidy, these materials are unsuitable for use in research to identify the gene responsible for pollen-part SI, and they are difficult to use in breeding to generate self-compatible cultivars by crossing with normal diploids. Therefore, it is necessary to develop diploid PPMs by using artificial mutation approaches.

Ionizing radiation is a useful method for inducing a mutation in plant genes (van Harten 1998). Such radiation produces DNA double-strand breaks, which can in turn produce deletions, insertions, replacement of nucleotides, or chromosomal rearrangements during the process of DNA repair. If a mutation occurs in an apical cell, the shoot from that apex may show modified characteristics. Selection of mutants with desirable traits from irradiated plants or their progeny is called “radiation breeding”. This process is highly effective for adding a refinement (e.g., disease resistance, dwarfness, or early flowering) to existing cultivars of perennial crops, especially fruit species (Jain 2002), because both the induced mutation and the original characteristics can be maintained through vegetative propagation (Predieri 2001). In most cases, either whole plants or plant parts are irradiated, and mutated shoots generated from the irradiated tissue are selected. However, chimeric plants consisting of both mutated and non-mutated cells are often produced from irradiated somatic cells (e.g., meristems or asexual embryos). In these cases, a stable mutant needs to be generated from the mutated region by allowing new growth after cutting-back or *in vitro* propagation (van Harten 1998; Predieri 2001). Further, a significant proportion of the mutations in somatic cells are non-heritable. These mutants are considered to have a large deletion involving genes essential for normal growth or seed development, making them unavailable for further use because they

cannot stay alive or reproduce (van Harten 1998). To avoid these problems, pollen irradiation has been used as a method for mutagenesis. Irradiated pollen grains are used to fertilize non-irradiated plants and generate seeds. The seedlings grown from these seeds each contain one or more mutations from a single irradiated pollen grain, but can often survive and reproduce because a complete haploid set of normal genes from the non-irradiated seed parent is also present (Yang et al. 2004; Naito et al. 2005; Ding et al. 2007).

Induction of SC in self-incompatible plants by the use of irradiated pollen was successful in *Oenothera organensis* (Onagraceae; Lewis 1949), sweet cherry (Rosaceae; Lewis and Crowe 1954), *Petunia inflata* (Solanaceae; Brewbaker and Natarajan 1960), and *Nicotiana alata* (Solanaceae; Pandey 1965). In these experiments, X-rays were used to irradiate whole plants containing young flower buds. Mature pollen from the X-rayed flower buds was then used to pollinate the styles of non-irradiated flowers containing the same *S* haplotypes. Only the pollen grains (M0 generation) with a self-compatible mutation in the gene encoding SI would be able to fertilize the style in the nominally incompatible cross, so the plants from any seeds produced (M1 generation) would be likely to be self-compatible, especially pollen-part self-compatible. This overall approach proved to be useful and effective for obtaining self-compatible mutants as a result of selective fertilizations with viable, self-compatible pollen from irradiated SI plants. Following pollen irradiation of sweet cherry, two PPM selections that retained normal stylar function were selected from M1 plants (Lewis and Crowe 1954; Matthews and Lapins 1967). The first pollen-part self-compatible sweet cherry cultivar, ‘Stella’, was developed through cross-breeding using one of the self-compatible mutant selections as the gene source (Lapins 1971). The successful breeding of this cultivar has led to the addition of SC to the objectives of cherry breeding programs in many countries

(Sonneveld et al. 2005).

The aim of the research described in this chapter was to develop a new self-compatible breeding selection of Japanese pear using pollen from a chronically γ-irradiated tree. An offspring from a cross of this pollen onto a style of a non-irradiated tree was identified by cross-compatibility experiments to be pollen-part self-compatible.

2–2 Materials and Methods

2–2–1 Plant materials

Five self-incompatible Japanese pear cultivars, 'Gold Nijisseiki' (*S* haplotype S_2S_4), 'Hosui' (S_3S_5), 'Kosui' (S_4S_5), 'Oushuu' (S_4S_5), and 'Syuugyoku' (S_4S_5), all maintained at the NARO Institute of Fruit Tree Science, were used in this experiment. The accession numbers (Conservation ID) of these cultivars in the National Institute of Agrobiological Sciences (NIAS) Genebank were 20004387, 20001512, 20001533, 20007847, and 20001621, respectively. The *S* genotypes listed for all except 'Oushuu' were previously determined by Cleaved amplified polymorphic sequence (CAPS) analysis (Ishimizu et al. 1999; Castillo et al. 2001); that of 'Oushuu' was initially estimated to be S_4S_5 as a result of cross-pollination experiments (Kotobuki et al. 2004) and confirmed to be S_4S_5 in the present study (section 2−3−3).

2–2–2 Development of progeny using pollen from a γ-irradiated tree

In 1962, a tree of 'Kosui' vegetatively propagated from a scion taken at NARO Institute of Fruit Tree Science was planted in the gamma field of the Institute of Radiation Breeding, NIAS (Hitachiomiya, Ibaraki Prefecture, Japan) at a distance of 77 m from a ^{60}Co source (Fig. 2.1). Thereafter, the tree has been irradiated chronically with γ-rays at

a dose rate of about 3×10^{-3} Gy/h for 19 h/day, 281 days/year from 1962 to 1988 and for 6 h/day, 221 days/year from 1989 to 1993.

In 1993, anthers of the γ-irradiated 'Kosui' were collected from balloon-stage flowers (one day before opening). The pollen was dried and stored at –20°C until use. The pollen germination rate was measured by testing *in vitro* pollen tube growth before storage and one year after storage at –20°C. Pollen was plated on medium (1% agar and 10% sucrose) and incubated according to the general method (Galletta 1983). After 24 h, the number of grains producing pollen tubes was counted under a microscope.

In 1994, 450 balloon-stage flowers of a non-irradiated 'Kosui' tree were pollinated with the pollen of chronically irradiated 'Kosui' collected in 1993. The flowers were covered with paper bags after crossing. In August 1994, the fully developed fruits were harvested and the seeds were collected. The seeds were stored at 4°C and planted the following spring. The single surviving seedling (see Results) was grown in a nursery and then transplanted to a breeding field in 1995. This tree, designated 415-1, was used for the present study.

2–2–3 Self- and cross-pollination tests

The seedling described in section 2–2–2 and self-incompatible pear cultivars whose *S* haplotype had been previously described were used for self- and cross-pollination tests each year from 2002 to 2005. Anthers from the pollen parents were gathered from balloon-stage flowers and dried to collect the pollen. Flowers used as seed parents were emasculated and then pollinated with the collected pollen. The pollinated flowers were covered with paper bags after crossing. About 70 days later, the developed fruits were harvested and seeds were counted. A plant was considered to be compatible if 30% or

more of the pollinated flowers set seeded fruit (Sato 1993).

2–2–4 *S-RNase* and SSR genotyping

Genomic DNA was extracted from fresh young leaves using a modified cetyltrimethylammonium bromide (CTAB) protocol (Yamamoto et al. 2000). The *S-RNase* genotypes of Japanese pear cultivars were determined by CAPS analysis according to the protocol of Ishimizu et al. (1999) with some modifications. PCR amplification of the *S-RNase* alleles was performed in a total volume of 20 µL of 1× *Ex Taq* PCR buffer (TaKaRa Bio, Japan) containing 0.2 mM of each dNTP and 0.5 µM each of the forward and reverse primers, namely, "FTQQYQ" (5′-TTTACGCAGCAATATCAG-3′) labeled with 5-carboxyfluorescein (FAM) and "anti-IIWPNV" (5′-AC(A/G)TTCGGCCAAATAATT-3′) labeled with hexachloro-6-carboxyfluorescein (HEX). PCR was carried out for 10 cycles of 94°C for 15 s, 48°C for 30 s, and 70°C for 2 min, followed by 20 cycles of 94°C for 15 s, 48°C for 30 s, and 70°C for 2.5 min, with a final extension at 70°C for 7 min. The amplified fragments were digested with *Nde*I for S_4, *Alw*NI for S_5, and *Ppu*MI for S_3 and S_5. The fragments before and after digestion with restriction endonucleases were separated and detected using an ABI PRISM 377 DNA sequencer (Applied Biosystems, USA). The size of fragments were calculated by comparison to an internal DNA marker standard (GeneScan-500TAMRA [Applied Biosystems]) with GeneScan software (Applied Biosystems).

Seventeen simple sequence repeat (SSR) markers previously developed and positioned on the genetic linkage maps of pear (BGT23b, NB103a, NB104a, NB105a, NB113a, NB114a, NB135a, NB141a, NH007b, NH009b, NH011b, NH025a, NH029a, NH039a, NH046a, NH201a and NH207a; Yamamoto et al. 2002a, 2002b, 2004, 2007)

were used for the analysis of 415-1. PCR amplification was performed in 10 μL of *Ex Taq* PCR buffer containing 0.2 mM of each dNTP, 0.5 μM of each forward primer labeled with a fluorescent chemical (5-FAM, tetrachloro-6-carboxyfluorescein (TET), HEX, or 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC)) and unlabeled reverse primer, 5 ng of genomic DNA, and 0.25 U of *Ex Taq* polymerase. PCR was carried out for 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, and extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min. The PCR products were separated and detected using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The sizes of the amplified bands were calculated by comparison to an internal DNA size standard (GeneScan 400 HD ROX Size Standard [Applied Biosystems]) with the GeneScan software. Allele size data (in bp) were rounded down to the nearest whole number.

2–3 Results

2–3–1 Breeding selection derived from pollen of a chronically γ-irradiated 'Kosui' tree

Pollen germination rates of the sample collected from chronically irradiated 'Kosui' were 77.0% just after collection and 55.3% after one year of preservation. Emasculated 'Kosui' flowers were pollinated using the preserved pollen after its viability was confirmed in the germination test. Of the 450 pollinated flowers, only two produced fruits with seeds, and each of these fruits had one seed. The two seeds were planted in the nursery in 1995, but only one seedling survived. This selection was named 415-1 and used in the studies reported here.

415-1 showed depressed vegetative growth compared with the seedlings from

crosses between non-irradiated self-incompatible cultivars, but it started to flower and fruit in 2000. The following characteristics of 415-1 are distinct from the parent cultivar ‘Kosui’: (1) small flower size, (2) late flowering time, (3) a low amount of pollen, (4) small fruit size, and (5) green fruit skin (Fig. 2.2).

2–3–2 Self-fertility of 415-1

In 2002, self-pollination tests of 415-1 were initiated to assess the level of SC. Over four years, 90 flowers of 415-1 were self-pollinated, and the rate of seeded fruit set was 74.4% (Table 2.1). In comparison, control self-pollination tests with the original ‘Kosui’ cultivar resulted in only 5.0% seeded fruit set.

2–3–3 *S-RNase* and SSR genotype of 415-1

The *S-RNase* genotypes of 415-1 and of the self-incompatible cultivar ‘Oushuu’ were determined by CAPS analysis (Table 2.2). Two PCR products of 368 bp and 376 bp were amplified with the primers ‘FTQQYQ’ and ‘anti-IIWPNV’ from 415-1 and ‘Oushuu’. Digestion with the S_4-specific endonuclease *Nde*I cut the 368-bp product into two fragments, 140 bp and 228 bp, respectively. Similarly, digestion with the S_3- and S_5-specific endonuclease *Ppu*MI or the S_5-specific endonuclease *Alw*NI cut the 376-bp product into two fragments, 113 bp and 263 bp, respectively. By digestion with a combination of *Nde*I and *Alw*NI, all of the amplified products of 415-1 and ‘Oushuu’ were cut into four fragments, 113 bp, 140 bp, 228 bp, and 263 bp, indicating that the *S-RNase* genotypes of 415-1 and ‘Oushuu’ were both S_4S_5.

Among the 17 SSR markers tested, seven (NB105a, NB113a, NB114a, NH007b, NH011b, NH025a, and NH046a) showed heterozygosity in ‘Kosui’. These seven markers

were used for parental analysis of 415-1 and ‘Kosui’. For three of the markers (NB114a, NH007b, and NH025a), 415-1 had the same genotypes as the parent. For the other four markers, 415-1 was homozygous for one of the two alleles of the parent ‘Kosui’ (Table 2.3). These results are consistent with 415-1 being derived from a (mutated) pollen grain of ‘Kosui’ pollinating a style of ‘Kosui’.

2–3–4 Stylar and pollen self-incompatibility phenotype of 415-1

Styles and pollen of 415-1 were used for pollination experiments to examine the self-(in)compatibility phenotype of each tissue. Styles of 415-1 (S_4S_5) were pollinated with pollen from ‘Syuugyoku’ (S_4S_5) and ‘Oushuu’ (S_4S_5). The following control crosses were also performed: ‘Kosui’ (S_4S_5) × ‘Oushuu’, ‘Gold Nijisseiki’ (S_2S_4) × ‘Oushuu’, and ‘Hosui’ (S_3S_5) × ‘Oushuu’ (30 crosses each). No seeded fruit were obtained from either the 415-1 × ‘Syuugyoku’ or the 415-1 × ‘Oushuu’ crosses, and only one seeded fruit was obtained from the ‘Kosui’ × ‘Oushuu’ crosses (Table 2.4). On the other hand, high rates of seeded fruit set were obtained from the ‘Gold Nijisseiki’ × ‘Oushuu’ and ‘Hosui’ × ‘Oushuu’ crosses, indicating that the pollen of ‘Oushuu’ was viable.

Pollen from 415-1 was used to pollinate 105 ‘Syuugyoku’ flowers and 42 ‘Oushuu’ flowers. After 70 days, these pollinations resulted in 60 seeded fruits from ‘Syuugyoku’ and 32 seeded fruits from ‘Oushuu’ (57.1% and 76.2% seeded fruit set, respectively) (Table 2.5). The control cross (‘Oushuu’ × ‘Kosui’; 80 flowers) resulted in only two fruits with seeds (2.5% seeded fruit set).

2–4 Discussion

2–4–1 Acquisition of a new self-compatible mutant from pollen of γ-irradiated tree

of Japanese pear

Only two seeds were obtained when pollen of chronically γ-irradiated ‘Kosui’ was used to pollinate non-irradiated ‘Kosui’, even though the pollen was viable. This result confirmed that the original ‘Kosui’ cultivar was strictly self-incompatible. The one surviving selection, named 415-1, was genotyped by *S-RNase* and SSR markers. The *S-RNase* genotype of 415-1 was determined to be S_4S_5, the same as that of the parent ‘Kosui’. Moreover, the SSR genotypes of 415-1 were heterozygous (like the parent) for three loci, and were homozygous for a parental allele at four other loci. These results indicate that 415-1 was generated as a result of selective fertilization with a rare self-compatible pollen grain from γ-irradiated ‘Kosui’ (S_4S_5) pollinating the style of non-irradiated ‘Kosui’. Thus, use of irradiated pollen to pollinate ‘‘cross-incompatible’’ styles (i.e., having the same *S* haplotypes as the male parent) successfully selected a self-compatible mutant pollen grain from among large numbers of non-mutant pollen grains in Japanese pear. This method can be applied to selection of self-compatible mutants in other self-incompatible fruits.

2–4–2 Pollen-type self-compatible phenotype of 415-1

Self-pollination tests revealed a high level of fruit set in 415-1 (74.4%), in comparison with the low level obtained in self-incompatible ‘Kosui’ (5.0%). Generally, compatible crosses in Japanese pear give 30% or more of fruit set (Sato 1993). Therefore, the fruit set percentage derived from the selfing of 415-1 was in the range for compatible crosses, and was enough for 415-1 to be characterized as self-compatible.

Cross-compatibility tests using the styles of 415-1 and the pollen of two self-incompatible cultivars containing the same *S* alleles revealed that the styles of 415-1 maintained the same *S*-allele-specific rejection of S_4 and S_5 pollen found in ‘Kosui’. On

the other hand, cross-compatibility tests revealed that the pollen of 415-1 was not rejected by the styles of self-incompatible cultivars containing the same *S* alleles. Thus, only the pollen function of SI was affected by γ-induced mutagenesis in 415-1. This situation differs from that of SPM cultivar 'Osa-Nijisseiki', a spontaneous mutant of 'Nijisseiki' that lacks the stylar function of SI but retains the pollen function (Sato 1993). In conclusion, the selection 415-1 is deemed to be a PPM. PPMs are expected to produce omni-potential pollen (i.e., pollen compatible with the self-incompatible styles of all *S* haplotypes including their own).

High frequencies of seeded fruit set were obtained by both self-pollination of 415-1 and cross-pollinations between pollen from 415-1 and the styles of self-incompatible cultivars with the S_4S_5 haplotype (Tables 2.1 and 2.5). In European pear (*Pyrus communis*), triploid cultivars were found to be slightly self-compatible, but exhibited poor seed development thought to be caused by the low fertility of both pollen and egg cells (Crane and Lewis 1942). Thus, 415-1 did not appear to be a triploid derived from unreduced diploid pollen of irradiated 'Kosui'. PPMs generated in previous studies using irradiated pollen in *S*-RNase–based self-incompatible plants in Solanaceae were diploid, aneuploid, or tetraploid (Pandey 1967; de Nettancourt et al. 1971). Hence, flow cytometric analysis was performed to determine whether 415-1 was diploid (Chapter 3), which would make it more useful as a genetic resource for breeding of self-compatible cultivars. Moreover, genetic analysis of the pollen-part SC of this mutant (Chapters 3–5) was performed to elucidate the integral factors controlling pollen-part SI in the Pyrinae.

Table 2.1 Evaluation of self-compatibility of 415-1 and ‘Kosui’ by self-pollination

Selection/cultivar	No. of pollinated flowers	No. of fruit with seeds	Rate of seeded fruit set (%)	Self-compatibility[a]
‘Kosui’	60	3	5.0	SI
415-1	90	67	74.4	SC

[a] SC, self-compatible (30% or more seeded fruit set); SI self-incompatible (less than 30% seeded fruit set).

Table 2.2 *S-RNase* genotypes of 415-1 and other Japanese pear cultivars, determined by CAPS

Selection/cultivar	*S*-allele-specific restriction endonucleases				*S-RNase* genotype
	*Ppu*MI (S_3, S_5 specific)	*Nde*I (S_4 specific)	*Alw*NI (S_5 specific)	*Nde*I and *Alw*NI (S_4 and S_5)	
415-1	+	+	+	++	S_4S_5
'Kosui'	+	+	+	++	S_4S_5[a]
'Syuugyoku'	+	+	+	++	S_4S_5[b]
'Oushuu'	+	+	+	++	S_4S_5

[a] Data from Ishimizu et al. (1999).

[b] Data from Castillo et al. (2001).

+: One of the two amplified *S*-RNase fragments was digested by the restriction endonuclease(s).

++: Both of the amplified *S*-RNase fragments were digested by the restriction endonuclease(s).

Table 2.3 SSR genotypes of 415-1 and its parent ‘Kosui’

Selection/cultivar	SSR name[a]							
	NB105a		NB113a		NB114a		NH007b	
‘Kosui’	147	161	138	150	129	131	126	154
415-1	161	161	150	150	129	131	126	154

Selection/cultivar	SSR name[a]					
	NH011b		NH025a		NH046a	
‘Kosui’	178	186	67	98	130	144
415-1	178	178	67	98	144	144

[a] Numbers below SSR names indicate allele sizes in bp.

Table 2.4 Evaluation of the stylar-part self-compatibility phenotype of 415-1

Seed parent (*S* haplotype)[a]	Pollen parent (*S* haplotype)	No. of pollinated flowers	No. of fruit with seeds	Rate of seeded fruit set (%)	Compatibility[b]
415-1 (S_4S_5)	'Syuugyoku' (S_4S_5)	32	0	0.0	IC
415-1 (S_4S_5)	'Oushuu' (S_4S_5)	40	0	0.0	IC
'Kosui' (S_4S_5)	'Oushuu' (S_4S_5)	30	1	3.3	IC
'Gold Nijisseiki' (S_2S_4)	'Oushuu' (S_4S_5)	30	28	93.3	C
'Hosui' (S_3S_5)	'Oushuu' (S_4S_5)	30	22	73.3	C

[a] *S* haplotype assigned by PCR amplification of *S-RNase* alleles (Table 2.2).

[b] C, cross-compatible (30% or more of seeded fruit set); IC, cross-incompatible (less than 30% of seeded fruit set).

Table 2.5 Evaluation of the pollen-part self-incompatibility phenotype of 415-1

Seed parent (*S* haplotype)[a]	Pollen parent (*S* haplotype)	No. of pollinated flowers	No. of fruit with seeds	Rate of seeded fruit set (%)	Compatibility[b]
‘Syuugyoku' (S_4S_5)	415-1 (S_4S_5)	105	60	57.1	C
‘Oushuu’ (S_4S_5)	415-1 (S_4S_5)	42	32	76.2	C
‘Oushuu’ (S_4S_5)	‘Kosui’ (S_4S_5)	80	2	2.5	IC

[a] *S* haplotype assigned by PCR amplification of *S-RNase* alleles (Table 2.2).

[b] C, cross-compatible (30% or more seeded fruit set); IC, cross-incompatible (less than 30% seeded fruit set).

Fig. 2.1 A chronically γ-irradiated tree of Japanese pear cultivar ‘Kosui’ treated at a distance of 77 m from a ^{60}Co source in the gamma field of the Institute of Radiation Breeding (IRB) of NIAS. In 1993, this tree provided the pollen used to generate 415-1. The photograph was taken by Dr. Haji of IRB on April 20, 2015.

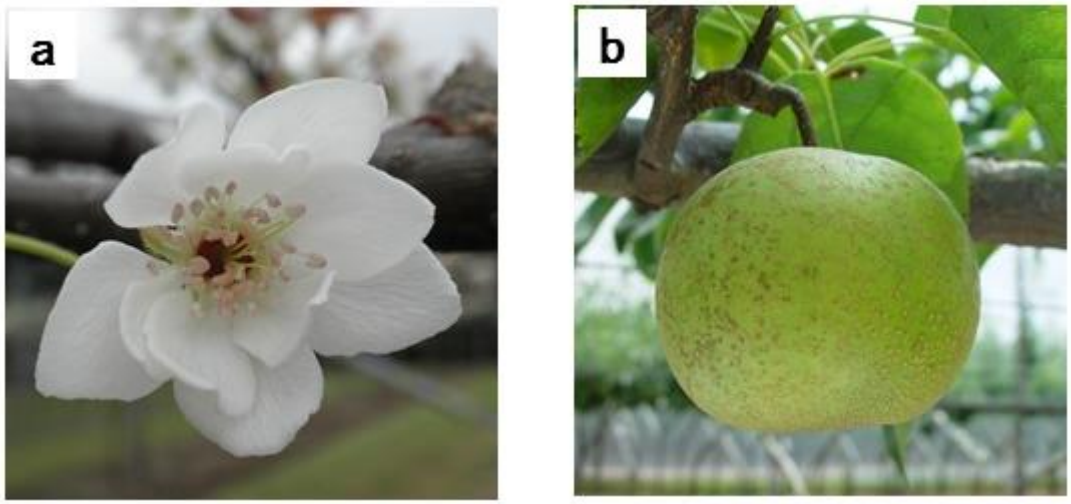

Fig. 2.2 A flower (a) and a mature fruit (b) of 415-1. The photographs were taken at NARO Institute of Fruit Tree Science on April 23, 2010, and August 18, 2010, respectively.

Chapter 3

A segmental duplication encompassing the *S* haplotype triggers pollen-part self-compatibility in Japanese pear

3–1 Introduction

A self-compatible mutant selection of Japanese pear (designated 415-1) with apparent S_4- and S_5-*RNase* genotypes was identified in the progeny of a cross using pollen from a tree continuously exposed to low-dose-rate γ-irradiation. Reciprocal crosses with self-incompatible cultivars with the same *S* alleles indicated that this mutant is a PPM that lost its pollen SI function but retained its stylar SI function (Chapter 2). However, since the pollen of a PPM is cross-compatible with the styles of all *S-RNase* genotypes, the identity of the pollen *S* allele mutated in 415-1 could not be determined by cross-compatibility tests.

In the Pyrinae (family Rosaceae) and Solanaceae, polyploid mutants with more than two *S* alleles exhibit pollen-part SC (Lewis and Modlibowska 1942; de Nettancourt et al. 1971; Adachi et al. 2009). Furthermore, diploid mutants with a duplicated *S* allele obtained in the progenies of irradiated *S*-heterozygous diploids also become pollen-part self-compatible in the Solanaceae (Brewbaker and Natarajan 1960; Pandey 1965). This phenomenon was termed “competition” or “competitive interaction (CI)” by Lewis and Modlibowska (1942) and Lewis (1952) because they hypothesized that both pollen *S* functions would be broken down when two different alleles existed in a single pollen grain. In the species that have PPM caused by a duplicated *S* haplotype, multiple pollen *S*-related F-box genes (called *SFBB*, *SLF*, or *SLF*-like genes) were identified. These genes exhibit specific expression in pollen and have allele-specific diversity among *S*

haplotypes (Sassa et al. 2007; Kubo et al. 2010; Minamikawa et al. 2010; de Franceschi et al. 2011).

Recently, Kubo et al. (2010) constructed petunia transformants that produced pollen with a transgenic copy of *S*$_7$*-SLF1* (one of the *SLF*-like genes of the *S*$_7$ haplotype) and reported that pollen with an *S*$_9$ haplotype (and also containing the *S*$_7$*-SLF1* transgene) induced SC whereas pollen with an *S*$_5$, *S*$_{11}$, or *S*$_{19}$ haplotype (and the *S*$_7$*-SLF1* transgene) did not. Furthermore, Kakui et al. (2011) and Saito et al. (2012) reported that the pollen of a stylar-part self-compatible mutant cultivar 'Osa-Nijisseiki' of Japanese pear that lacks both *S*$_4$*-RNase* and *PpSFBB*$^{4–d1}$ (one of the *Pyrus pyrifolia SFBB* genes) (Okada et al. 2008, 2011) and contains a mutated *S*$_4$ (*S*$_4^{sm}$) haplotype showed cross-incompatibility with a style harboring a non-*S*$_4$ (e.g., *S*$_1$) haplotype.

From these results, Kubo et al. (2010) hypothesized the existence of a "collaborative non-self recognition system" in which, in species within the Solanaceae and Pyrinae, each of the SLFs/SFBBs can degrade only some of the non-self *S*-RNases, but in which only the complete set of SLFs/SFBBs in the *S* haplotypes of a given species is capable of degrading all of the non-self *S*-RNases (Fig. 1.2). If this hypothesis is correct, PPMs with a duplicated *S* haplotype could produce pollen grains harboring two different *S* haplotypes that together can degrade all *S*-RNases, including their own, and become self-compatible.

On the other hand, there are some reports in tetraploid species in *Prunus* that pollen with two different *S* haplotypes does not show CI (Bošković et al. 2006; Hauck et al. 2006b). Instead, PPMs in *Prunus* have mutations in one of the *SLF* genes (Hauck et al. 2006a; Hanada et al. 2009). Thus, in these instances the functional SLF/SFB is considered to be the sole pollen *S* factor, and a specific *S* haplotype can become self-compatible by silencing or dysfunction of the pollen *S* factor (Yamane et al. 2003; Ushijima et al. 2004).

Until now, all of the known PPMs in the Pyrinae have been tetraploids (Crane and Lewis 1942; Adachi et al. 2009; Tahira et al. 2010). Because of their ploidy, these materials are unsuitable for use in research to identify the gene responsible for pollen-part SI, and they are difficult to use in breeding to generate self-compatible cultivars by crossing with normal diploids. On the other hand, diploid PPMs with mutations in the gene for pollen recognition or with segmental duplications involving the *S*-locus could contribute to the identification of genomic sequences and gene functions that control pollen-part SC as well as providing suitable material for cultivar improvement.

The purpose of the studies in this chapter was to elucidate the cause for SC in 415-1 by determining whether it was caused by a loss of function of a pollen *S* factor or by CI in *S*-heteroallelic diploid pollen (produced by polyploidization or segmental duplication involving the *S*-locus. The following analyses were performed: (1) DNA ploidy analysis, (2) segregation analysis of *S* haplotypes, and (3) segregation analysis of genetic markers around the *S*-locus.

3–2 Materials and methods

3–2–1 Preparation of self and outcross progenies

The PPM selection 415-1 was generated as a result of selective fertilization with self-compatible pollen from γ-irradiated 'Kosui' (S_4S_5) by pollinating the style of non-irradiated 'Kosui' (Chapter 2). Then, three populations were generated from crosses using 415-1 as the pollen parent in order to identify the pollen-part mutated allele (Table 3.1). A breakdown of pollen-part SI in 415-1 is postulated to be brought by either a mutation in a non-*S*-locus gene, or in a pollen-determinant of the *S*-locus or a duplication of either the S_4 or S_5 haplotype.

First, if 415-1 is self-compatible by a mutation in a non-*S*-locus gene, compatible pollen would contribute to fertilization independent of their *S* haplotype in a cross with a cultivar containing the same haplotypes, *S* haplotypes of the progeny would segregate approximately 1 S_4S_4 : 2 S_4S_5 : 1 S_5S_5.

Second, if 415-1 is self-compatible because of a mutation in one of the putative pollen factors of either the S_4 or S_5 haplotype, only pollen grains having a self-compatible allele would contribute to fertilization in a cross with a cultivar containing the same haplotypes, and the apparent *S* haplotypes of the progeny (estimated from the genotype of markers representative of each *S* haplotype), would segregate 1:1 for "homozygous" individuals (actually containing one mutant allele and one wild-type allele of the same *S* haplotype) and heterozygous individuals (containing one mutant and one wild-type allele of different *S* haplotypes).

Finally, if 415-1 is self-compatible because of duplication of the *S* haplotype, only the S_4S_5 heteroallelic pollen grains would contribute to fertilization of a cultivar containing the same (S_4 and S_5) haplotypes, and all of the progeny would be S_4S_5 heterozygous. In addition, some plants with three *S* haplotypes would be obtained in the progeny of crosses to cultivars that share no *S* haplotype with 415-1 (i.e., that contain neither the S_4 nor the S_5 haplotype).

Therefore, two populations, (1) 23 self-progeny of 415-1 and (2) 63 F_1 plants obtained from a cross of 415-1 to the self-incompatible cultivar 'Syuugyoku' (S_4S_5), were used to determine the type of self-compatible allele present in 415-1. In addition, 103 F_1 plants obtained from a cross to the self-incompatible cultivar 'Niitaka' (S_3S_9), which does not have any *S* haplotypes in common with 415-1, were used to further examine the inheritance of the self-compatible allele (Table 3.1).

The accession number (Conservation ID) of 'Niitaka' in the NIAS Genebank is 20001544. Two *S*-homozygous selections 421-6 (S_4S_4) and 421-24 (S_5S_5), self-progeny of 'Shinsui' (S_4S_5) (Saito et al. 2005), were used as a control for complete digestion of S_4- and S_5-specific fragments, respectively.

3–2–2 DNA extraction

Genomic DNA was extracted using a FastDNA kit (MP Biomedicals, USA) according to the manufacturer's instructions, except that 10 mg polyvinylpyrrolidone (insoluble) and 30 µL 2-mercaptoethanol were added to Cell Lysis Solution (0.8 mL CLS-VF, 0.2 mL PPS) [reagents in the FastDNA kit] in the initial homogenization step.

3–2–3 Determination of the *S* haplotypes of self and outcross progenies

CAPS analysis was used to determine the *S-RNase* and *PpSFBB*$^{-\gamma}$ (one of the *PpSFBB* genes) alleles present in the *S* haplotypes of the parents and progenies described above. The results of this analysis are referred to here as "electrophoretic *S*-phenotypes".

CAPS analysis of *S-RNase* followed the procedure of Takasaki et al. (2004). Partial sequences of the *S-RNase* gene were amplified by PCR using FTQQYQ and anti-(I/T)IWPNV primers (5′-AC(A/G)TTCGGCCAAATA(A/G)TT-3′). *S-RNase* allele-specific fragments were detected by digestion with *Nde*I for S_4, *Alw*NI for S_5, and a combination of *Nde*I and *Alw*NI for S_3 and S_9.

CAPS analysis of *PpSFBB*$^{-\gamma}$ followed the procedure of Kakui et al. (2007). Partial sequences of *PpSFBB*$^{-\gamma}$ were amplified by PCR using the primers PpFBXf7 and PpFBXr3. *PpSFBB*$^{-\gamma}$ allele-specific fragments were detected by digestion with *Nsp*I for S_4, *Afl*II for S_5, *Hae*III for S_9, and *Hpy*CH4IV for S_3 (Dr. H. Kakui [University of Zurich], personal

communication).

PCR amplification was performed in a total volume of 20 μL of 1× *Ex Taq* PCR buffer containing 15 ng of genomic DNA, 1 U of TaKaRa *Ex Taq* polymerase, 0.2 mM of each dNTP, and 0.5 μM of each forward primer and reverse primer. PCR was carried out for 30 cycles of denaturation at 94°C for 15 s, annealing at 54°C for 15 s for *S-RNase* or 59°C for 15 s for *PpSFBB*$^{-\gamma}$, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 7 min. The PCR products were incubated with the specified endonucleases for 16 h at 37°C. These products were separated on 2% agarose gels in TBE buffer and visualized with ethidium bromide. The goodness of fit of the progeny segregation ratios to the expected Mendelian ratios was tested using binomial or multinomial exact tests.

3–2–4 Estimation of ploidy level

Flow cytometry was performed to measure the relative nuclear DNA content of 415-1 and 'Kosui' (diploid) using maize (*Zea mays*) inbred line B73, which has an actual nuclear DNA content of 2.3 Gbp (Schnable et al. 2009), as an internal standard. Cuttings of pear and seedlings of maize were grown in a growth chamber (20°C, 12-h light period). About 0.15 cm^2 of pear leaf and 1.5 cm^2 of maize leaf were placed together in 400 μL of extraction buffer from the Partec CyStain UV Precise P kit (Sysmex Partec GmbH, Germany) with 1% polyvinylpyrrolidone K-30 (Wako Pure Chemical, Japan) and chopped using a razor blade. The suspension was incubated for 1 min at 4°C and then filtered through a 30-μm nylon mesh (Sysmex Partec GmbH). Then, 1.6 mL of staining buffer containing 4,6-diamidino-2-phenylindole (DAPI) was added and the mixture was incubated for 5 min at 4°C in the dark. Data were collected for approximately 5000 nuclei

per sample using a flow cytometer (Ploidy Analyzer PA-II, Sysmex Partec GmbH) with UV excitation at 366 nm from a mercury arc lamp. The histograms were generated on a linear scale, and 15 measurements with coefficients of variation (CVs) smaller than 7% were obtained by using five different leaves from each pear cultivar on three different days. Comparisons of ploidy levels between the two pear cultivars were expressed in arbitrary units (AU) that represent the ratio (%) of the mode value of fluorescence intensity of the G_0/G_1 peak of pear to that of the internal standard (maize) in each measurement.

3–2–5 SSR genotyping

Twenty-seven SSR markers, previously developed and mapped to the same linkage group as the *S* haplotype (linkage group [LG] 17) of pear or apple (*Malus* × *domestica*), were tested for the analysis of 415-1. These SSR markers consisted of 15 pear SSRs (HGT6, NH008b, NH014a, NH015a, NB125a, TsuENH002, TsuENH026, TsuENH028, TsuENH033, TsuENH071, and TsuENH080 [Yamamoto et al. 2002a, 2002b, 2007; Nishitani et al. 2009], and TsuENH104, TsuENH114, TsuENH154, and TsuENH163 [GenBank accession numbers AB735182, AB735183, AB735184, and AB735185, respectively]) and 12 apple SSRs (CH01b12, CH01h01, CH05g03, CH04c10, CN444542SSR, AF527800SSR, AT000174SSR, AJ001681SSR, AY187627SSR, NZmsMDAJ1681, NZmsEB137525, and NZmsEE663955 [Gianfranceschi et al. 1998; Liebhard et al. 2002; Silfverberg-Dilworth et al. 2006; Celton et al. 2009]).

PCR amplification was performed in 10 μL *Ex Taq* PCR buffer containing 0.2 mM of each dNTP, 0.5 μM of each forward primer labeled with a fluorescent chemical (FAM, TET, HEX, or VIC) and unlabeled reverse primer, 5 ng of genomic DNA, and 0.25 U of

Ex Taq polymerase. PCR was carried out for 30 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 15 s, and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 7 min. The PCR products were separated and detected using an ABI PRISM 3100 Genetic Analyzer. The sizes of the amplified bands were calculated by comparison to an internal DNA size standard (GeneScan 400 HD ROX Size Standard) with the GeneScan Software. Allele size data (in bp) were rounded down to the nearest whole number. The frequencies of recombination between the *S-RNase* gene and SSR markers were translated into genetic distances using the Kosambi map function (Kosambi 1944), and the linkage phase of markers within LG 17 was determined.

3–3 Results

3–3–1 Segregation of *S-RNase* and *PpSFBB*$^{-\gamma}$ in the progenies of crosses involving 415-1

All 23 self-progeny of 415-1 were S_4S_5 heterozygous for both *S-RNase* (Fig. 3.1a). Out of 63 F_1 progeny of 'Syuugyoku' (S_4S_5) × 415-1, 60 were S_4S_5 and 3 were homozygous for S_5 (Fig. 3.1b). In short, almost all progenies from either self-pollination of 415-1 or cross-pollination with a cultivar with the same *S* haplotypes (S_4S_5) were heterozygous. These segregation patterns did not fit the hypothesis that the PPM in 415-1 was caused by a loss of function of pollen *S* factor S_4 or S_5 (Table 3.1).

In 103 F_1 progeny obtained from a cross with the self-incompatible cultivar 'Niitaka' (S_3S_9), there was no recombination between the two CAPS markers (*S-RNase* and *PpSFBB*$^{-\gamma}$) in the *S* haplotype. The electrophoretic *S*-phenotypes segregated in a ratio of 2 S_3S_4 : 40 S_3S_5 : 0 S_4S_9 : 47 S_5S_9 : 7 $S_3S_4S_5$: 7 $S_4S_5S_9$ (Table 3.1, Fig. 3.2). Thus, 14% of the plants were triallelic for the *S* haplotype.

3–3–2 Ploidy level

Simultaneous analysis of pear and maize (internal standard) DAPI-stained nuclei in suspension produced histograms of fluorescence intensity with two peaks corresponding to the relative DNA content of the G_0/G_1 nuclei of both plants. The mean AU of 415-1 was 34.97 ± 1.36% (CV = 5.80 ± 0.91%), and the mean AU of diploid cultivar 'Kosui' was 34.51 ± 1.81% (CV: 5.55 ± 0.75%). There was no significant difference in the ploidy status between 415-1 and 'Kosui' (Student's *t*-test, Table 3.2).

3–3–3 Segregation of SSR markers on LG 17

Among the 27 SSR markers we tested in LG 17, five (TsuENH154, CH04c10, NH014a, TsuENH028, and NZmsEB137525) showed heterozygosity in 415-1. These five markers were used to genotype the progeny from the cross of 'Niitaka' (S_3S_9) × 415-1. From this analysis, linkage maps of the S_4 and S_5 haplotypes were constructed (Fig. 3.3).

All 14 plants that were triallelic for the *S* haplotype contained two alleles of NZmsEB137525 from the pollen parent 415-1 and one allele from the seed parent. For SSR markers NH014a and TsuENH028, 13 of the 14 plants contained two alleles from 415-1 and 1 contained only one allele from 415-1. On the other hand, at loci TsuENH154 and CH04c10, all 14 plants had just two alleles, one from the pollen parent and the other from the seed parent.

Two-point genetic linkage analysis was performed with the *S-RNase* gene and the five informative SSR markers by using the 89 progeny from the cross of 'Niitaka' (S_3S_9) × 415-1 that each had only two *S* alleles, one from each parent. With this information, local haplotype maps of the region around the *S*-locus of 415-1 were constructed (Fig.

3.3). In 415-11, SSR markers NZmsEB137525, NH014a, and TsuENH028 were completely linked to the *S*-locus (i.e., no recombination was observed between the *S* haplotype from 415-1 and these markers). In contrast, CH04c10 and TsuENH154 were located 63.5 cM and 67.9 cM, respectively, from the *S*-locus. The genetic distance between CH04c10 and TsuENH154 was 4.5 cM. In 'Niitaka', NZmsEB137525 was completely linked to the *S*-locus. However, segregation between the *S*-locus and markers NH014a and TsuENH028 was observed; these markers were estimated to be 2.2 cM from the *S*-locus. Genetic distances between the other two markers (CH04c10 and TsuENH154) and the *S*-locus could not be calculated for haplotypes from 'Niitaka' because their genotypes were homozygous.

3–4 Discussion

3–4–1 Pollen-part self-compatibility of 415-1 caused by duplication of an *S* haplotype

In this study, the reason for the SC of 415-1 was determined by analyzing the segregation of *S* haplotypes in the progeny and comparing the segregation ratios with the hypothesis of a pollen S_4 or S_5 mutation. All 23 progeny from self-pollination of 415-1 (S_4S_5) and 60 out of 63 progeny from cross-pollination to the cultivar 'Syuugyoku' (also S_4S_5) were heterozygous S_4S_5 (Table 3.1); only 3 plants (from the cross) were homozygous (S_5S_5). These relatively rare homozygous plants are assumed to be from S_5 pollen that escaped degradation, as was previously reported in PPMs of *Nicotiana alata* (Pandey 1967), rather than from self-compatible pollen containing a mutated S_5 factor. Instead, the observed segregation patterns support the hypothesis that PPM of 415-1 was caused by the duplication of an *S* haplotype.

The inheritance of *S*-locus alleles from both parents was determined (Table 3.1) by analyzing the progeny obtained from a cross with the self-incompatible cultivar 'Niitaka' (S_3S_9), which does not share any *S* haplotypes with 415-1. In the progeny, some plants contained three *S* alleles: one allele (either S_3 or S_9) from the seed parent and both of the alleles (S_4S_5) from the pollen parent. Therefore, 415-1 had the ability to produce S_4S_5 pollen as well as S_4 and S_5 pollen. When 415-1 was used to pollinate a cultivar having the same *S* haplotype (S_4S_5), only the S_4S_5 heteroallelic pollen would have been able to complete fertilization; thus, the S_4S_5 heteroallelic progeny produced in such crosses are assumed to have been the product of an S_4 or S_5 egg cell and S_4S_5 pollen. Such progeny would in fact have three *S* alleles and their true genotypes would be $S_4S_4S_5$ or $S_4S_5S_5$.
In summary, the pollen-part SC of 415-1 was not caused by a mutation of a pollen *S* factor in either *S* haplotype, but instead because a duplicated *S* haplotype made it possible for 415-1 to produce *S*-heteroallelic pollen that expresses CI.

3–4–2 Segmental duplication of an LG 17 region containing the *S* haplotype

415-1 was estimated to be diploid because it showed no significant difference in relative nuclear DNA content from the diploid cultivar 'Kosui' (Table 3.2). As a diploid, 415-1 would be expected to produce haploid pollen and diploid progeny. Nevertheless, two *S* alleles from 415-1 were detected in some of its progeny (Table 3.1). This indicates that at least part of the chromosome corresponding to LG 17, including the *S* haplotype, was duplicated, though the duplication was undetectable by flow cytometric analysis.

The haplotype linkage maps of LG 17 in 415-1 were very similar to that of the normal self-incompatible cultivar 'Hosui' (Terakami et al. 2009) (Fig. 3.3). Therefore, the chromosome corresponding to LG 17 of 415-1 is considered to be similar in overall

structure to that of normal cultivars.

Three of the five informative SSR markers in LG 17 showed complete or very close linkage to the *S*-locus in 415-1. For these three markers, both of the alleles from 415-1 were detected in the progeny with the duplicated *S* haplotype. This indicates that the duplicated part of the chromosome includes at least the *S*-locus and these three markers. In a previous molecular genetic analysis of *S* haplotypes of Japanese pear (Kakui et al. 2011), *SFBB* genes were mapped within a 560-kb chromosome region containing *S-RNase* genes. In European pear, *SFBB-gamma* genes were located 0.4 or 0.5 cM distal to *S-RNase* genes (de Franceschi et al. 2012). In the present study, no recombination between *S-RNase* and *PpSFBB*$^{-\gamma}$ was observed in 103 F_1 plants from the 'Niitaka' × 415-1 cross. Therefore, based on the results from our crossing experiments, the duplicated region that encompasses recombination between the *S*-locus and the SSR loci would contain all of the genes involved in CI between the S_4 and S_5 haplotypes.

Partial chromosomal duplication involving an *S*-locus has been reported in PPMs in petunia (Brewbaker and Natarajan 1960) and tobacco (Pandey 1965; Golz et al. 1999); these duplications were produced by X-ray or γ-ray irradiation of pollen. PPMs with an extra *S* allele often had an additional small chromosome, called a centric fragment (Brewbaker and Natarajan 1960). More recently, the presence of an *S*-gene in a centric fragment has been detected by fluorescence *in situ* hybridization (FISH) analysis (Golz et al. 2001). On the other hand, PPMs without centric fragments have also been found (Pandey 1965; Golz et al. 1999); in these cases, the extra *S* allele was either translocated to a non-homologous chromosome or inserted next to the original *S*-locus (Golz et al. 2001). Consequently, these three cases (i.e., existence as a centric fragment, translocation to a non-homologous chromosome, or linkage to the original *S*-locus) should be

considered for the interpretation of our results.

If the duplicated segment identified in this study is present as a centric fragment or as part of a non-homologous chromosome, it would be inherited independently from LG17, and offspring inheriting the original S_4 or S_5 haplotype from 415-11 would occur in equal frequencies. For example, if 415-1 has a single copy of a duplicated S_5 haplotype (designated dS_5) unlinked to the normal *S*-locus, the expected segregation ratio of the pollen *S* haplotype would be S_4: S_5: S_4dS_5: S_5 dS_5 = 1 : 1 : 1 : 1 (Table 3.3). However, the probability of inheriting a duplicated *S* haplotype is often less than that of a normal *S* haplotype (Pandey 1967). Thus, if the probability of inheriting dS_5 is represented as x ($0 \leq x \leq 1$, where 1 means transmission comparable to that of a non-duplicated allele), and the deletion of dS_5-containing pollen occurs with probability $(1–x)$, the segregation ratio of pollen *S* haplotype would be $S_4 : S_5 : S_4dS_5 : S_5dS_5 = 1+(1–x) : 1+(1–x) : x : x$. Because dS_5 and S_5 cannot be distinguished electrophoretically, the segregation of pollen electrophoretic haplotypes under this scenario would be $S_4 : S_5 : S_4S_5 = (2–x) : 2 : x$. Because $x \leq 1$, the frequency of offspring inheriting only an electrophoretic S_4 haplotype from 415-1 will be more than half the frequency of those inheriting only an electrophoretic S_5 haplotype.

If the duplicated S_5 haplotype is translocated or inserted very close to the original S_4 haplotype (i.e., the chromosomes with *S* haplotypes contain either S_4dS_5 or S_5), there would be little or no recombination between dS_5 and the original *S*-locus, and the segregation ratio of pollen *S* haplotypes would be $S_4 : S_5 : S_4dS_5 : S_5dS_5 = 0 : 1 : 1 : 0$ (Table 3.3).

In our study, very few plants (<2%) inherited the S_4 haplotype alone from 415-1 (Table 3.3). Therefore, the chromosomal segment containing the duplicated S_5 haplotype

was usually inherited together with the S_4 chromosome. Consequently, it is reasonable to infer that LG 17 of 415-1 is represented by one chromosome containing an S_5 haplotype and a homologous chromosome containing both an S_4 haplotype and a duplicate of the S_5 haplotype (Fig. 3.3).

However, there remains a question as to why the frequency of plants from S_4S_5 pollen (16% of the frequency of progeny from S_5 pollen) is so low, despite the prediction that S_4S_5 pollen grains might be produced at the same frequency as S_5 pollen. One possibility is that duplication of the *S* haplotype or other adjacent genes might be detrimental to the production or growth of pollen. In trisomics of barley and tobacco, pollen grains with an extra chromosome were less frequent and smaller than normal grains, and were not fully mature at anthesis (Tsuchiya 1960; Niizeki and Saito 1988). Thus, reduced viability of heteroallelic pollen by segmental duplication could reduce the probability of inheriting the duplicated S_5 haplotype. It is also possible that the duplicated S_5 haplotype and the S_4 haplotype are not very tightly linked (i.e., that recombination occurs at some frequency). Hence, at least three factors—the probability of deletion of the duplicated *S* haplotype, the reduction of viability of heteroallelic pollen, and the possibility of recombination between S_4 and dS_5—are required to estimate the location of the duplicate S_5 haplotype by segregation analysis of progeny from crosses involving 415-1. Therefore, our segregation data cannot be statistically compared with the basic models presented in Table 3.3, because one or more of these complicating factors affect the inheritance of the duplicated region. The two plants that inherited the S_4 haplotype alone from 415-1 are assumed to be the product of "S_4 pollen" that lost the duplicated S_5 segment by deletion or unequal crossing-over.

3–4–3 Variation among SI/SC systems in the Rosaceae

In the present study, a segmental duplication including the *S* haplotype was found to induce pollen-part SC in Japanese pear. Pollen-part SC in the Pyrinae can also be caused by tetraploidization, as was reported for European pear (Crane and Lewis 1942), apple (Adachi et al. 2009), and Japanese pear (Tahira et al. 2010). Thus, acquisition of one or more extra *S* haplotypes by either tetraploidization or segmental duplication can produce pollen-part SC in the Pyrinae, as is the case in the Solanaceae. Therefore, the mechanism of SI in the Pyrinae may be identical or closely related to that found in the Solanaceae (de Franceschi et al. 2012).

On the other hand, there are contradictory findings from two different tetraploid species of *Prunus* (Rosaceae), sour cherry (*Prunus cerasus*) and Chinese cherry (*Prunus pseudocerasus*), that carry functional *S* haplotypes. Sour cherry is not self-compatible (Hauck et al. 2006b) whereas Chinese cherry is (Huang et al. 2008). These examples raise the question of whether the SI regulation mechanism is different in different species of *Prunus*, or whether the regulation mechanism is the same but leads to different responses (Tao and Iezzoni 2010).

In a recent study of tetraploid Chinese cherry, Gu et al. (2013) showed that *S*-heteroallelic pollen with particular combinations of two different *S* haplotypes, each containing a functional *SFB* gene, gave rise to SC whereas other combinations did not, and they concluded that the capability of the pollen to degrade *S*-RNase in the self style and to grow into the ovaries depends on the combination of *S* haplotypes. Lewis (1943) had previously reported that heteroallelic pollen gave rise to SC in some cases but not in others, depending on the particular combination of *S* haplotypes, in tetraploid *Oenothera organensis* Munz (Onagraceae). Under the CI hypothesis, *S*-heteroallelic pollen produced

by a biallelic plant should be self-compatible whether one or more *S*-related F-box proteins recognize and degrade self or non-self *S*-RNase. Therefore, the failure of breakdown of SI in *Prunus cerasus* might be the result of an incompatible combination of *S* haplotypes and subsequent lack of *S*-RNase degradation, even though (as a general rule) CI occurs in *S*-heteroallelic pollen. Consequently, further investigations of PPMs with duplicated *S* haplotypes or tetraploid PPMs will be required to determine whether all possible combinations of *S* haplotypes in *S*-heteroallelic pollen give rise to CI in Japanese pear, as is seen for the S_4S_5 pollen of 415-1.

3–4–5 Conclusions

415-1 is the first diploid PPM with a duplicated *S* haplotype to be identified in the Pyrinae. 415-1 can cross with normal diploid cultivars of any *S* genotype, and an additional *S* haplotype is transmitted to the progeny. More information about the chromosome status of the duplicated segment will be obtained by analyzing the inheritance of the duplicated *S* allele. Moreover, it is essential to analyze the phenotypes of different combinations of normal *S* haplotypes and extra *S* haplotype copies in the progeny of 415-1, for the genetic analysis and selection of pollen-part SC by using the markers associated with the duplicated *S* haplotype as indicators.

Based on the data reported here, the duplicated S_5 chromosomal segment of 415-1 contains the genes necessary to cause CI with the S_4 haplotype. In a case such as this which involves a relatively small duplicated region, the effect of genetic factors outside of the *S* haplotype itself (e.g., modifying factors) would be much more limited than in the case of whole-genome duplication, as is found in tetraploid PPMs. Therefore, materials derived from 415-1 will be valuable for elucidating the mechanisms of regulation and

function of SI, and especially that of CI caused by a duplicated *S* haplotype. Genome information of apple (Velasco et al. 2010), Chinese pear (Wu et al. 2013) and European pear (Chagné et al. 2014) may allow us to define the genes involved in SI and SC in the duplicated region. Further studies of 415-1 and its offspring will help to elucidate the self/non-self recognition mechanism between pollen and pistil in pear and its relatives.

Table 3.1 Discrepancy between observed segregation of the electrophoretic *S*-phenotype and the expected *S* haplotype under the hypothesis of pollen *S*-factor mutation

Parents and *S* haplotypes			Number of seedlings	Hypothesized 415-1 *S* haplotype[a]	Expected segregation of *S* haplotype	Observed segregation of electrophoretic *S*-phenotype	Goodness of fit (p)[b]
415-1 (S_4S_5) PPM	×	self	23	$S_4^{pm}S_5$	$S_4^{pm}S_4^{pm}$: $S_4^{pm}S_5$ = 1:1	S_4S_5 = 23	**
				$S_4S_5^{pm}$	$S_4S_5^{pm}$: $S_5^{pm}S_5^{pm}$ = 1:1		**
Syuugyoku (S_4S_5) SI	×	415-1 (S_4S_5) PPM	63	$S_4^{pm}S_5$	$S_4S_4^{pm}$: $S_4^{pm}S_5$ = 1:1	S_4S_5 : S_5S_5 = 60:3	**
				$S_4S_5^{pm}$	$S_4S_5^{pm}$: $S_5S_5^{pm}$ = 1:1		**
Niitaka (S_3S_9) SI	×	415-1 (S_4S_5) PPM	103	$S_4^{pm}S_5$	$S_3S_4^{pm}$: S_3S_5 : $S_4^{pm}S_9$: S_5S_9 = 1:1:1:1	S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : $S_3S_4S_5$: $S_4S_5S_9$ = 2:40:0:47:7:7	**[c]
				$S_4S_5^{pm}$	S_3S_4 : $S_3S_5^{pm}$: S_4S_9 : $S_5^{pm}S_9$ = 1:1:1:1		**[c]

[a] S_4^{pm}, hypothesized pollen-part mutant of S_4; S_5^{pm}, hypothesized pollen-part mutant of S_5.

[b] p, p value for the binomial goodness-of-fit tests.

[c] Multinomial goodness-of-fit tests were performed excluding the unexpected classes (triallelic electrophoretic *S*-phenotypes).

** Significantly different from the expected segregation ratio (p<0.01).

Table 3.2 Relative nuclear DNA contents of 415-1 and ‘Kosui’ determined by flow cytometric analysis

Cultivar	*n*[a]	AU (%)[b]		CV[c]	Ploidy
		Mean	SD	(%)	level
415-1	15	35.1	1.19	5.80	2*n*
Kosui	15	34.8	1.36	5.55	2*n*

[a] *n*, number of samples (each from an individual leaf).

[b] AU (arbitrary unit), calculated as (peak value of relative fluorescence intensity of Japanese pear / peak value of relative fluorescence intensity of the internal standard, *Zea mays* B73) × 100.

[c] CV, coefficient of variation of peak intensity.

Table 3.3 Comparison between observed and expected segregation of the electrophoretic *S*-phenotype under two hypotheses of S_5 haplotype duplication

Parents and *S* haplotypes			Number of seedlings	Hypothesized chromosomal location of duplicated *S* haplotype[a]	Expected segregation of *S* haplotype in pollen[b]	Expected segregation of *S* haplotype in progeny[b]	Expected segregation of electrophoretic *S*-phenotype[b]	Observed segregation of electrophoretic *S*-phenotype
Niitaka (S_3S_9) SI	×	415-1 (S_4S_5) PPM	103	$S_4\|S_5\ dS_5\|–$	$S_4 : S_5 : S_4dS_5 : S_5dS_5$ = 1:1:1:1	$S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : S_3S_5dS_5 : S_5dS_5S_9 : S_3S_4dS_5 : S_4dS_5S_9$ = 1:1:1:1:1:1:1:1	$S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : S_3S_4S_5 : S_4S_5S_9$ = 1:2:1:2:1:1	$S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : S_3S_4S_5 : S_4S_5S_9$ = 2:40:0:47:7:7
				$S_4dS_5\|S_5$	$S_4 : S_5 : S_4dS_5 : S_5dS_5$ = 0:1:1:0	$S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : S_3S_5dS_5 : S_5dS_5S_9 : S_3S_4dS_5 : S_4dS_5S_9$ = 0:1:0:1:0:0:1:1	$S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : S_3S_4S_5 : S_4S_5S_9$ = 0:1:0:1:1:1	

[a] dS_5, duplicated S_5 haplotype; $S_4|S_5\ dS_5|–$, duplicated S_5 haplotype present as a centric fragment or translocated to a non-homologous chromosome; $S_4dS_5|S_5$, duplicated S_5 haplotype tightly linked to S_4 haplotype of homologous chromosome

[b] Expected segregation in the case that the probability of inheriting the duplicated *S* haplotype is 1 (i.e., not reduced relative to that of a non-duplicated haplotype)

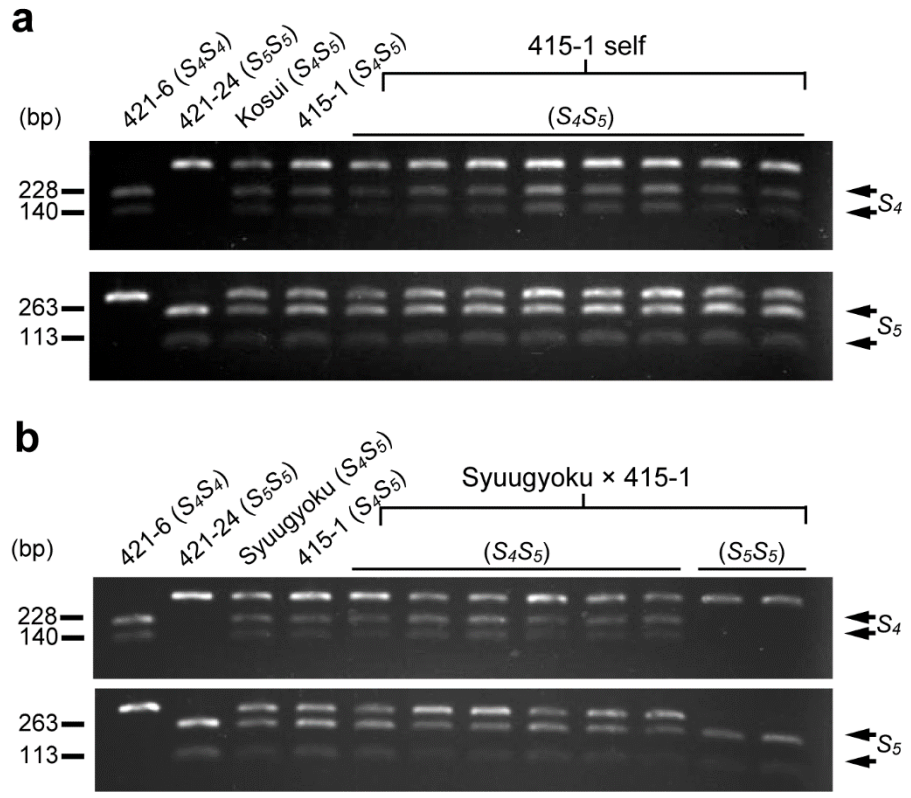

Fig. 3.1 Segregation analysis of *S* haplotypes by means of CAPS analysis of *S-RNase* in (a) 'Kosui', 415-1, and eight self-progeny plants of 415-1 and (b) the parents and eight progeny plants of 'Syuugyoku' × 415-1. Lines 421-6 and 421-24 appear in both figure parts as indicators of complete digestion of S_4 *a*nd S_5 fragments, respectively.

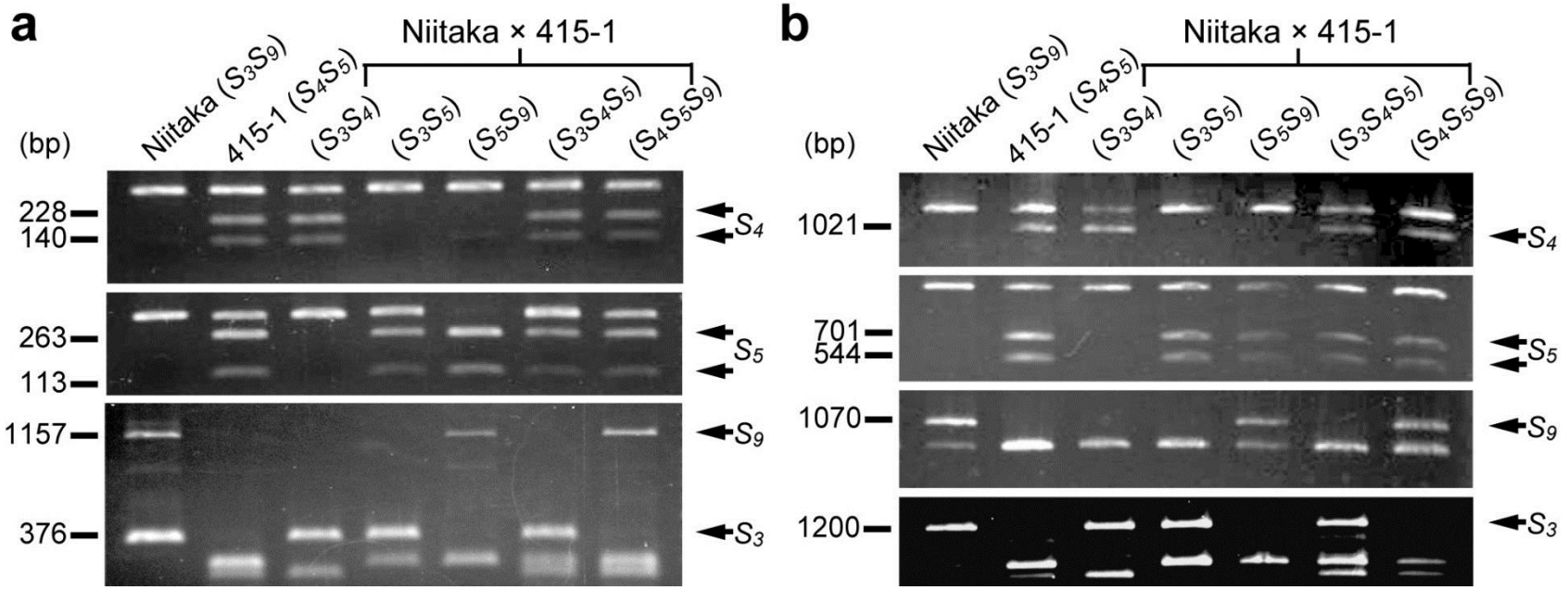

Fig. 3.2 Segregation analysis of *S* haplotypes by means of CAPS analysis of *S-RNase* (a) and *PpSFBB*$^{-\gamma}$ (b) alleles in the parents and five progeny plants of 'Niitaka' × 415-1. The same plants are represented in both gels.

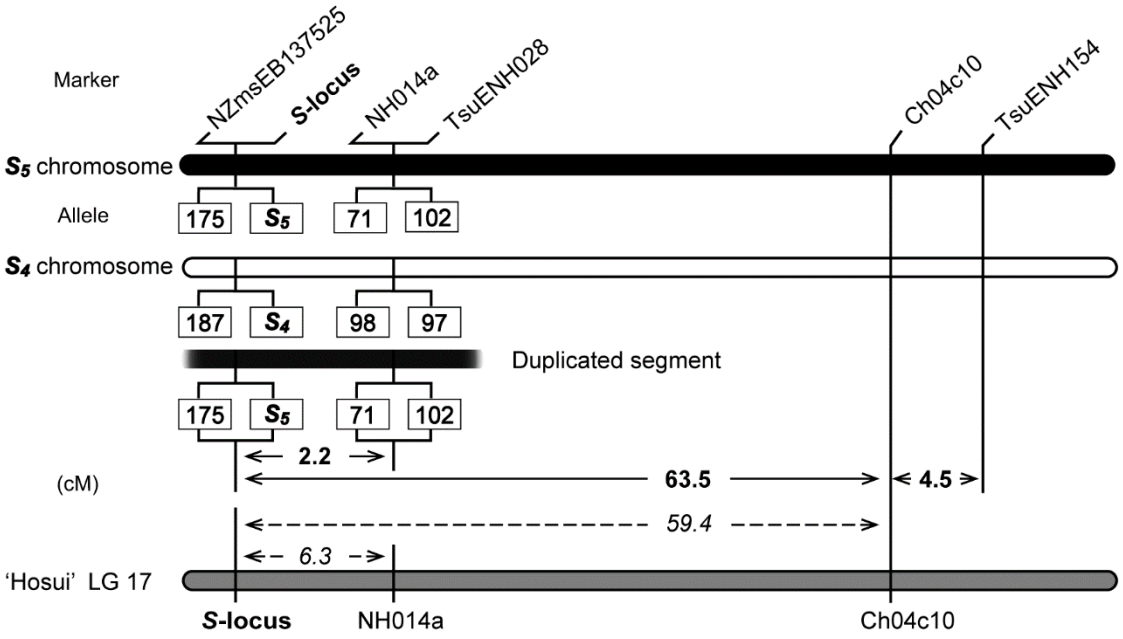

Fig. 3.3 Local haplotype maps of 415-1 estimated from intermarker distances in the progeny of the 'Niitaka' × 415-1 cross. Marker haplotypes are displayed as a pair of homologous LG 17 chromosomes and a duplicated segment of LG 17. Marker/allele designations connected by horizontal lines indicate that no recombination was observed between those markers. SSR alleles are designated by size (bp). On the basis of the genetic data, the duplicated segment is estimated to be contained within the chromosome containing *S*$_4$. Only map distances are displayed for Ch04c10 and TsnENH154 because their linkage phases could not be inferred from the data. Solid-line arrows indicate the genetic distances calculated for the 89 biallelic progeny of the 'Niitaka' × 415-1 cross. Dashed-line arrows indicate genetic distances from a high-density genetic map of the normal (self-incompatible) cultivar 'Hosui' (Terakami et al. 2009).

Chapter 4

Direct genotyping of single pollen grains of a self-compatible mutant of Japanese pear revealed inheritance of a duplicated chromosomal segment containing a second *S* haplotype

4–1 Introduction

According to the available evidence, a PPM can produce omni-potential pollen (i.e., pollen compatible with self-incompatible styles of all *S* haplotypes, including its own). A PPM that produced enough viable pollen could be used as a pollinator by planting it in a limited part of an orchard. However, all hitherto-known PPMs in the Pyrinae have been polyploids that exhibit a reduction in pollen tube growth and pollen germination rate (Lewis and Modlibowska 1942; Adachi et al. 2009). Hence, they are not suitable for use as pollinators or for breeding material.

Breeding selection 415-1, a PPM derived by using pollen from γ-irradiated 'Kosui' (self-incompatible) to pollinate non-irradiated 'Kosui' was deduced to be a diploid by means of flow cytometry, but genetic analysis indicated that it has a segmental duplication encompassing the S_5 haplotype that is usually inherited together with the S_4 chromosome (Chapter 3). Thus, this selection is predicted to produce *S*-heteroallelic pollen grains that are capable of breaking down SI by competitive interaction (CI) between the two different *S* factors in the pollen grain (Chapter 3). It is expected to be an important material for breeding as well as for analyzing the mechanism of CI, which is now hypothesized to be caused by non-self recognition of *S*-RNase by two different sets of pollen *S*-determinants in a single pollen tube (de Franceschi et al. 2012). However, the direct detection of two *S* haplotypes in a single pollen grain of a PPM plant has not previously been achieved.

To be of practical use, PPM cultivars should produce fairly large amounts of self-compatible pollen. However, pollen-sterile or low-fertility mutants are often found in progeny derived from irradiated pollen (Vizir et al. 1994; Naito et al. 2005). Moreover, inbreeding can induce pollen sterility through accumulation of deleterious genes. Indeed, the pollen production of 415-1 is lower than that of normal (pollen-fertile) cultivars. To address this limitation, we plan to cross 415-1 with fertile cultivars and select progeny that produce large quantities of pollen, especially with a high frequency of the duplicated *S* haplotype.

In most studies, the segregation ratio of alleles in germ cells has been estimated from the ratio of corresponding genotypes in the progeny. The frequency of 415-1 progeny with a duplicated *S* haplotype is low (14%) (Chapter 3). This decrease in viability could be occurring at one or more stages from meiosis to the 1-year-old seedling stage, which we used for progeny analysis. Thus, the *S* haplotype frequency in the pollen of PPM mutant 415-1 could be different from that of the progeny. For these reasons, it is important to determine the frequency of the duplicated *S* haplotype in pollen directly, enabling us to select the most appropriate individual or individuals without genotyping a large number of progeny.

Single-pollen genotyping has been performed successfully by some research groups (Petersen et al. 1996; Suyama et al. 1996; Aziz et al. 1999; Matsunaga et al. 1999) based on the procedure of PCR amplification of DNA from a single animal cell or sperm (Li et al. 1988; Cui et al. 1989). The original methods are laborious and time-consuming, especially in the pollen manipulation and DNA extraction steps. Recently, a simpler method for direct genotyping of SSR markers in single pollen grains was developed by Matsuki et al. (2007). This research group revealed that strict SI maintains the genetic

diversity of wild Japanese chestnut by determining the SSR alleles of individual pollen grains on the styles and thus the genotypes of the seeds produced (Hasegawa et al. 2009).

In the study described in this chapter, we identified the *S* haplotype and SSR genotype of individual pollen grains from 415-1, and we observed the pollen morphology of 415-1 and a normal (self-incompatible) cultivar, 'Kosui'. We compared the occurrence of the duplicated *S* haplotype in pollen and progeny and we examined the possible involvement of the duplicated haplotype in the pollen morphology and pollen-producing capability of 415-1.

4–2 Materials and methods

4–2–1 Collection of pollen grains

The PPM selection 415-1 and the normal cultivar 'Kosui' were sampled from the orchard of the NARO Institute of Fruit Tree Science. Anthers of 415-1 and 'Kosui' were collected from flower buds 1 d prior to opening and dried in an incubator (25°C, 35% relative humidity) until dehiscence, which usually took about 40 h, and then stored at –80°C until used.

4–2–2 DNA extraction from individual pollen grains

The DNA extraction method was that of Suyama (2011), with some modifications. In a clean booth (ACR-321C [Airtech Japan, Japan]), dried pollen grains (with anthers) of 415-1 were suspended in a drop (20 µL) of 2× PCR buffer (20 mM Tris-HCl [pH 8.0], 100 mM KCl, 3 mM $MgCl_2$) with 0.005% sodium dodecyl sulfate (SDS) and 0.04% 2-mercaptoethanol in a disposable Petri dish. Under a stereomicroscope with a 6–50× zoom lens, a few pollen grains in 2× PCR buffer were transferred into a clean drop of 2× PCR

buffer using a micropipette and tips with 0.5–10 µL capacity. For each reaction, a single pollen grain, filled with cytoplasm (pale yellow) and not abnormally small (Fig. 4.1a), was picked up in 2× PCR buffer (less than 2 µL) and put into a 0.2-mL PCR tube. The tube was then checked to ensure that only a single grain had been transferred (Fig. 4.1b), 2 µL of lysis buffer containing 0.015% SDS and 0.2 µg/µL proteinase K was added, and the tube was incubated at 50°C for 60 min followed by 95°C for 10 min.

4–2–3 Determination of *S-RNase* and SSR genotypes

Multiplex PCR was performed in a 10-µL volume with a QIAGEN Multiplex PCR kit (QIAGEN, Germany) containing total DNA extracted from a single pollen grain (up to 4 µL), 1× QIAGEN Multiplex PCR mix, and the following six pairs of primers. A 6-FAM-labeled FTQQYQ primer (Ishimizu et al. 1999) and a newly designed non-labeled primer (5′-(A/G)(C/T)GTGCATGAAAATCTATG-3′) were used to amplify a 254- or 262-bp fragment of the S_4- or S_5-*RNase* gene, respectively (Fig. 4.2). Five sets of fluorescently (6-FAM, HEX, or 2'-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED)) labeled forward primers and non-labeled reverse primers were used to genotype SSR loci mapped to the same linkage group as the *S* haplotype (LG 17) of pear or apple. These SSR markers consisted of three pear SSRs, NH014a (Yamamoto et al. 2002b), TsuENH028 (Nishitani et al. 2009), and TsuENH154 (GenBank accession number AB735184), and two apple SSRs, NZmsEB137525 (Celton et al. 2009) and CH04c10 (Liebhard et al. 2002). To establish the reaction conditions, 0.2 µM of each primer set was used in a single reaction, and then the concentration of each primer was adjusted to improve amplification efficiency. Amplification was carried out using a Veriti Thermal Cycler (Applied Biosystems) under the following conditions: initial activation

at 94°C for 15 min, followed by 37 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 90 s, and extension at 72°C for 60 s, followed by a final extension at 72°C for 10 min. The PCR products were separated and detected using an ABI PRISM 3100 Genetic Analyzer. The sizes of the amplified bands were determined based on an internal DNA size standard (GeneScan 400 HD ROX Size Standard) with the GeneScan Software. Allele size data (in bp) were rounded down to the nearest whole number.

4–2–4 Microscopic observations

Dried pollen of 415-1 and 'Kosui' was suspended in *n*-hexane and filtered through a 50-μm nylon mesh (Sysmex Partec GmbH) to remove anther tissues. After drying at room temperature, pollen was suspended in aniline blue lactophenol stain (Hauser and Morrison 1964), and then incubated for 30 min at room temperature. Twenty μL of suspension was dropped onto a glass slide and observed at 100× magnification under a light microscope (Axioskop 50 [Carl Zeiss, Germany]). The images were captured with an AxioCam Mrc5 camera and processed with the AxioVision 4.5Ac software (Carl Zeiss). Cross-sectional areas were measured for approx. 200 pollen grains of each cultivar with the *SmartGrain* software (Tanabata et al. 2012).

4–3 Results

4–3–1 *S-RNase* and SSR genotyping from single pollen grains

We successfully amplified and genotyped DNA fragments representing the *S-RNase* gene and three SSR loci. The optimized concentrations of primers in each pair were 0.15 μM for *S-RNase*, 0.08 μM for NH014a, 0.06 μM for NZmsEB137525, and 0.2 μM for

CH04c10. The amplifications of TsuENH154 and TsuENH028 were unstable, so these two primer sets were excluded from the genotyping.

PCR reactions were performed with 316 pollen grains from 415-1, and amplification of all four loci (*S-RNase* and three SSR loci) was successful for 206 of the grains (65%). Success in isolating single pollen grains with a single *S* haplotype per haploid genome was confirmed by detection of only one allele of each of the four loci in a single amplification reaction. On the other hand, successful isolation of an *S*-heteroallelic pollen grain was confirmed by detection of two alleles of *S-RNase* and of two SSR loci (NH014a and NZmsEB137525), all on the duplicated chromosomal segment in 415-1, and detection of one allele of a non-duplicated SSR locus (CH04c10) farther away on the same chromosome (Fig. 4.2).

Five samples (1.6%) that had two alleles of CH04c10 (non-duplicated locus) were determined to be from having two different pollen grains in one amplification product. This frequency was very low and nearly the same (2.2%) as in the previous study (Hasegawa et al. 2009), and these samples were considered to be insignificant in our determination of allelic frequencies. We could not decide the genotype of 33 pollen grains that had allelic compositions inconsistent with the expectations for either the *S*-homoallelic or *S*-heteroallelic situation. This result could be due to failure of amplification of one of two alleles, recombination between alleles of duplicated loci in the *S*-heteroallelic pollen, or amplification of DNA from two different pollen grains.

Collectively, the haplotypes of 173 individual pollen grains were successfully analyzed. The allelic frequency of *S-RNase* in pollen from 415-1 was S_4 : S_5 : S_4S_5 = 10 : 135 : 28 (Table 4.1). In 103 F_1 progeny obtained from a cross with the self-incompatible cultivar ‘Niitaka’ (S_3S_9), the *S*-phenotypes from 415-1 segregated in a ratio of 2 S_4 : 87

S_5 : 14 S_4S_5 (recalculation data of Table 3.1). There was no significant difference in the allelic frequency of *S-RNase* in pollen and offspring from 415-1 (p = 0.25 for Fisher's exact test, Table 4.1).

4–3–2 Pollen morphology

We compared the size and viability of pollen grains from normal cultivar 'Kosui' and PPM selection 415-1. Pollen grains of 'Kosui' were uniform in size and shape: 95% were large, viable (fully stained) and roundish-triangular, and the mean cross-sectional area was 114.2 ± 10.5 μm^2, whereas 5% were relatively small (47.1 ± 3.1 μm^2), shrunken, and unstained or partially stained (Fig. 4.3a, c). In contrast, pollen grains of 415-1 were non-uniform in size, shape, and staining intensity. The mean area of fully stained and round or roundish-triangular pollen (65%) was 104 ± 21.6 μm^2, whereas the mean area of unstained or partially stained and dimpled or shrunken pollen (35%) was 49.7 ± 8.9 μm^2 (Fig. 4.3b, d).

4–4 Discussion

4–4–1 DNA amplification from a single pollen grain

In this study, DNA of pollen grains of 415-1 was readily isolated by suspending pollen in 2× PCR solution containing a low concentration of SDS and 2-mercaptoethanol, instead of sterile water or sterile water-based solution as in previous methods (Hasegawa et al. 2009; Suyama 2011). This improvement was observed to reduce the frequency of pollen bursting (data not shown), probably leading to less DNA degradation. It takes only 60 to 90 min to collect 45 pollen grains and transfer individual grains into different PCR tubes

for one experiment. It was important to keep the time before DNA extraction as short as possible to succeed in PCR amplification from pollen grains of Japanese pear.

We had only six markers available to test (only four of which gave reliable amplification in this experiment) because of the high homozygosity in 415-1 and a consequent paucity of heterozygous SSR loci. However, more SSR markers will be available for pollen genotyping of later progenies, as successful amplification of a maximum of 104 AFLP markers was reported by Aziz and Sauve (2008).

4–4–2 Pollen size and fertility of 415-1

Most pollen grains of 'Kosui' were uniform in size, shape, and viability and had the standard morphology of mature pollen of Japanese pear (Okusaka and Hiratsuka 2009). On the other hand, the histogram of pollen size of 415-1 showed two approximately uniform peaks, one at the standard size and the other at a smaller size (Fig. 4.3). The former consisted of standard viable pollen, and the latter contained sterile pollen that was empty or only partially full. In many cases, aborted pollen comes from abnormal meiosis during gamete formation due to polyploidy, aneuploidy, or a genetic defect that controls the distribution of chromosomes (Ramsey and Schemske 2002; Consiglino et al. 2007). Consequently, the aborted grains do not have a complete haploid genome (Deng and Wang 2007). In this study, we isolated and used only larger, filled pollen grains (presumed to have a complete haploid genome) for PCR amplification.

This procedure enabled us to successfully detect normal-size pollen with a duplicated *S* haplotype. Overall, the segregation ratio of markers in the normal pollen grains of 415-1 was approximately the same as that observed in the progeny ($p = 0.25$ for Fisher's exact test; Table 4.1). Looking more specifically at each class, the frequencies of the single-

allelic (S_5) and heteroallelic (S_4S_5) classes were approximately the same in pollen and offspring. However, the frequency of S_4 pollen grains (5.8%), which are assumed to be produced by loss of the duplicated S_5 segment by deletion or recombination from meiotic cells with the S_4S_5 chromosome, appeared to be higher than that of S_4 offspring (1.9%). If real, this difference may indicate that the recombination frequency between the duplicated S_5 and the original *S* haplotype during meiosis is higher than that originally predicted by segregation in the offspring. Another possibility is that the viability of S_4 pollen (meiotic cells) is higher than that of S_4S_5 during the processes of meiosis and pollen formation because of one or more detrimental genes in the duplicated S_5 segment. Either of these explanations require the assumption that S_4 pollen is less viable than S_4S_5 pollen in the steps following pollen production (i.e., from pollen germination to seed germination), because the frequencies of pollen and offspring containing S_4S_5 were similar. In other words, the segmental duplication does not decrease the likelihood of fertilization when present in an otherwise normal pollen grain. This indicates that the decrease in frequency of pollen with the duplicated *S* haplotype occurred during meiosis or pollen formation, but it is unclear whether this duplication is the only reason for the defect in pollen production seen in 415-1. Because the paternal parent tree of 415-1 had been irradiated an average of 18 h/day, 280 days/year, for more than 30 years prior to pollen collection, many other detrimental mutations are expected to have accumulated throughout the genome that may play a negative role in pollen formation, regardless of the pollen *S* haplotype.

4–4–3 Conclusions

Using single-pollen genotyping, we succeeded in a straightforward proof of the presence of *S* haplotype duplication in pollen grains of 415-1 that did not require analysis of progeny. We also showed that the segregation ratio of marker alleles in the pollen was nearly identical to that in the progeny. Under such circumstances, it is possible through pollen genotyping to reveal the segregation ratio of alleles at each locus rapidly and without generating progeny. On the other hand, a difference in the segregation ratio of alleles between pollen and progeny would suggest that pollen with a specific allele at the locus of interest might be less competitive. Single-pollen genotyping could also be used to perform fine-scale mapping in the flanking region of a gene of interest in a fruit or forest tree species without spending the enormous time, cost, and effort to obtain a sufficient number of progeny; this approach has been used with sperm cells of large animal species and humans (Cui et al. 1989; Windemuth et al. 1998).

Utilization of this method will simplify the selection of breeding materials with a duplicated *S* haplotype that produce adequate amounts of pollen and are thus suitable for use in the production of pollen-part self-compatible cultivars.

Single-pollen genotyping and sequencing have so far been utilized mainly in basic science such as molecular taxonomy and ecological genetics (Zhou et al. 2007; Ito et al. 2008; Hirota et al. 2013). However, the present results suggest that single-pollen analysis has the potential for important applications in breeding science, and we expect further utilization of this method.

Table 4.1 Frequency of *S-RNase* alleles in pollen and 1-year-old offspring of 415-1

Genotype	S_4	S_5	S_4S_5	Total
Pollen	10	135	28	173
Offspring[a]	2	87	14	103

[a]F_1 plants obtained from a cross to the self-incompatible cultivar 'Niitaka' (S_3S_9); data were recalculated from Table 3.1.

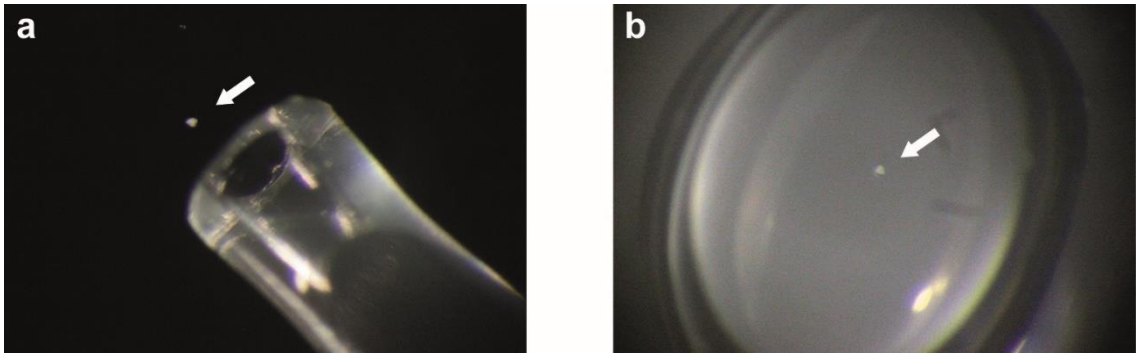

Fig. 4.1 Manipulation of a single pollen grain (indicated by arrow). (**a**) A single pollen grain before transfer to a PCR tube. Micropipette tips with outer and inner diameter of 750 and 450 µm, respectively, were used for transfer. (**b**) A single pollen grain of 415-1 transferred to the inner wall of a PCR tube.

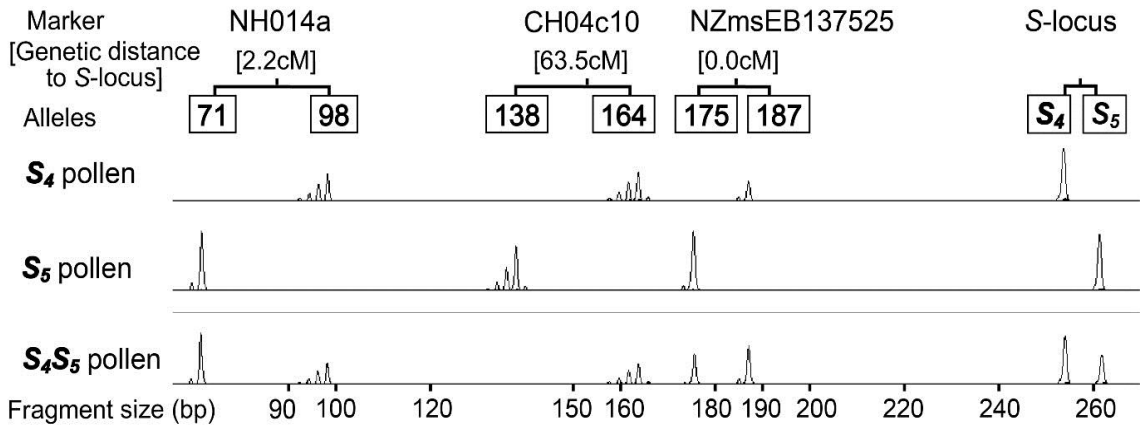

Fig. 4.2 *S-RNase* and microsatellite alleles detected by multiplex PCR from single pollen grains of 415-1. Fragment sizes (bp) of an internal DNA size standard (GeneScan 400 HD ROX Size Standard) are indicated below.

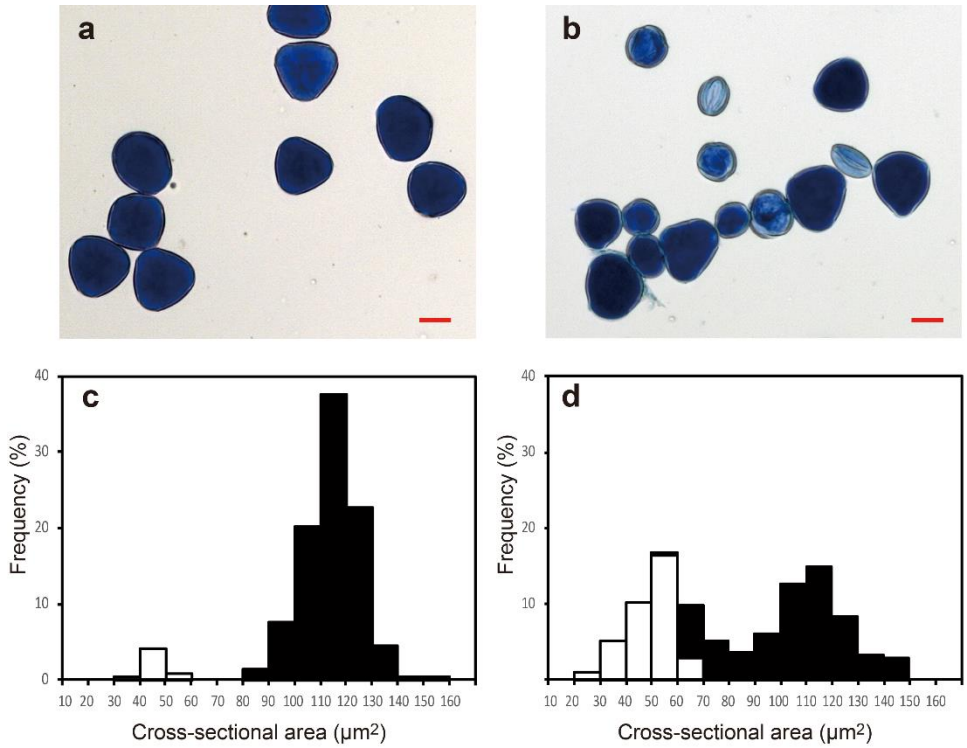

Fig. 4.3 Pollen viability and size of 415-1 and 'Kosui'. Aniline blue staining of pollen grains from normal (self-incompatible) cultivar 'Kosui' (**a**) and PPM selection 415-1 (**b**); scale bar, 20 μm. Histogram of cross-sectional areas of pollen grains (μm^2) of 'Kosui' (**c**) and 415-1 (**d**). Fully stained grains are shown in filled bars and unstained or partially stained grains are shown in open bars. The frequency of pollen grains of each size is expressed as a percentage of the total number of pollen grains [*n*=222 in (**c**); *n*=214 in (**d**)].

Chapter 5

Evaluation of self-compatibility by detection of a duplicated *S* haplotype by TaqMan real-time quantitative PCR in the progeny of a pollen-part self-compatible mutant of Japanese pear

5–1 Introduction

The *S* haplotype of a self-compatible mutant selection of Japanese pear (designated 415-1) was initially identified to be S_4S_5 by CAPS analysis of the *S-RNase* gene (Chapter 2). Later, it was revealed that the accurate *S* haplotype of 415-1 is $S_4S_5S_5$, resulting from the presence of a duplicated chromosomal segment containing an S_5 haplotype on the chromosome carrying the S_4 haplotype. The accurate haplotype was determined by segregation analysis of *S* haplotypes and SSR markers in the progeny obtained from a cross with the self-incompatible cultivar 'Niitaka', which does not share any *S* haplotypes with 415-1 (Chapter 3). Single-pollen genotyping confirmed that 415-1 produces normal S_4, S_5, and S_4S_5 heteroallelic pollen grains (Chapter 4). Only S_4S_5 pollen grains were predicted to be able to complete fertilization in a cross with the self-incompatible S_4S_5 cultivar 'Syuugyoku' as the seed parent, and in fact, almost all progeny of this cross were S_4S_5 heterozygous according to CAPS analysis (Chapter 3). These progeny are assumed to have been the product of an S_4 or S_5 egg cell from 'Syuugyoku' and S_4S_5 pollen from 415-1; each one would in fact have three *S* haplotypes and their true genotypes would be $S_4S_4S_5$ or $S_4S_5S_5$ (Chapter 3). To use these progeny in breeding, the actual *S* haplotypes would need to be determined. For example, the frequency of progeny with a duplicated *S* haplotype would vary depending on whether the selection possesses two S_4 or two S_5 alleles when the seed parents possess S_4 or S_5. Therefore, a simple method is required for

determining of the copy number of the *S*-locus and the allelic composition and dosage of each *S* haplotype in the progeny of 415-1.

Allelic composition and dosage (allele copy number) have been studied in polyploid crop species because they are known to affect phenotypic traits such as disease resistance and male sterility (Meyer et al. 1998; Ward 2000). Although a diploid plant with two different alleles (A and B) at a given locus exhibits only one heterozygous genotype (AB), a tetraploid plant with these same two alleles may exhibit three different heterozygous genotypes (AAAB, AABB, or ABBB). For efficient accumulation of desirable alleles in breeding selections of polyploid crops, the breeding materials need to be selected according to their allelic dosage at the locus of interest. Although segregation analysis using control lines with known genotypes has been the standard way to determine allele copy number (Mendoza et al. 1996; Meyer et al. 1998), it is very laborious and time-consuming. Recently, allelic composition and dosage have been successfully estimated in some polyploid crops by comparison of the peak area of a test allele relative to that of a reference allele detected using SSR markers (Esselink et al. 2004), or determined by quantitative detection of single-nucleotide polymorphisms (SNPs) through methods such as pyrosequencing (Rickert et al. 2002), TaqMan assay (De Jong et al. 2003), or mass spectrometry (Oberacher et al. 2004).

This chapter describes the evaluation of allelic composition and dosage of the *PpSFBB*$^{-\gamma}$ gene, one of the F-box genes within the *S*-locus, in progeny derived from a cross between 'Syuugyoku' (S_4S_5) and 415-1 ($S_4S_5S_5$). This analysis was performed using a hydrolysis-probe-based real-time PCR assay (the TaqMan assay), a useful tool for discrimination of SNPs between alleles with high sequence homology. Furthermore, SC was investigated by self-pollination, and the correlation between SC and the presence of

a duplicated *S* haplotype was evaluated in these progeny. Finally, the suitability of 415-1 and its progeny with a duplicated *S* haplotype for use as breeding material for development of PPM cultivars was assessed.

5–2 Materials and methods

5–2–1 Examination of outcross progeny

Out of 63 F_1 plants obtained from a cross of 415-1 ($S_4S_5S_5$) to the self-incompatible cultivar 'Syuugyoku' (S_4S_5) derived in Chapter 3 (section 3–2–1), 61 plants grew to bloom and were used to determine the copy number of S_4 and S_5 haplotypes in a diploid genome. The electrophoretic *S*-phenotypes of these 61 plants, which had been determined as part of the CAPS analysis of *S-RNase* and *PpSFBB*$^{-\gamma}$ markers in Chapter 3 (section 3–3–1), segregated in a ratio of 59 S_4S_5 : 2 S_5S_5.

Two *S*-homozygous selections, 421-6 (S_4S_4) and 421-24 (S_5S_5), obtained from progeny of self-pollinated 'Shinsui' (S_4S_5) (Saito et al. 2005), were used as controls for S_4 and S_5-specific amplification, respectively.

5–2–2 DNA extraction

Genomic DNA was extracted using a FastDNA kit (MP Biomedicals, USA) according to the manufacturer's instructions, except that 10 mg polyvinylpyrrolidone (insoluble) and 30 µL 2-mercaptoethanol were added to Cell Lysis Solution (0.8 mL CLS-VF, 0.2 mL PPS [reagents in the FastDNA kit]) in the initial homogenization step.

5–2–3 Design of TaqMan primer and probe set

For discrimination of a SNP in the sequences of the *PpSFBB*$^{-\gamma}$ alleles, a specific primer

pair and two TaqMan minor groove binder (MGB) probes were designed with the online Custom TaqMan Assay Design Tool (Applied Biosystems) and synthesized by Applied Biosystems. The primer pair yielded 145-bp amplicons from both alleles. The TaqMan MGB probes were each bound with a fluorescent reporter dye at the 5′-end and a non-fluorescent quencher dye at the 3′-end. The first probe, for detecting *PpSFBB*$^{-4\gamma}$ (GenBank accession: AB270799), was labeled with 6-carboxyfluorescein (FAM), and the second probe, for detecting of *PpSFBB*$^{-5\gamma}$ (AB270802), was labeled with 6-carboxyrhodamine (VIC). The primer and probe sequences are indicated in Table 5.1.

5–2–4 TaqMan SNP genotyping

SNP genotyping of *PpSFBB*$^{-\gamma}$ was performed using TaqMan SNP Genotyping Master Mix (Applied Biosystems) and the TaqMan allelic discrimination assay with an ABI 7300 real-time PCR system (Applied Biosystems). The final reaction volume for PCR was 20 μL, which contained 8 ng of genomic DNA, 10 μL of 2× TaqMan SNP Genotyping Master Mix, and 0.5 μL of 40× genotyping assay mix (Custom TaqMan SNP Genotyping Assays [Applied Biosystems]) containing 36 μM of specific primers and 8 μM of TaqMan MGB probes as determined by the manufacturer. The holding stage before PCR was performed at 60°C for 1 min. PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The final extension after PCR was performed at 60°C for 1 min. PCR amplifications for the 61 progeny plants were carried out in two batches of DNA samples (21 and 40 plants, respectively) and tested in duplicate in 96-well plates, each containing homozygous samples 421-6 (S_4S_4) and 421-24 (S_5S_5), heterozygous sample ‘Syuugyoku’ (S_4S_5), and PPM 415-1 ($S_4S_5S_5$) as controls, and no-template controls (NTCs). Reactions were analyzed using the TaqMan

allelic discrimination assay software (Applied Biosystems). Copy number of the S_4 and S_5 haplotypes was estimated from the ratio of the delta Rn (ΔRn) value (the fluorescence signal intensity of the reporter dye normalized to the fluorescence signal intensity of the passive reference ROX) of allele Y (the S_4 haplotype) to that of allele X (the S_5 haplotype). From the ΔRn value of x and y, the relative X allele dosage [$x' = x/(x+y)$] of each sample was calculated according to the method of Cuenca et al. (2013). Samples were grouped by x' value, and the S haplotype of each cluster was estimated. Comparisons between groups were carried out using one-way analysis of variance (ANOVA).

5–2–5 Self-pollination tests

The 61 F_1 plants obtained from a cross of 415-1 ($S_4S_5S_5$) to the self-incompatible cultivar 'Syuugyoku' (S_4S_5) and described in section 5–2–1 were also used for self-pollination tests. Anthers were gathered from flower buds one day before opening and dried to collect the pollen. Flower buds one day before opening were pollinated with the collected pollen. The pollinated flowers were then covered with paper bags after crossing. The developed fruits were harvested about 70 days later and seeds were counted. Plants were considered to be compatible if 30% or more of the pollinated flowers set seeded fruits (Sato 1993).

5–3 Results

5–3–1 Estimation of allelic composition and dosage for *PpSFBB*$^{-y}$ in the progeny of 415-1 by TaqMan allelic discrimination assay

The *S*-haplotype-specific fluorescence signals of *PpSFBB*$^{-y}$ were detected by the TaqMan allelic discrimination assay in all 61 F_1 progeny plants obtained from a cross of 415-1 ($S_4S_5S_5$) to the self-incompatible cultivar 'Syuugyoku' (S_4S_5), in both parents, and in two

S-homozygous selections used as controls [421-6 (S_4S_4) and 421-24 (S_5S_5)]. This assay revealed five clusters in the allelic discrimination plot of normalized *x* and *y* signals (Fig. 5.1). The first cluster, consisting only of 421-6 (S_4S_4), and the second cluster, consisting of 421-24 (S_5S_5) and two progeny plants, were automatically genotyped (98.0% call rate) as S_4- and S_5-homozygous, respectively, by the TaqMan allelic discrimination assay software. The three other clusters consisted of 'Syuugyoku' (S_4S_5) and 4 progeny plants, 415-1 and 18 progeny plants, and 37 progeny plants.

The relative *x* allele dosages [$x' = x/(x+y)$] of the progeny plants were calculated, and the four clusters containing progeny plants were more clearly defined (Fig. 5.2). The cluster including 'Syuugyoku' (S_4S_5 heterozygous) was assigned to have one copy each of the S_4 and S_5 haplotypes. The x' values of the cluster including 415-1 were larger, and this cluster was assigned to have one copy of the S_4 haplotype and two copies of the S_5 haplotype. The x' values of the cluster including 421-24 (S_5S_5) were even larger, and this cluster was assigned to have two copies of the S_5 haplotype. The x' values of the rest were smaller than those of the 'Syuugyoku' cluster, forming a cluster that was assigned to have two copies of the S_4 haplotype and one copy of the S_5 haplotype. There were significant differences among the genotyped clusters by ANOVA [p=2.1E-143 (p<0.001)].

The actual *S* haplotypes of the progeny between 'Syuugyoku' and 415-1 segregated in a ratio of 0 S_4S_4 : 4 S_4S_5 : 2 S_5S_5 : 37 $S_4S_4S_5$: 18 $S_4S_5S_5$ (Table 5.2). Thus, 55 out of 61 offspring (90%) had two copies of an S_4 or S_5 haplotype and one copy of the other haplotype.

5–3–2 Self-fertility in the progeny of 415-1

From 2008 to 2010, the SC of each progeny plant of 415-1 was investigated by self-

pollination tests at least once, and the highest score for each plant was used. By visual observation, different amounts of pollen were released after anthesis among the progeny, but the differences were unrelated to the presence or absence of a duplicated *S* haplotype (data not shown). The rates of seeded fruit set in the 61 F_1 plants ranged from 0 to 100%. Among these, 47 progeny plants, all of which had a duplicated *S* haplotype, exhibited SC (rate of seeded fruit set ≥30%). The remaining 14 plants (8 plants with a duplicated *S* haplotype and 6 plants without a duplicated *S* haplotype) exhibited SI (<30% seeded fruit set after self-pollination; Fig. 5.3).

5–4 Discussion

5–4–1 Selective fertilization by *S*-heteroallelic pollen of 415-1 in a cross with a cultivar containing the same haplotypes

In this study, 61 progeny from cross-pollination to the cultivar 'Syuugyoku' (S_4S_5), which had been identified as 59 S_4S_5 plants and 2 S_5S_5 plants by CAPS analysis of the *S-RNase* gene (Chapter 3), were genotyped for the relative dosages of the S_4 and S_5 haplotypes by a TaqMan allelic discrimination assay. This assay enabled accurate *S*-haplotyping of the 59 S_4S_5 progeny without making the next generation. The TaqMan assay revealed that 55 of the 59 S_4S_5 progeny had three *S* haplotypes ($S_4S_4S_5$ or $S_4S_5S_5$), thus containing a duplicated S_5 haplotype, and 4 were S_4S_5 heterozygotes without a duplicated S_5 haplotype (Fig. 5.2). This result demonstrated that *S*-heteroallelic pollen from 415-1 has SC and selectively fertilized a self-incompatible cultivar that shares both *S* haplotypes with 415-1. The relatively rare S_4S_5 heterozygous plants are assumed to have been derived from S_4 or S_5 pollen that escaped degradation. Similarly, the rare S_5S_5 homozygous plants from this cross are assumed to have been derived from S_5 egg cells and (undegraded) S_5 pollen

(Chapter 3).

5–4–2 Inheritance of pollen-part self-compatibility in progeny with a duplicated *S* haplotype

A large proportion (47 out of 55) of the progeny with a duplicated *S* haplotype from the cross between ‘Syuugyoku’ and 415-1 were self-compatible. However some plants did not demonstrate SC even though they had a duplicated *S* haplotype. A possible cause is reduced pollen viability. As discussed in Chapter 4, 415-1 showed reduced pollen viability (Fig. 4.3), which appears to be caused by harmful effects of gene duplication or detrimental mutations caused by chronic γ-irradiation. The effects leading to reduced pollen viability might be expressed more strongly in some progeny with a duplicated *S* haplotype than in 415-1 itself, resulting in low fertility (seeded fruit set) of those progeny. Another explanation might be the accumulation through inbreeding of genetic aberrations that affect fertilization and seed development. In fact, this population [‘Syuugyoku’ (‘Kikusui’ × ‘Kosui’) × 415-1 (‘Kosui’ × γ-irradiated ‘Kosui’)], which has a high inbreeding coefficient (*F*=0.375), had already exhibited reduced initial growth similar to that in other (non-irradiated) populations with a similar inbreeding coefficient (Sato et al. 2008). Inbreeding could negatively affect the ability of both pollen and style to produce seeds by self-pollination in self-compatible lines. Alonso and Socias i Company (2005) studied the flower sterility in almond selections possessing a stylar-part self-compatible allele. These selections were bred from a cross between a self-compatible cultivar and an inbred (*F*=0.25) self-incompatible cultivar that originated from a landrace in the same region of Italy as the self-compatible cultivar. A considerable number of the selections showed reduced fertility upon self-pollination, but both pollen and styles of these

selections functioned normally when crossed with unrelated cultivars possessing different *S* genotypes. From these results, Alonso and Socias i Company (2005) concluded that inbreeding depression may mask SC in some genetically self-compatible genotypes of almond. For the progeny of 415-1, there is not yet any genetic information on the effects of radiation-induced mutations, inbreeding depression, or copy number changes other than those described in this thesis for the *S* haplotype, so the reason for variation in SC is unknown. However, plants that are self-sterile despite possessing a duplicated *S* haplotype are presumed to have the potential for SC, so they could be tested as seed parents for breeding with pollen from unrelated cultivars, with the idea that these steps might purge radiation-induced mutations and inbreeding depression and restore SC phenotype.

5–4–3 Conclusions

The inheritance of a duplicated *S* haplotype and SC in the progeny from a cross between 'Syuugyoku', a diploid self-incompatible cultivar containing the same two *S* haplotypes as 415-1, and 415-1 was examined by *S*-locus allelic discrimination and copy number determination using TaqMan analysis and by self-pollination tests. These tests demonstrated that 415-1 has the potential to serve as breeding material for pollen-part self-compatible cultivars, because 90% of the progeny had a duplicated *S* haplotype (Table 5.2) even though only 16% of the pollen grains from 415-1 had a duplicated *S* haplotype (Chapter 4). In contrast, only 14% of the progeny from a cross between 'Niitaka' (S_3S_9) and 415-1 had a duplicated *S* haplotype (Chapter 3; Table 5.2). These results suggest that crosses between self-incompatible S_4S_5 cultivars and 415-1 ($S_4S_5S_5$) may be more efficient for producing pollen-part self-compatible progeny than those using self-incompatible cultivars possessing neither S_4 nor S_5 haplotypes, such as 'Niitaka', as the

female parent. However, some progeny with a duplicated *S* haplotype exhibited self-sterility or weaker SC than 415-1 owing to low pollen fertility or other undetermined causes. Assuming that inbreeding depression is expressed in various characteristics of the population bred from 'Syuugyoku' × 415-1, crosses using cultivars possessing an S_4 haplotype, an S5 haplotype, or both haplotypes simultaneously might not perform well, at least for a few generations, because these cultivars may have the same genetic background as 'Kosui', the parent of 415-1. Therefore, in breeding programs for PPM cultivars, it is necessary to develop the next generation from crosses between the PPM selections and self-incompatible cultivars with genetic backgrounds different from that of 'Kosui'. This strategy will remove the negative effect of detrimental genetic factors other than the *S* haplotype itself, such as radiation-induced aberrations and mutations accumulated through inbreeding. Further studies of genotype and phenotype in subsequent generations will help to determine the precise region in the segmental duplication of 415-1 that is necessary for pollen-part SC and the importance of segmental duplications in the breeding of self-compatible cultivars of Japanese pear.

Table 5.1 The primer and probe set used for TaqMan assays

Primer/probe	Sequence 5'→3' (length)	Reporter (5')	Quencher (3')
Forward primer	TGAAATATCTGATATGATAGAATTGCCTTTTAGGG (35)	NA	NA
Reverse primer	CGTCATCGTAGTCCATTACCCATAT (25)	NA	NA
Probe for *S*$_4$ haplotype	AAGGCTCTTC<u>A</u>TAACTAC (18)	6-FAM	NFQ
Probe for *S*$_5$ haplotype	AAGGCTCTTC<u>G</u>TAACTAC (17)	VIC	NFQ

NA=not applicable; 6-FAM=6-carboxyfluorescein; VIC=6-carboxyrhodamine; NFQ=non-fluorescent quencher.

The underlined letter in each probe sequence is the single-nucleotide polymorphism (SNP) in the *PpSFBB*$^{-\gamma}$ gene.

Table 5.2 Comparison between observed and expected rate of the progeny with a duplicated *S* haplotype using seed parents either possessing both S_4 and S_5 haplotypes ('Syuugyoku') or neither of these haplotypes ('Niitaka')

Parents and *S* haplotypes[a]			Expected segregation ratio of *S* haplotype in progeny	Expected frequency of progeny with a duplicated *S* haplotype (%)	Number of seedlings	Observed segregation of *S* haplotype in progeny[b]	Observed frequency of progeny with a duplicated *S* haplotype (%)
Syuugyoku (S_4S_5) SI	×	415-1 ($S_4S_5dS_5$) PPM	S_4S_5 : S_5S_5 : $\boldsymbol{S_4S_4dS_5}$: $\boldsymbol{S_4S_5dS_5}$ = 0 : 0 : **1** : **1**	100	61	S_4S_5 : S_5S_5 : $\boldsymbol{S_4S_4dS_5}$: $\boldsymbol{S_4S_5dS_5}$ = 4 : 2 : **37** : **18**	90
Niitaka (S_3S_9) SI	×	415-1 ($S_4S_5dS_5$) PPM	S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : $\boldsymbol{S_3S_4dS_5}$: $\boldsymbol{S_4dS_5S_9}$ = 0 : 1 : 0 : 1 : **1** : **1**[c] (=3 : 39 : 3 : 39 : **8** : **8**)[d]	50[c] (16)[d]	103	S_3S_4 : S_3S_5 : S_4S_9 : S_5S_9 : $\boldsymbol{S_3S_4dS_5}$: $\boldsymbol{S_4dS_5S_9}$ = 2 : 40 : 0 : 47 : **7** : **7**	14

[a] dS_5, duplicated S_5 haplotype tightly linked to the S_4 haplotype of the homologous chromosome.

[b] Observed segregation of *S* haplotype was determined by TaqMan allelic discrimination assay or CAPS analysis (Chapter 3) of the *PpSFBB*$^{-\gamma}$ gene.

[c] Expected segregation ratio in the case that the probability of inheriting the duplicated *S* haplotype is 1 (i.e., not reduced relative to that of a non-duplicated haplotype), and the maximum frequency of progeny with a duplicated *S* haplotype.

[d] Expected segregation ratio in the case that the probability of inheriting the duplicated *S* haplotype is 0.16 (the ratio of pollen with a duplicated *S* haplotype detected by direct genotyping of single pollen grains of 415-1 in Chapter 4), and the maximum frequency of progeny with a duplicated *S* haplotype.

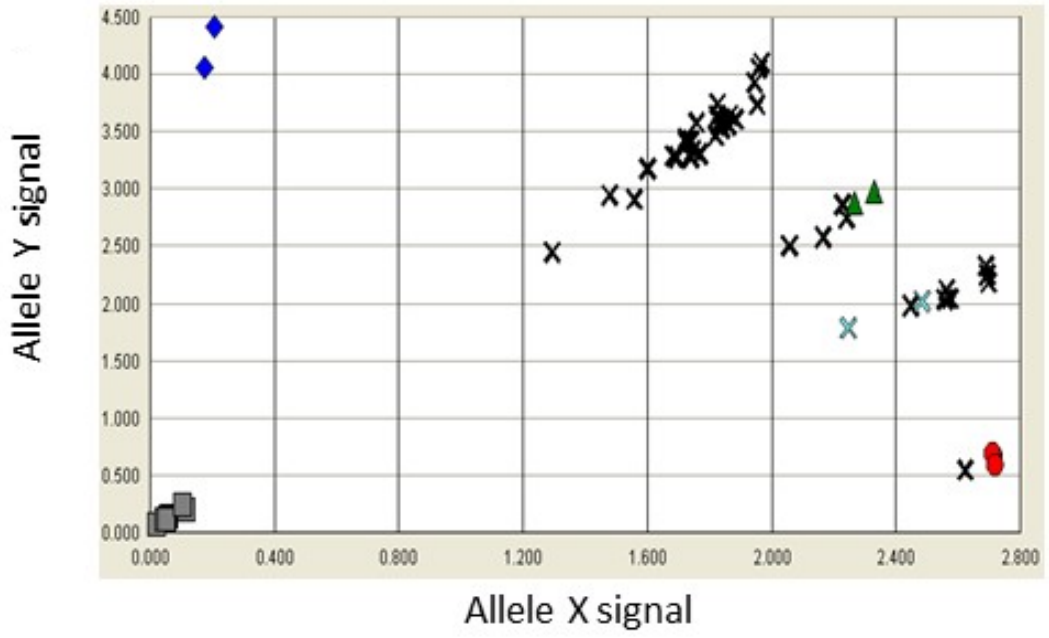

Fig. 5.1 Allelic discrimination plot of *PpSFBB*$^{-\gamma}$ in the progeny of a cross between 'Syuugyoku' and 415-1 by TaqMan assay. The horizontal axis (*x*) indicates the signal intensity of the VIC-labeled probe used to detect the SNP corresponding to the S_5 haplotype (allele X). The vertical axis (*y*) indicates the signal intensity of the 6-FAM-labeled probe used to detect the SNP corresponding to the S_4 haplotype (allele Y). Gray squares indicate no-template controls. Blue diamonds indicate S_4S_4-homozygous standards (421-6). Red circles indicate S_5S_5-homozygous standards (421-24). Green triangles indicate S_4S_5-heterozygous sample ('Syuugyoku'). Light blue crosses indicate 415-1, which has one copy of S_4 and two copies of S_5. Black crosses indicate cross-progeny. The experiment shown in this graph included 21 out of the 61 total progeny plants that were analyzed. Each DNA sample was analyzed in duplicate.

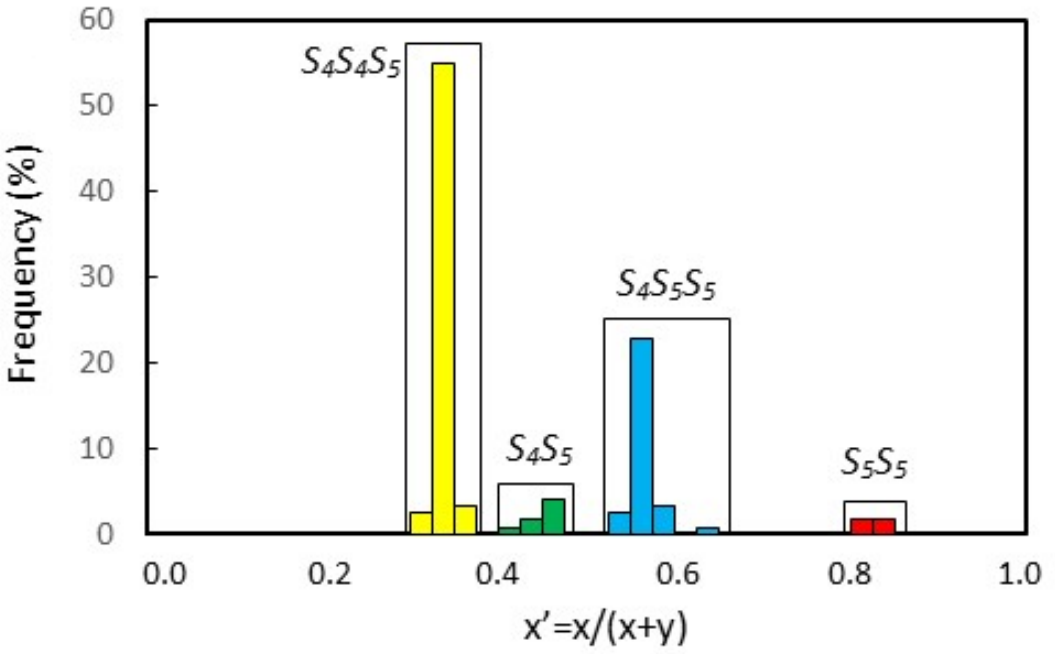

Fig. 5.2 Frequency histogram for relative X allele (S_5 haplotype) dosage [$x' = x/(x+y)$] of 61 progeny of a cross between 'Syuugyoku' and 415-1. The progeny were clustered with the *S* haplotype estimated from the x' parameter.

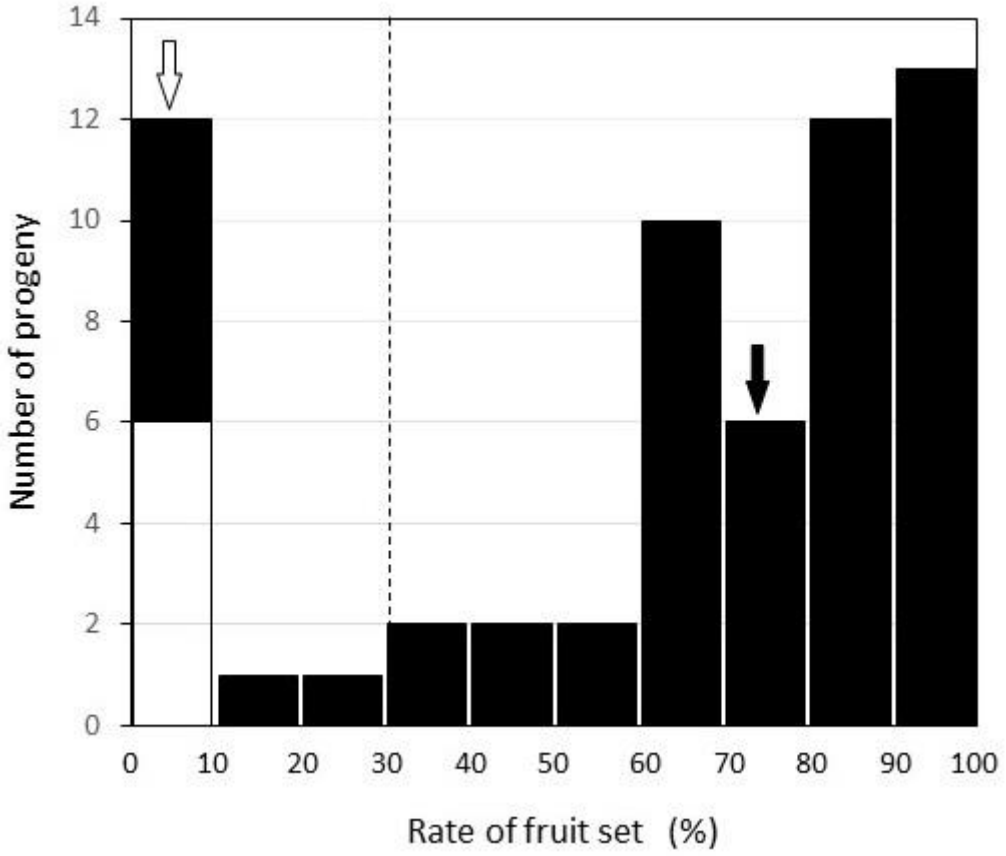

Fig. 5.3 Self-compatibility of progeny with the presence or absence of a duplicated *S* haplotype derived from a cross between 'Syuugyoku' and 415-1 (*n*=61). Rate (%) of fruit set = (number of seeded fruits)/(number of self-pollinated flowers) × 100. Progeny with a duplicated *S* haplotype are shown as filled bars; progeny without a duplicated *S* haplotype are shown as open bars. The black arrow indicates the average fruit set rate derived from the selfing of PPM selection 415-1 (74.4%; Table 2.1); the open arrow indicates the average fruit set rate derived from the selfing of 'Kosui' (5.0%; Table 2.1). SC were determined by whether the seeded fruit set rate was 30% or higher (dashed line).

Chapter 6

General discussion

6–1 Acquisition of self-compatibility by pollination with γ-irradiated pollen in Japanese pear

Despite the long-established and widespread cultivation of apple and pear, there have been no spontaneous self-compatible mutants available until recently. However, since the first report of a self-compatible bud mutant of Japanese pear, 'Osa-Nijisseiki' (Furuta et al. 1980), many physiological, biochemical, and genetic studies of self-incompatibility genes in *Pyrus* have been performed. Molecular comparisons between the mutant and the original cultivar revealed that a highly polymorphic gene at the *S*-locus, called the *S-RNase* gene, controls the stylar function of SI interactions in Japanese pear. Furthermore, it was revealed that the deletion of an S_4-RNase gene occurred in an *S* haplotype of 'Osa-Nijisseiki', causing its stylar-part SC (Norioka et al. 1996). Moreover, a selectable marker for this deletion has been developed (Okada et al. 2008) and used for selection of self-compatible progeny of 'Osa-Nijisseiki'. However, no pollen-part self-compatible mutants (PPMs) with an altered *S* haplotype have been discovered in the Pyrinae (Hegedűs et al. 2012) until the studies described here.

On the other hand, PPM selections of sweet cherry were generated by using pollen collected from an X-ray-irradiated tree in the early years of the application of radiation for mutation breeding (Lewis 1949). These mutants were used to breed PPM cultivars (Lapins 1971) and to study the pollen-part *S*-determinants and DNA sequence of the *S*-locus of sweet cherry (Ushijima et al. 2004). Subsequently, many spontaneous self-compatible cultivars, either SPM or PPM, were discovered in other self-incompatible

species of *Prunus*, and the pollen and stylar functions of the responsible genes in the SI system were estimated using information about the *S*-locus in sweet cherry (Hauck et al. 2006a; Hanada et al. 2009). Furthermore, a selectable marker for each self-compatible *S* haplotype was developed to use for breeding of self-compatible cultivars (Ikeda et al. 2004; Zhu et al. 2004; Tsukamoto et al. 2008). Notably, in the cultivation of sweet cherry, the PPM cultivars are usually planted in mixtures with other self-incompatible cultivars; this practice promotes stable fruit production because the PPM cultivars are able to fertilize not only themselves but also all of the self-incompatible cultivars (Granger 1997). As illustrated by these previous results in Japanese pear and sweet cherry, once a self-compatible mutant has been discovered or generated artificially, it has played important roles in understanding gene function as well as contributing to breeding programs and production system improvements.

Following the development of practical self-compatible cultivars in cherry and almond, production of new self-compatible bud (somatic) mutants of Japanese pear cultivars by continuously exposing trees to low-dose-rate γ-irradiation had been tried in vain, although a bud mutant resistant to black spot disease of Japanese pear had been successfully obtained by that method at the Institute of Radiation Breeding of NIAS (Sanada et al. 1988). However, previous studies for obtaining self-compatible mutations by X-rays or fast neutrons revealed that higher efficiencies for producing self-compatible mutation were obtained when plants with immature flower buds were exposed to radiation as a means of producing mutations in the pollen. Specifically, irradiation of meiotic cells in the prophase I to metaphase I stages was more effective than irradiation of meiotic cells at other stages or of somatic cells (Lewis 1949; van Gastel 1976). In these studies, pollen from irradiated plants was crossed to the styles of non-irradiated plants possessing the

same *S* genotype as the irradiated ones because, theoretically, only pollen containing a self-compatible mutation could grow and fertilize the style. In fact, a large fraction of seedlings from these crosses were revealed to be self-compatible; of these, almost all were pollen-part self-compatible (Lewis 1949; van Gastel 1976).

By using a similar strategy in these study, we were able to obtain the first PPM pear by pollination using pollen from a chronically γ-irradiated pear tree (Chapter 2). Since the pollen parent 'Kosui' had been irradiated intermittently for more than 30 years, the pollen-part self-compatible mutation in a pollen grain of 'Kosui' was presumed to be generated in somatic cells during the vegetative growth stage or in a germ cell during pollen development. Regardless of the stage at which the mutation was introduced, this strategy for obtaining self-compatible mutants, especially PPM, by using pollen from irradiated plants was demonstrated to be effective.

In conclusion, for acquisition of a new character using radiation mutagenesis, pollination with pollen from mutagenized plants is worth trying, not only in seed-propagated plants but also in perennial fruit trees. More than a half-century after the first PPM was generated using X-ray-irradiated pollen in *Prunus*, the successful obtaining of a PPM in Pyrinae by using γ-irradiated pollen as the second case in self-incompatible fruit crops has great significance for research and breeding. By using the same methods as in these experiments, PPMs could potentially be developed not only in other cultivars of Japanese pear, but also in European pear or apple. In recent years, heavy-ion radiation has been revealed to be more effective than γ-rays for inducing mutations in plants because of its higher ability to produce chromosomal rearrangements involving segmental duplications (Tanaka et al. 2010). Therefore, pollination using pollen from heavy-ion-irradiated plants may be an even better option for obtaining PPMs with a segmental

duplication encompassing *S* haplotype.

6–2 Availability of pollen *S*-determinant mutations that trigger pollen-part SC under different recognition systems in *S*-RNase–based SI in Rosaceae

The pollen determinants of SI in Rosaceae were first identified in almond (*Prunus dulcis*; Ushijima et al. 2003). In *Prunus* (e.g., apricot, sweet cherry, and Japanese apricot), all PPMs except a non-*S*-linked PPM (Cachi and Wünsch 2011; Zuriaga et al. 2013) displayed inactivation of one of two *SLFs*/*SFBs* by the deletion or non-transcriptional mutation of *SLF*/*SFB* in an *S* haplotype. Thus, SLF/SFB is considered to be the sole pollen *S* factor for recognition and activation of the cytotoxic function of self *S*-RNase in the SI system in *Prunus* (Tao and Iezzoni 2010). However, the PPM of Japanese pear obtained in this study did not show any deletion in an *S* haplotype; rather, it was caused by duplication of an *S* haplotype (Chapter 3). Furthermore, this PPM was shown to be diploid, but was able to produce *S*-heteroallelic pollen grains capable of breaking down SI. Therefore, the competitive interaction (CI) hypothesis (Lewis and Modlibowska 1942; Lewis 1952) is a possible explanation of our findings. This hypothesis was originally proposed as an explanation of SC in tetraploid pears; that is, it was proposed that two different *S*-determinants in a diploid *S*-heteroallelic pollen grain could break down the pollen function of SI (Lewis and Modlibowska 1942). This hypothesis is adaptable to haploid pollen grains with two different *S* haplotypes from diploid pear, such as those with a duplication of an *S* haplotype. Recently, this CI hypothesis was studied in detail in petunia (Solanaceae) and redefined as a “collaborative non-self recognition system” by Kubo et al. (2010). According to their current hypothesis, each SLF can degrade only a subset of the non-self *S*-RNases, even though the complete set of SLFs in an *S* haplotype

can degrade all except self-*S*-RNase (Fig. 1.3). In this model, the two different sets of SLFs in *S*-heteroallelic pollen will together contain the SLFs necessary to degrade the two *S*-RNases encoded by the two alleles. Consequently, these pollen lead to SC, because all *S*-RNases (including those from self *S* haplotypes) are degraded. Kubo et al. (2010) raised the possibility that the system for SI/SC system in Pyrinae, including Japanese pear, is also regulated by a collaborative non-self recognition system (Fig. 1.3) on the basis of the following three lines of evidence: (1) Japanese pear and apple have clusters of pollen *S*-haplotype-specific F-box proteins, as in the Solanaceae (Okada et al. 2008; Minamikawa et al. 2010); (2) apple was found to contain almost-identical *SFBB* sequences across different S-haplotypes (Minamikawa et al. 2010); and (3) SPM cultivar 'Osa-Nijisseiki' of Japanese pear lacks both S_4-*RNase* and one of the *SFBB* genes (Okada et al. 2008), but maintains pollen-part SI and shows cross-incompatibility with a style harboring a non-S_4 (e.g., S_1) haplotype (Saito et al. 2002). Our finding that addition of a chromosomal segment involving an extra *S* haplotype to a diploid genome leads to a breakdown in pollen function of SI in Japanese pear provides further support for the hypothesis that the SI/SC system is regulated by collaborative non-self recognition in the Pyrinae. Under this hypothesis, a pollen grain of Japanese pear that has lost all functional SFBBs of an *S* haplotype would not be self-compatible; rather, such pollen would be incapable of detoxifying any non-self *S*-RNases and would thus be incompatible with styles of any *S* haplotype. Therefore, when a new PPM is discovered in the Pyrinae, there is no need to look for a deletion in the *S* haplotype; instead, the first step should be to determine the *S* haplotype copy number. Recently, copy number variation of genes in the human genome has been effectively revealed by comparative genomic hybridization arrays and SNP arrays (Redon et al. 2006). By using microarrays made from the genome

sequence information of *Pyrus*, the type and number of *SFBB* genes in the duplicated S_5 haplotype in 415-1 will be determined precisely.

6–3 Utility of a PPM with a duplicated *S* haplotype for determining the factors associated with pollen-part SC in Pyrinae

Most progeny from cross of self-incompatible 'Syuugyoku' (S_4S_5) × 415-1 ($S_4S_5S_5$) inherited the duplicated *S* haplotype from 415-1 (Chapter 5), even though *S*-heteroallelic pollen represented a low proportion (16%) of the total (Chapter 4). This indicates that the progeny containing the duplicated *S* haplotype were generated as a result of selective fertilization. The S_4S_5 heteroallelic pollen grains were capable of breaking down SI in S_4S_5 styles by two non-self recognitions: one between S_5-RNase and a particular type of S_4-PpSFBB expressed by the original S_4 haplotype, and another between S_4-RNase and a particular type of S_5-PpSFBB expressed by the duplicated S_5 haplotype (Fig. 6.1). However, it has yet not been identified which one of the *S_5-PpSFBB* genes in the duplicated S_5 haplotype caused pollen-part SC in the S_4S_5 style.

Unlike the previously described tetraploid PPMs, in which the whole genome was duplicated, 415-1 is diploid with a relatively small duplicated region encompassing a duplicated *S* haplotype. Thus, this mutant will be useful for elucidating the functions of duplicated *PpSFBB* genes. Two genetic approaches to identify the function of each duplicated *PpSFBB* gene can be considered. One approach is to transform a single *PpSFBB* of the duplicated S_5 haplotype into an S_4-homozygous genotype and then evaluate whether the transformants express SC or SI. The transformation approach was performed in petunia, where it provided information on the functions of some *SLF* genes (Kubo et al. 2010, 2015). Another approach is to narrow down the duplicated region of

415-1 by searching for recombinants with even smaller duplicated regions, and then assessing whether the recombinants retain SC. In tobacco, some self-incompatible plants with a centric fragment were obtained from a cross using pollen of PPMs that had a centric fragment containing a duplicated *S* haplotype; these plants were presumed to have a smaller duplicated region than that of their PPM parents (Golz et al. 2001). By understanding which PpSFBB is required to degrade *S*-RNase in certain *S* haplotypes of Japanese pear, pollen-part self-compatible plants could be generated by transforming the needed *PpSFBB* gene into normal self-incompatible cultivars with the corresponding *S* haplotypes.

6–4 Availability of a PPM with a duplicated *S* haplotype for breeding of self-compatible cultivars using marker-assisted selection and single-pollen genotyping

PPM selection 415-1 is not promising for use as a practical self-compatible cultivar, because it has unremarkable characteristics in terms of fruit quality. Furthermore, the selection is not suitable to use as a pollinator because of its low production of mature pollen grains (Chapter 4). However, 415-1 is suitable for use in breeding of pollen-part SC cultivars not only in Japanese pear but also in European and Chinese pear. Since the selection is diploid, with a limited segmental duplication, it is possible to cross it with any self-incompatible cultivar to obtain PPM progeny. In the progeny between 415-1 and a self-incompatible cultivar that contain neither the S_4 nor the S_5 haplotype, offspring with three *S* haplotypes (including the duplicated *S* haplotype), which have a high possibility of being pollen-part self-compatible, could be selected by CAPS analysis using an *S-RNase* or *PpSFBB*$^{-\gamma}$ marker. In progeny from crosses with cultivars that have S_4 haplotype, a S_5 haplotype, or both haplotypes, plants with a duplicated *S* haplotype could

be selected by determining the *S* haplotype allelic composition and dosage with the TaqMan allelic discrimination assay (Chapter 5). Moreover, the productivity of *S*-heteroallelic pollen in the selected progeny plants with a duplicated *S* haplotype could be evaluated by direct genotyping of the *S* haplotype or haplotypes in a single pollen grain. By using this technique, PPM progeny with higher production of *S*-heteroallelic pollen can be selected without the need to produce the next generation for genetic analysis. At present, the PPM selection 415-1 is the sole genetic resource for breeding of pollen-part self-compatible cultivars in Japanese pear.

6-5 Future strategies for sustainable breeding of pollen-part self-compatible cultivars of Japanese pear

One of the important limitations in breeding use of self-compatible mutants is inbreeding. Self-incompatible plants have maintained genetic heterozygosity and diversity through cross-pollination, but self-compatible mutations promote a transition from outcrossing to selfing. Therefore, the progeny of self-compatible mutants of wild self-incompatible species may have narrowed genetic diversity and thus a risk of inbreeding depression owing to the homozygosity of detrimental recessive alleles; this is particularly true for accessions such as 415-1 that resulted from decades of exposure to mutagenic radiation. For this reason, SC genes tend to be eliminated by natural selection (Goldberg et al. 2010), although they have been maintained in some fruit species by artificial selection. In cultivated fruit trees, modern artificial cross-breeding has been apt to use particular founders and their offspring, so genetic diversity in cultivars of many species has decreased (Choi and Kappel 2004; Mariette et al. 2010).

For example, in almond, a species in which breeding has been performed using stylar-

part self-compatible cultivars, the genetic diversity of recent cultivars has become narrower (Fernández i Martí et al. 2014). In almond breeding programs, the self-compatible landrace ‘Tuono’, which has almost the same genetic background as existing breeding materials, was used frequently. As a result of this breeding strategy, both self-compatible and self-incompatible cultivars with low genetic diversities have been bred (Lansari et al. 1994). Consequently, a decrease in productivity was observed in inbred self-compatible cultivars (Socias i Company 1990). It was later found that low or null fruit set of highly inbred self-compatible selections is caused by delay of pollen tube growth or abortion of the endosperm after self-pollination (Alonso and Socias i Company 2005; Martínez-García et al. 2012). Martínez-García et al. (2012) concluded that inbreeding depression negatively affects embryonic development in the initial growth stage of progeny. These results indicate that genetic diversification will be needed in breeding of self-compatible cultivars.

In Japanese pear, the inbreeding coefficient (F) had increased among a large number of recently released cultivars and breeding selections because of frequent use of economically important cultivars bred from a limited number of ancestors, such as ‘Nijisseiki’ (Onoue et al. 2015). Furthermore, only one self-compatible mutant, ‘Osa-Nijisseiki’, which was derived from ‘Nijisseiki’, has been used for breeding of self-compatible cultivars in the last 20 years (Tanabe et al. 2001). Further continuous breeding using these materials may cause inbreeding depression in both self-incompatible and self-compatible cultivars. Although 415-1 can contribute to enlarging the genetic diversity of self-compatible cultivars of Japanese pear, two sources of SC may not be enough. 415-1 has some defects, such as low tree vigor and pollen fertility, that may be caused by detrimental mutations induced during long-term γ-irradiation and by inbreeding

depression. Furthermore, since ‘Kosui’ (the parent of 415-1) was derived as a second-generation progeny of ‘Nijisseiki’, this mutant might not significantly increase the genetic diversity of self-compatible breeding selections. Thus, for breeding of pollen-part self-compatible cultivars using 415-1, it should not be crossed only with current commercial cultivars. Instead, crossing it with native cultivars that have not been used much in breeding programs may remove many genetic defects from the progeny. However, because these native cultivars may have less favorable characteristics, this strategy may take some time to produce acceptable results.

Using a similar strategy to that used for production of PPM 415-1, it should be possible to obtain various PPM selections of pears from cultivars with different genetic backgrounds. As one feasible option for obtaining PPMs with a duplicated *S* haplotype, heavy-ion irradiation might be used, as it generates segmental duplications more frequently but induces fewer harmful mutations than γ-irradiation (Tanaka et al. 2010). The mutagenized pollen should be used to pollinate cultivars that share the same *S* haplotypes as the pollen donor but are of a different genetic background, so as to reduce the risk of inbreeding depression in the progeny. Subsequently, crossing with selected commercial cultivars, ideally those with a low inbreeding coefficient, will enable development of pollen-part self-compatible cultivars with high fruit productivity and quality. Various pollen-part self-compatible cultivars will be needed to ensure stable production of Japanese pear in the future.

6–6 Conclusions

In this study, a novel PPM (415-1) of Japanese pear was generated by fertilization with pollen from γ-irradiated ‘Kosui’ (S_4S_5) (SI) onto the style of non-irradiated ‘Kosui’.

Genetic analysis of the *S* haplotypes and SSR markers in the same linkage group revealed that the pollen-part SC of breeding selection 415-1 is caused by duplication of an *S* haplotype. These results provided new evidence that the recognition between pollen and pistil in *S*-RNase–based SI in Pyrinae is controlled by a collaborative non-self recognition system. Furthermore, the effectiveness of using *S* haplotype markers to select for progeny with a duplicated *S* haplotype was demonstrated. Moreover, the *S* haplotypes of individual pollen grains from 415-1 were successfully genotyped, providing a direct measure of the proportion of pollen with a duplicated *S* haplotype. These achievements are important for the breeding of pollen-part self-compatible cultivars in *Pyrus* and will contribute to future research on the exact mechanisms of *S*-RNase–based SI in Pyrinae.

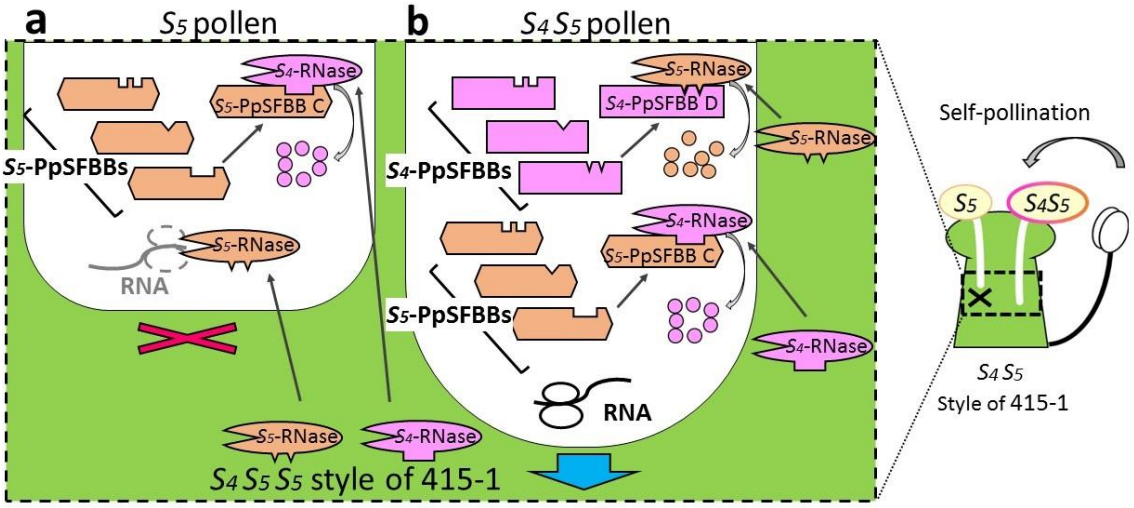

Fig. 6.1 Mechanism of pollen rejection and acceptance in 415-1 ($S_4S_5S_5$) according to the collaborative non-self-recognition model. (a) Pollen tube growth of S_5 pollen is assumed to be arrested because there is no PpSFBB that can recognize and degrade self (S_5)-RNase. (b) Pollen tube growth of S_4S_5 pollen (pre-existent S_4 haplotype and a duplicated S_5 haplotype) is assumed to continue and enable fertilization because one of the S_4-PpSFBBs expressed by the original S_4 haplotype is capable of degrading S_5-RNase, and one of the S_5-PpSFBBs expressed by the duplicated S_5 haplotype is capable of degrading S_4-RNase.

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