



Reproduction in woody perennial Citrus: an update on nucellar embryony and self-incompatibility

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Key message Review on citrus reproduction.

Abstract Citrus is one of the most important and widely grown fruit crops. It possesses several special reproductive characteristics, such as nucellar embryony and self-incompatibility. The special phenomenon of nucellar embryony in citrus, also known as the polyembryony, is a kind of sporophytic apomixis. During the past decade, the emergence of novel technologies and the construction of multiple citrus reference genomes have facilitated rapid advances to our understanding of nucellar embryony. Indeed, several research teams have preliminarily determined the genetic basis of citrus apomixis. On the other hand, the phenomenon of self-incompatibility that promotes genetic diversity by rejecting self-pollen and accepting non-self-pollen is difficult to study in citrus because the long juvenile period of citrus presents challenges to identifying candidate genes that control this phenomenon. In this review, we focus on advances to our understanding of reproduction in citrus from the last decade and discuss priorities for the coming decade.

Keywords Citrus · Reproduction · Polyembryony · Apomixis · Self-incompatibility · Self- and non-self-recognition

Introduction

Citrus, including oranges, mandarins, lemons, and grapefruits, is some of our planet's highest-yielding fruit crops and is distributed worldwide. The top ten citrus-producing countries are China, India, Nigeria, Brazil, Mexico, the USA, Spain, Egypt, Italy and Argentina. According to FAOSTAT, in 2014, the worldwide cultivation of citrus utilized more than 9.08 million hectares and yielded more than 139.79 million tons, with a gross production value of 66.44 billion US dollars. Most citrus species are xylophytas that

are evergreen with juvenile periods that range from five to eight years. Although the long juvenile period hinders the breeding and research process, a series of unique reproduction features in citrus are attracting more and more interest from developmental biologists. During the last decade, research on reproduction in citrus has focused on three areas of reproductive development: gene-cytoplasmic interactions in mandarin that induce pollen sterility during gametogenesis (Zheng et al. 2014), production of seedless fruits in several pummelo and mandarin-like varieties with self-incompatibility (Ollitrault et al. 2007), and nucellar embryo development from somatic embryos derived from maternal nucellus tissue surrounding the sexual embryo sac. This last process is a kind of apomixis and is found in many citrus cultivars (Bicknell and Koltunow 2004).

Apomixis has been observed in more than 400 plant species spanning approximate 61 families, such as Gramineae, Compositae, Cruciferae, Roaceae, and Rutaceae (Ozias-Akins 2006). Except for apple and citrus, apomixis is rarely found in agricultural crop plants. The apomictic progeny inherit the mother's genotype. Thus, apomixis can fix desirable genotypes for research and breeding (Spillane et al. 2001). Mechanisms of apomixis vary widely in woody plants and herbages. Mechanisms of apomictic reproduction are classified as either gametophytic or sporophytic according

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to their origin. In gametophytic apomixis, which is widely observed in grass species, the apomictic embryo develops from the female gametophytic structure through apospory or diplospory (Koltunow and Grossniklaus 2003). In adventitious embryony, typical in citrus and mango, somatic embryos initiate directly from nucellus or integument cells in the ovule. The offspring derived from nucellar embryony in *Citrus* possess the same genetic constitution as the female parent. The phenomenon of nucellar embryony hinders the formation of hybrid offspring and the progress of cross-breeding. However, it greatly benefits the production of offspring for true-to-type rootstock with good unity and yields virus-free seedlings. Therefore, investigating the mechanism of nucellar embryony in citrus will provide deeper insight into the processes controlling apomictic reproduction and facilitate the transfer of apomixis into other crops.

Another significant trait in citrus breeding is self-incompatibility (SI). SI is associated with parthenocarpy, which yields seedless citrus fruit (Gambetta et al. 2013; Honsho et al. 2009). Many commercial cultivars of citrus are self-compatible and seedy. However, seedlessness is an important commercial feature in many fruits. Recently, the self-incompatibility response was found to underpin seedlessness in a new seedless mutant of citrus—the mandarin cultivar ‘Wuzishatangju’ (Ye et al. 2009). The list of self-incompatible accessions in citrus is growing. Additionally, the bud mutants of cultivated varieties provide pairs of phenotypes that promote the study of self-incompatibility and provide a new approach for breeding seedless fruit in citrus. In addition to the normal outcrossing, apomixis and self-incompatibility are two major pathways of reproduction in angiosperms (Hörandl 2010). However, these mechanisms of reproduction remain poorly understood in citrus. This review mainly describes advances on the mechanisms of nucellar embryony and self-incompatibility, specifically focusing on advances in the mapping and cloning of genes controlling asexual reproduction and the identification of the potential *S*-locus of self-incompatibility.

Nucellar embryony in citrus

Many members of the genus *Citrus* and some closely related genera belonging to Rutaceae reproduce apomictically by nucellar embryony, including *Zanthoxylum*, *Clausena*, *Aegle*, *Feroniella*, *Murraya*, *Ptelea*, *Ruta*, and *Triphasia* (Carman, 1997). The presence of extra embryos from nucellar tissue gives rise to polyembryonic seeds. The closely related term polyembryony refers to the development of two or more embryos in one seed. A polyembryonic variety is commonly considered to be apomictic, as a monoembryonic variety is sexual or zygotic. However, the extra embryos are occasionally produced by the division of the zygote embryo,

usually in polyploidy cultivars (Webber 1940; Aleza et al. 2010). Various polyembryonic resources in *Citrus*, *Fortunella*, and *Poncirus* provide excellent germplasm for studying this trait. We summarize the germplasm of citrus polyembryonic and monoembryonic cultivars, based on a survey by the Chinese Academy of Agricultural Sciences Institute of Citrus (Zhou and Ye 2010) and our own examination and previous reports (Table 1). The citrus relatives in *Poncirus* and *Fortunella* are mostly polyembryonic, except for *F. margarita* and *F. japonica*. In *Citrus*, most cultivars in *C. paradise*, *C. sinensis*, and *C. reticulata* are polyembryonic. However, several types of citrus with ancient origins always produce monoembryonic seeds, such as *C. medica*, *C. grandis*, *C. mangshanensis*, and *Papeda*. The exceptions are mainly derived from hybridization, such as ‘Kiyomi,’ which is derived from a cross between *C. unshiu* ‘Miyagawa wase’ and *C. sinensis* ‘Trovia’ orange—two polyembryonic cultivars (Nakano et al. 2008b). The diverse varieties of citrus germplasm provide rich resources for breeding and for studying the evolution of polyembryony in citrus.

The phenomenon of polyembryony was first reported by Leeuwenhoek in 1719, when he found that orange seeds each contain more than one embryo. Following this initial observation, numerous histological and genetic studies have advanced our understanding of this special phenomenon (Koltunow et al. 1995; Wakana and Uemoto 1987). The initial nucellar embryo cells have characteristic angular thick-walled cells with enlarged nuclei and a condensed cytoplasm. These cells are derived from one or two cell layers of the nucellus, which surround the embryo sac from the chalazal end to the micropyle prior to anthesis (Fig. 1) (Koltunow et al. 1995; Wakana and Uemoto 1987). The nucellar tissue acquires the potential for nucellar embryogenesis at the binucleate embryo sac stage, before the initial cells are evident (Koltunow et al. 1995). Subsequently, the initial cells become larger, rounded, thin walled, and vacuolated. Then, they divide and expand. The division and expansion of the embryonic cell groups is suspended as the zygotic embryo enters dominant period. As the zygotic embryo develops, these embryonic cell groups grow into the position of embryo sac and develop into mature nucellar embryos that frequently supersede the zygotic embryo (Koltunow 1993; Koltunow et al. 1995; Wakana and Uemoto 1987).

Genetic and molecular studies of nucellar embryogenesis

The genetic characteristics of apomixis are complex. Additionally, gametophytic and sporophytic apomixis may utilize distinct genetic mechanisms. During the last decade, several research groups have used segregating populations and molecular markers to develop several hypotheses that

Table 1 Embryony and self-incompatibility type in citrus and relatives of citrus

Genus	Classification	Species	Embryony type	Monoembryonic cultivars	Self-incompatibility cultivars
<i>Fortunella</i>	Kumquat	<i>Fortunella hindsii</i>	Polyembryony, monoembryony occasionally		
		<i>F. cassifolia</i>	Polyembryony		
		<i>F. margarita</i>	Monoembryony		
		<i>F. japonica</i>	Monoembryony		
<i>Poncirus</i>	Trifoliolate	<i>Poncirus trifoliata</i>	Polyembryony	'Guanyun' (4X) 'Changyangzhicheng'	
		Hybrid of <i>P. trifoliata</i>	Polyembryony		
<i>Citrus</i>	Papedocitrus	<i>C. ichangensis</i>	Monoembryony generally		
		<i>C. junos</i>	Mixture		
	Papada	<i>C. hongheensis</i>	Monoembryony	'Yunnanhonghe'	
		<i>C. macropera</i>	Monoembryony		
Citrophorum	<i>C. medica</i>		Monoembryony or 2-embryo occasionally		
			Monoembryony	'Binchuan,' 'Cang Yuanye,' 'Daxiangyuan,' 'Hongpidaguo,' 'Zhoupi,' 'Yunnan'	
			Polyembryony	'Mangshi'	'Xiangshui,' 'Kagzi Kalan'
			Mixture	'Meyer,' 'Honglimeng E-4-123'	
Cephalocitrus	<i>C. grandis</i>	<i>C. aurantifolia</i>	Polyembryony		
		<i>C. limonia</i>	Mixture		
		<i>C. limon</i>	Polyembryony	'Daningmeng,' 'Magan'	
		Hybrid of <i>C. limon</i>	Mixture	'Bergamot'	
Aurantium	<i>C. aurantium</i>		Mixture		
			Monoembryony		
			Polyembryony		
			Polyembryony		
Sinocitrus	<i>C. mangshanensis</i>		Polyembryony mostly		
			Mixture		
			Polyembryony		
			Polyembryony		
Hybrid of <i>C. reticulata</i>	Mixture				

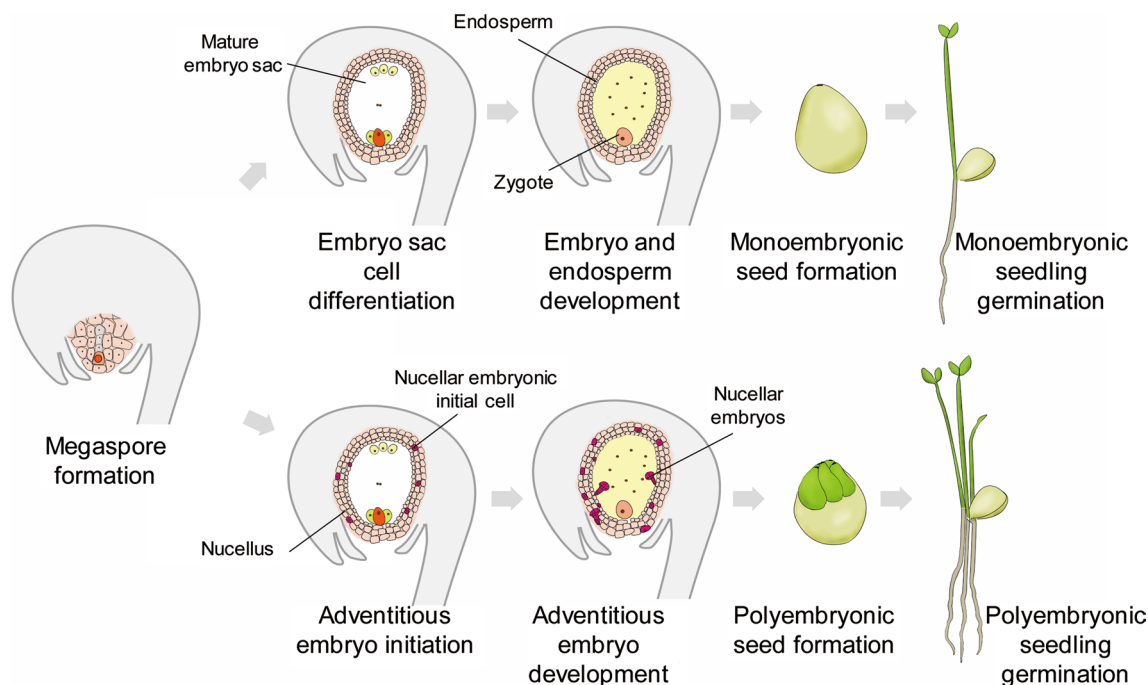


Fig. 1 Overview of sexual and asexual seed development in citrus. Major processes of seed development are depicted during the formation of monoembryonic and polyembryonic seeds. Sexual reproduction pathways including meiosis, mitosis, and double fertilization occur in both monoembryonic and polyembryonic seeds. In apomictic ovules, nucellar cells may possess the ability to form nucellar embryos, which is histologically invisible, when the megaspore is divided into the binuclear embryo sac. Several nucellar embryo ini-

tial cells (purple) emerge and develop into adventitious embryos in the nucellus tissue (pink) surrounding the sexual embryo sac. In the depicted adventitious embryogenesis pathway, parts of the nucellar embryo initial cells begin to divide and launch embryogenesis when the zygote remains dormant after fertilization. Multiple adventitious embryos develop within the ovule forming a polyembryonic seed. The monoembryonic seed contains only the zygotic embryo

have substantially advanced our knowledge of the genetic basis and the locus controlling polyembryony in citrus (Table 2). Population construction and genetic mapping of polyembryony utilizes the ‘double pseudo-test cross’ theory and an F1 population because citrus plants have a long juvenile phase and high degrees of heterozygosity. Based on the ratios of phenotypes that Parlevliet and Cameron (1959) observed in several segregating populations, they proposed that the initiation of nucellar embryony is controlled by a single dominant gene in the *Citrus* genus and the *Poncirus* genus. Subsequently, Iwamasa et al. (1967) found that the one gene hypothesis could not explain the ratios of phenotypes in every segregating population. They proposed that duplicated genes or modified genes complicated the ratios of segregating phenotypes. Cameron and Soost (1979) proposed that two dominant heterozygous genes control polyembryony and that the genotype was *P1p1 P2p2* in *Poncirus*. Based on an analysis of several populations and previously published results, Hong et al. (2001) hypothesized that two complementary dominant genes control apomixis in *Citrus* and *Poncirus* and that the dominant homozygous genotype *A1A1* is lethal. Based on the ratios of segregating phenotypes, a unanimous

conclusion on the genetic basis of this trait does not seem possible.

García et al. (1999) provided the first genetic analysis of nucellar embryony using molecular markers to analyze a hybrid population derived from *C. volkameriana* and *P. trifoliata*. They reported that apomixis in citrus is controlled by six quantitative trait loci that either positively or negatively affect apomixis. Based on these results, it appeared that different genetic mechanisms control the production of nucellar embryos and proportion of polyembryonic seeds. Furthermore, this research group investigated two complex rootstock populations and detected only three previously reported QTLs that control the type of embryony (Raga et al. 2012). Among these QTLs, Apo2 contributed to a greater degree than the other QTLs, consistent with the previous report by García et al. (1999). CR14,290 and TAA15 are two markers that were useful for the early selection of polyembryonic rootstocks in the progeny derived from *C. reshni*, *C. aurantium*, and *C. volkameriana* (García et al. 1999; Raga et al. 2012). Polyembryony in *P. trifoliata* was also investigated with a population derived from a cross between *C. maxima* and *P. trifoliata* using AFLP markers that were tightly linked to this trait (Roose and Kepiro 2010). These

Table 2 The study of apomixis in citrus

	Finding or hypothesis	Markers or genes of interest noted by author	Year of publication
Parlevliet and Cameron	Nucellar embryony is controlled by a major dominant gene <i>P</i> and minor or modifier genes that influence the level of expression.		1959
Iwamasa et al.	Duplicate genes or modifying genes complicate the phenotypic ratios of nucellar embryony phenotypes		1967
Cameron and Soost	Two dominant heterozygous genes control polyembryony		1979
Deidda and Chessa	Three principal dominant genes control polyembryony		1982
García et al.	Six QTL loci (two in <i>P. trifoliata</i> and four in <i>C. volkameriana</i>) contribute to apomixis	<i>Apo2</i>	1999
Hong et al.	Two complementary dominant genes <i>A1</i> and <i>A2</i> control apomixis, and in the homozygous state, <i>A1</i> is lethal		2001
Roose and Kepiro	Five markers tightly linked to a potential gene controlling polyembryony in <i>P. trifoliata</i>	EMB-6P	2010
Raga et al.	<i>Apo2</i> is the QTL locus that contributes the most to embryony	CR14,290 and TAA15	2012
Nakano et al.	Polyembryony is mainly determined by a single 380-kb locus containing 70 predicted open reading frames (ORFs)	70 ORFs	2008a, b, 2012
Nakano et al.	Two apomictic and three non-apomictic genotype-specific genes may be associated with polyembryony	msg-2	2013
Kumar et al.	Upregulation of transcripts associated with abiotic stress and downregulation of signaling genes is probably associated with polyembryony	orfs 21, 22, and 25 (unknown proteins)	2014
Long et al.	Oxidative stress-responsive genes are over-represented in polyembryonic ovules	miRN23-5p	2016
Wang et al.	A transposon insertion in the promotor of <i>CitRWP</i> upregulated its expression in polyembryonic ovules.	CitRWP	2017

data also indicated that the proportion of polyembryonic seeds was probably dependent on distinct genes. Nakano et al. (2008a, b) constructed genetic and physical maps of the chromosomal region flanking the nucellar embryony locus using an F1 population derived from the monoembryonic cultivar *C. unshiu* × *C. sinensis* ‘Kiyomi’ backcrossed to its parent *C. unshiu* ‘Miyagawa wase.’ This work independently localized the polyembryony locus to an approximately 380-kb interval containing 70 genes in mandarin (Nakano et al. 2012).

Mechanisms that contribute to nucellar embryogenesis were suggested to depend on genes that are differentially expressed in closely related cultivars with different modes of reproduction. Thus, the expression of genes in the ovules of poly- and mono-embryonic cultivars was analyzed with suppression subtractive hybridization techniques and microarrays (Kumar et al. 2014; Nakano et al. 2013). These studies provided evidence that stress-responsive genes may contribute to nucellar embryony. A conjoint analysis of mRNA and

microRNA was performed on the ovules from species with different reproductive modes prior to nucellar embryogenesis and during different stages of nucellar embryo initiation using two pairs of related citrus cultivars (Long et al. 2016). Results from these experiments indicated that the expression of the novel miRN23-5p is downregulated and its targets are upregulated in polyembryonic ovules. It was also shown that genes associated with stress responses were over-represented in polyembryonic cultivars, which is consistent with previous analyses.

Next-generation sequencing has fueled integrative projects on multiple citrus genomes to gain more insight into the development of citrus (Xu et al. 2013; Wang et al. 2017). The genetic locus responsible for citrus polyembryony was narrowed to an 80-kb region containing 11 candidate genes including *CitRWP*, which encodes a RWP-RK transcription factor that regulates cell differentiation during female gametophyte development and pattern formation in embryos (Wang et al. 2017; Jeong et al. 2011; Koi et al. 2016;

Rovekamp et al. 2016; Waki et al. 2011). The expression of *CitRWP* is higher in polyembryonic citrus, probably because of a miniature inverted repeat transposable element (MITE) that is inserted into the promoter region of *CitRWP*. This MITE insertion cosegregated with polyembryony in 786 citrus accessions. This is the first study to provide strong evidence for a candidate gene responsible for the development of nucellar embryos in citrus. The reduced expression of a *Marchantia polymorpha* RWP-RK domain gene (*MpRKD*) leads to parthenogenetic-like egg cell divisions (Rovekamp et al. 2016). The *PsASGR-BABY BOOM-like* (*PsASGR-BBML*) gene in *Pennisetum squamulatum* induces parthenogenesis and produces haploid offspring in transgenic sexual pearl millet, rice, and maize (Conner et al. 2015, 2017). *CitRWP* seems to act in a different way than *PsASGR-BBML* because it is expressed only in the egg cell. It is still unclear how these genes induce cells to form embryos in the absence of fertilization. Nonetheless, it is obvious that significantly different developmental mechanisms induce parthenogenesis in haploid egg cells and embryogenesis in diploid nucellar cells. Therefore, *CitRWP* is probably functionally distinct from *PsASGR-BBML*.

Previous studies of polyembryony have revealed the preliminary genetic and molecular mechanism of polyembryony. However, when studying citrus, the long juvenile phase and the considerable space required for growing citrus trees constrain the size of populations. Additionally, the initial aim for the construction of these populations is always for breeding rather than for locating genes. These issues lead to population structures that are relatively complex, distorted segregation, and increased difficulty for genetic analysis and mapping. Based on genetic analyses of *Poncirus* and *Citrus* populations, the polyembryony trait of *Citrus* is probably controlled by one major gene. In contrast, other genes may regulate polyembryony in *Poncirus* because serious distortions were observed in the segregating *Poncirus* hybrid population and the genetic basis of these distortions is not known (Roose and Kepiro 2010). Additionally, the location of a quantitative trait that controls the proportion of apomictic seed is not known. Larger population sizes are required to mine these QTLs. Since the candidate gene in *Citrus* has been identified, it would seem important to test whether the same mechanism drives the development of polyembryony in *Poncirus* and *Fortunella*. It seems that polyembryony may have evolved from monoembryony because most species in the subgenus *Metacitrus* are polyembryonic and the species in *Archicitrus* are monoembryonic. This becomes problematic when considering the breadth of citrus relatives because *Poncirus* and *Fortunella* are generally polyembryonic. Further study of the evolution of polyembryony may lead us to the mechanism that led to the emergence of sporophytic apomixis during the domestication and evolution of citrus. The cosegregation of the MITE insertion in the promoter

of *CitRWP* with polyembryony makes a strong case that the activities of transposable elements are coupled with these evolutionary events. High genome heterozygosity may also influence this phenomenon during domestication and evolution because of the feasibility of distant hybridization between different citrus species or relatives.

To unambiguously determine the contribution of the candidate gene *CitRWP* for nucellar embryony, it is necessary to knock out *CitRWP* in citrus. The CRISPR-Cas9 system may provide an excellent means for accomplishing this goal. The Hongkong kumquat, a wild citrus, should facilitate these experiments because it provides a short juvenile transformation platform (Zhang et al. 2009). The pathways that respond to *CitRWP* and the gene regulatory network that promotes the development of nucellar embryos will be explored using a delicate transcriptional approach, such as microdissection. Testing whether there are some distinct loci that interact with *CitRWP* in either *Poncirus* or *Fortunella* is also important to learn more about mechanisms that drive nucellar embryony. To understand the genetic basis of this process, several segregating populations using either *Poncirus* or *Fortunella* as parents should be constructed and analyzed. Fully understanding this process will not only provide information on how cell fate transitions take place during this particular reproductive process, but also provide some innovative and remarkable ideas for the utility of the mechanism of apomixis for the seed industry.

The S-RNase type of self-incompatibility

In contrast to apomixis, sexual reproduction promotes genetic diversity in citrus. Indeed, a species with higher rate of polymorphism has the ability to adapt to a wider range of environmental conditions. The SI system contributes to genetic diversity by preventing inbreeding and promoting outcrossing because this system recognizes and rejects pollen with same genotype (De Nettancourt 2001). This system includes two forms, the sporophytic SI (SSI) and the gametophytic SI (GSI). In SSI, the pollen S-genotype is determined by the diploid genotype of the parent. In contrast, in GSI, the genotype is determined by the haploid pollen. Currently, three different SI types have been described at the molecular level: the Brassicaceae type, Solanaceae type, and Papaveraceae type. The Solanaceae and Papaveraceae both use the GSI form of SI (Takayama and Isogai 2005).

The Solanaceae SI type is believed to be the most widely used mechanism of GSI, which is shared with two other families, Rosaceae and Plantaginaceae (McClure et al. 2011). This review will focus on the identification of the S-determinants specifying Solanaceae SI here. Classical genetic studies have established that this type of SI is usually controlled by a single polymorphic locus, termed the SI S-locus,

which is organized in a haplotype fashion with at least two separate genes, the female determinant and the male determinant. These *S*-determinant genes are always tightly linked and also are expressed in a tissue-specific and developmentally controlled manner in the pistil and pollen. The female determinant of the Solanaceae-type SI was first identified in *Nicotiana glauca* (Bredemeijer and Blaas 1981). A 32-kDa glycoprotein that segregated with *S*₂-alleles was identified in the mature upper style segment (Anderson et al. 1986). Similar glycoproteins were identified among the *S* stylar proteins of tomato (Mau et al. 1986), potato (Kirch et al. 1989), and petunia (Broothaerts et al. 1990). Loss- and gain-of-function transformation experiments provided unambiguous evidence that these glycoproteins are the female determinant of recognition specificity in the style (Lee et al. 1994; Murfett et al. 1994). Because these glycoproteins share a homologous domain with the T2 and Rh ribonucleases from fungi (Horiuchi et al. 1988; Kawata et al. 1990) and were confirmed to be ribonucleases (McClure et al. 1989), they are named S-RNases. The Solanaceae type of SI is also called the S-RNase type. When the ribonuclease activity is abolished, the style fails to reject self-pollen (Huang et al. 1994). Moreover, McClure et al. (1990) reported that pollen RNAs were degraded by RNases after incompatible pollinations. Thus, it is believed that S-RNases serve as cytotoxins that inhibit the growth of pollen tubes by specifically degrading the RNA of incompatible pollen.

The *S*-locus contains another gene that specifically triggers the Solanaceae-type SI response that is expressed in the pollen as the male determinant. Earlier multiple attempts were made to identify this pollen *S* gene without success. These experiments utilized RNA differential display (McCubbin et al. 2000), subtractive hybridization (Dowd et al. 2000), and the yeast two-hybrid assay (Sims and Ordanic 2001). Lai et al. (2002) identified the first promising male determinant in *Antirrhinum hispanicum* using the bacterial artificial chromosome (BAC) approach. They found that this *F-box* gene located in the *S*₂-haplotype of *Antirrhinum* was specifically expressed in pollen. Transformation experiments in *Petunia inflata* were used to demonstrate that the SLF (S-locus F-box protein) serves as the pollen *S*-determinant in the SI system (Sijacic et al. 2004). Since then, the polymorphic *F-box* genes were also identified within the *S*-locus regions of *Prunus* (Ushijima et al. 2004; Yamane et al. 2003) and *Maleae* (Sassa et al. 2007), which are referred to as SFB (*S*-haplotype-specific F-box protein) and SFBB (*S*-locus F-box brothers), respectively (McClure et al. 2004). SLF/SFB/SFBBs are thought to function as E3 ubiquitin ligases because they have a conserved F-box motif in the N terminus. E3 ubiquitin ligases interact with E2 ligases and ubiquitinate target proteins, which in many cases targets these proteins for degradation by the 26S proteasome pathway (Hua and Kao 2006; Huang et al. 2006). It remains

to be clarified whether SLF ubiquitinates S-RNase. SLF was also proposed to ubiquitinate a general inhibitor gene (GI) which inhibits the activity of all S-RNases. These two different models will be discussed in the next paragraph. The observation that S-RNases are taken up by both self and non-self-pollen tubes provides evidence that SLF/SFB/SFBBs recognize S-RNases inside pollen tubes. Subsequently, cognate S-RNases function as cytotoxins that specifically degrade and arrest the growth of pollen tubes (Fig. 2a, b).

RNase-based SI in the xylophyta

There is no doubt that the identification of *S* glycoproteins in xylophyta is more difficult because the long juvenile period. They flower in 5–8 years. In contrast, Solanaceae can flower in few months. The long juvenile phase hinders the confirmation of phenotypes and the construction of populations. Nevertheless, considerable progress has been made in several species, particularly in *Maleae* and *Prunus* species. A self-compatible (SC) variety of Japanese pear (*Pyrus pyrifolia*), ‘Osa-Nijisseiki,’ was derived from the ‘Nijisseiki’ variety (Hirata 1989). The style proteins of the SI Japanese pear varieties with known *S*-genotypes and this pair of SI and SC pears were analyzed using isoelectric focusing–polyacrylamide gels and two-dimensional gel electrophoresis. As a result, a 30-kDa basic protein that segregated with the *S*-genotype was identified. This protein was expressed at much lower levels in the SC mutant than in the original SI variety (Sassa et al. 1992, 1993). Broothaerts et al. (1995) purified and identified a 29-kDa ribonuclease (S-RNase) from the pistil tissue of apple (*Malus domestica*). Subsequently, using the conserved sequences of the known S-RNases in Rosaceae, additional female S-RNases have been found in almond (*Prunus dulcis*) (Ushijima et al. 1998), plums (*Prunus salicina*) (Beppu et al. 2002), sweet cherry (*Prunus avium*) (Sonneveld et al. 2003), and apricot (*Prunus armeniaca*) (Romero et al. 2004).

Sequencing the chromosomal region of the *S*-haplotype was also found to be an effective way to identify the candidate male determinants in Rosaceae because the male and female *S*-determinants are tightly linked. An approximately 70-kb cosmid contig containing the *Sc* haplotype of almond was constructed that contains 12 open reading frames. But only one of them, *SFB*, was specifically expressed in the pollen (Ushijima et al. 2001, 2003). The function of the *SFB* gene was investigated in a pollen mutant from *Prunus*. Ushijima et al. (2004) found that the *SFB* gene in the SC mutant of *Prunus* lacks two hypervariable regions, HVa and HVb, and that the deletions of these hypervariable regions were responsible for loss of self-incompatibility. Researchers found that the *S*-locus from *Maleae* and *Prunus* are distinct and that the former contains multiple SFBs, referred to as

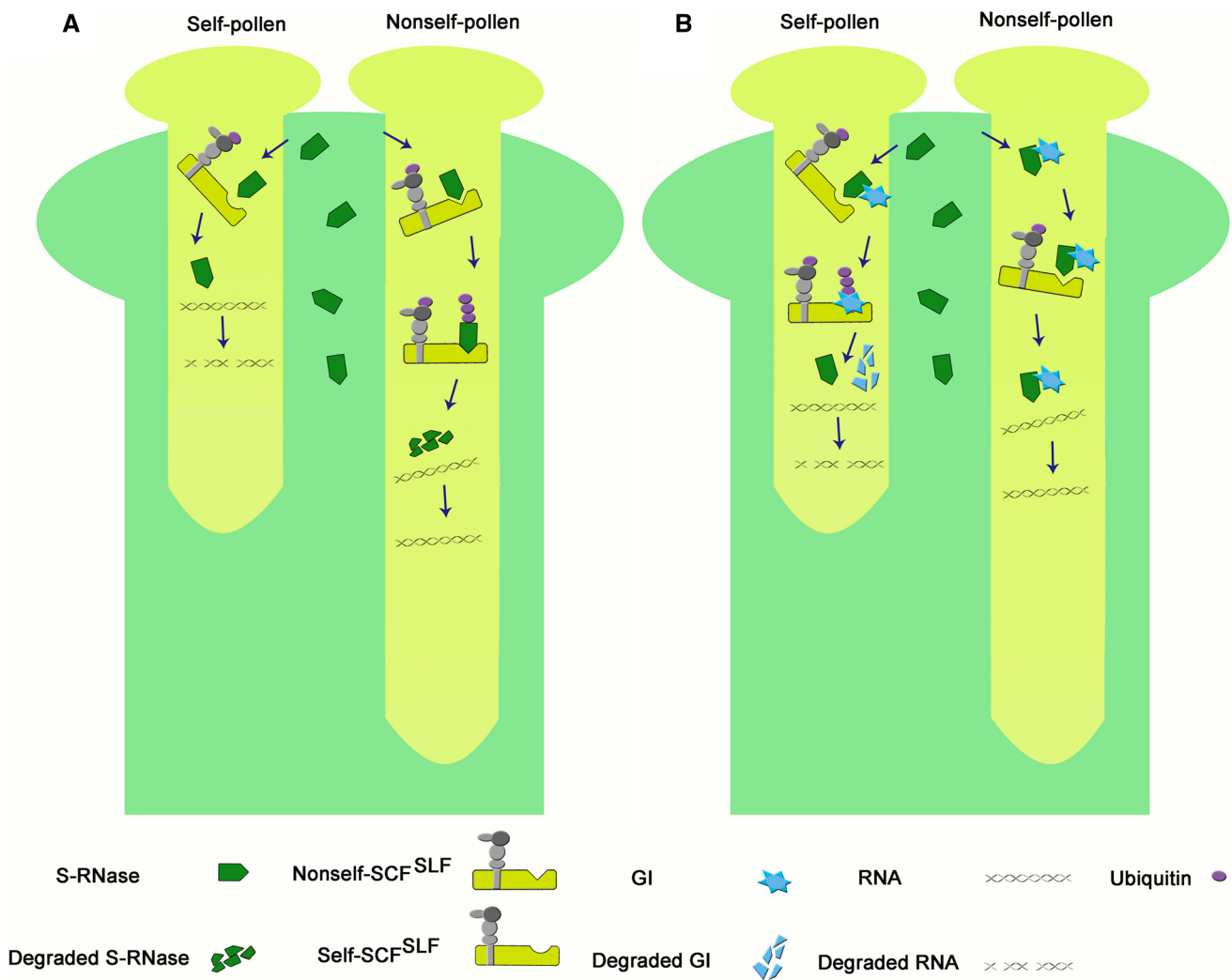


Fig. 2 Two molecular models of the RNase-type self-incompatibility. **a** Non-self-recognition system. In the self-pollen tube, the self-SCF^{SLF} complex is unable to recognize S-RNase. Therefore, S-RNase functions as cytotoxicity to degrade the RNA of pollen tube and induce the self-incompatibility reaction. In the non-self-pollen tube, the non-self-SCF^{SLF} recognizes and polyubiquitinates S-RNase, resulting that S-RNase is degraded by the 26S proteasome. **b** Self-

recognition system involved the general inhibitor (GI). The activity of S-RNase is inhibited by GI in pollen tube. Because of the inhibition, the RNA of non-self-pollen tube is able to escape the cytotoxicity of S-RNase and remain integrity. But the self-SCF^{SLF} complex degrades GI by the ubiquitin proteasome system, which enables S-RNase to degrade RNA

SFBB (Matsumoto and Tao 2016). But the distinct evolution of the *S*-locus in Maleae and *Prunus* remains obscure. Minamikawa et al. (2010) identified 13 *SFBB* genes from the *S*₃-haplotype and 12 from the *S*₉-haplotype in apple. In Japanese pear, 10, 6, and 3 *SFBB* genes were isolated from the *S*₂, *S*₄, and *S*₅-haplotypes, respectively (Sassa et al. 2007).

Later, two distinct systems were proposed: the non-self-recognition system and the self-recognition system. A pool of *F-box* genes are confirmed to localize at the *S*-locus of Maleae, Solanaceae, and Plantaginaceae, which are assumed to adopted the non-self-recognition system. The pool of *F-box* proteins targets all non-self S-RNases in a collaborative action for proteolytic degradation by polyubiquitinating

them. This mechanism allows the non-self-pollen tubes to escape the cytotoxic activity of the S-RNase. In contrast, each functional *F-box* fails to ubiquitinate the self S-RNase, which allows the S-RNases to degrade the RNAs of self-pollen tubes (De Franceschi et al. 2012; Kubo et al. 2010) (Fig. 2a). There is only one *F-box* in the *Prunus S*-locus. This SI type employs a self-recognition system to trigger the S-RNase cytotoxin. In this system, a general inhibitor (GI) is hypothesized to inhibit the activity of all S-RNases. When the *F-box* protein recognizes the self S-RNase-GI complex, it polyubiquitinates and degrades the GI. The degradation of the GI allows the S-RNases to function as cytotoxins and to inhibit the growth of self-pollen tubes (Matsumoto and Tao

2016) (Fig. 2b). Thus, studies in Rosaceae species (i.e., all woody species with some similar problems to citrus) have progressed well and provide a solid framework for studies in citrus.

Self-incompatibility in citrus

The SI varieties of citrus are mainly pummelo and mandarin (Ngo et al. 2001; Ngo et al. 2010; Yamamoto et al. 2006). The site of self-pollen tube rejection after pollination can serve as a good indicator of SI. In SSI, rejection generally occurs at or before pollen germination. In contrast, in GSI, the incompatible pollen is arrested within the style, rather than on the stigmatic surface (Newbigin et al. 1993). For example, in *Nicotiana*, the growth of the pollen tube is inhibited in the upper and lower parts of the transmitting tract (Lush and Clarke 1997). However, the GSI of poppies and grasses provides an exception to this rule (Lawrence 1975; Lewis 1956). ‘Shatian’ pummelo is a traditional cultivar of the *Citrus* genus that exhibits self-incompatibility. In self-pollination experiments with this cultivar, the pollen tubes penetrated the stigma and were rejected after growing through the top one-third of the style (Liang et al. 2017) (Fig. 3). The growth of self-pollen on ‘Comune’ clementine exhibited the same behavior (Distefano et al. 2009). The inhibition site for the self-pollen tubes of ‘Kagzi Kalan’ lemon was in the middle of the style (Kakade et al. 2017). Although the self-pollen tubes of ‘Wuzishatangju’ grew to the basal end of style, they became twisted and stopped growth once the tubes penetrated the ovary (Ye et al. 2009). Therefore, these studies are consistent with gametophytic rather than sporophytic control of the pollen rejection in citrus and this slower inhibition is also consistent with the proposed cytotoxic effect of S-RNases on pollen tubes.

It is vital to identify the *S*-haplotypes in citrus because linkage to the *S*-haplotypes is required to identify the candidate *S* genes. Although there is an ever-growing list of SI citrus accessions, the reported *S*-genotypes of these SI varieties are limited. The *S*-genotype of ‘Banpeiyu’ was defined as S_1S_2 (Ngo et al. 2010), which was used to produce self-fertilized seedlings (S1) with homozygous genotypes (Wakana et al. 2004). Homozygous S1 seedlings (S_1S_1 and S_2S_2) were identified by pollinated with ‘Banpeiyu’ (S_1S_2) pollen, and 55 pummelo accessions were separately pollinated with the pollen of S_1S_1 and S_2S_2 homozygous seedlings, indicating that 23/55 accessions had the S_1 allele and 16 of them had the S_2 allele (Kim et al. 2011). These studies provided a starting point for the identification of the *S*-determinants and the molecular mechanism of the SI response in citrus.

S-RNases share conserved sequence motifs and a similar topology with RNase T2, although their amino acid sequences have diverged substantially. A large number of *S*-genotypes in Rosaceae were identified based on these conserved motifs (Ushijima et al. 1998; Sonneveld et al. 2003). Many researchers tried to clone the homologous *S* genes from citrus using the same method, but none of the genes identified with this approach exhibited tissue-specific expression (Chai et al. 2011a, b; Miao et al. 2011a). By performing amino acid sequence similarity searches of the genomic databases of sweet orange (*C. sinensis*) and clementine mandarin (*C. clementine*), 16 genes encoding homologs of S-RNases were obtained. Among the 16 candidates, the amino acid sequence of only the protein encoded by CgRNS3 (*Citrus grandis* RNase 3) and the female determinant of SI were highly conserved. The CgRNS3 protein inhibited the growth of self-pollen tubes in an in vitro culture system, but after a heat treatment, this protein did not significantly inhibit the elongation of pollen tubes (Liang et al. 2017). We prepared multiple sequence alignments for the amino acid sequences

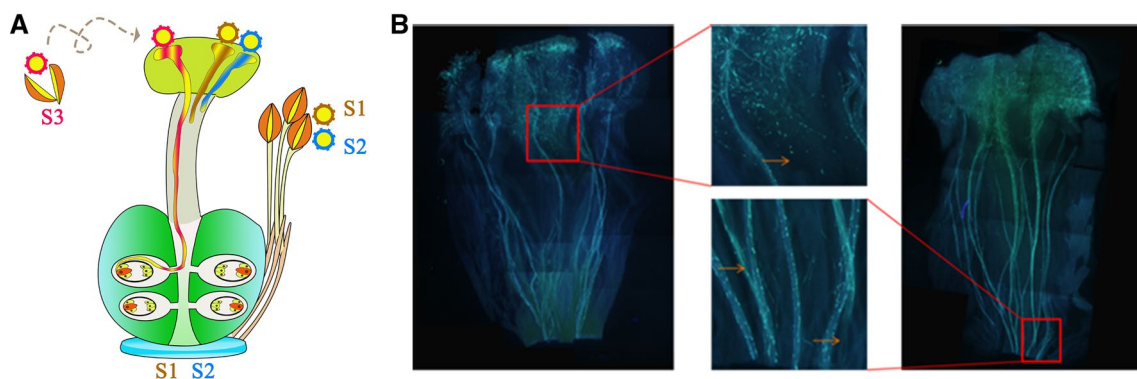


Fig. 3 The characteristics of self-incompatibility in citrus. Model for self-incompatibility in citrus. **a** Growth of a self-pollen tube with the genotype S1 or S2 is inhibited after penetrating the top one-third of the style. In contrast, a pollen tube with the S3 genotype can grow

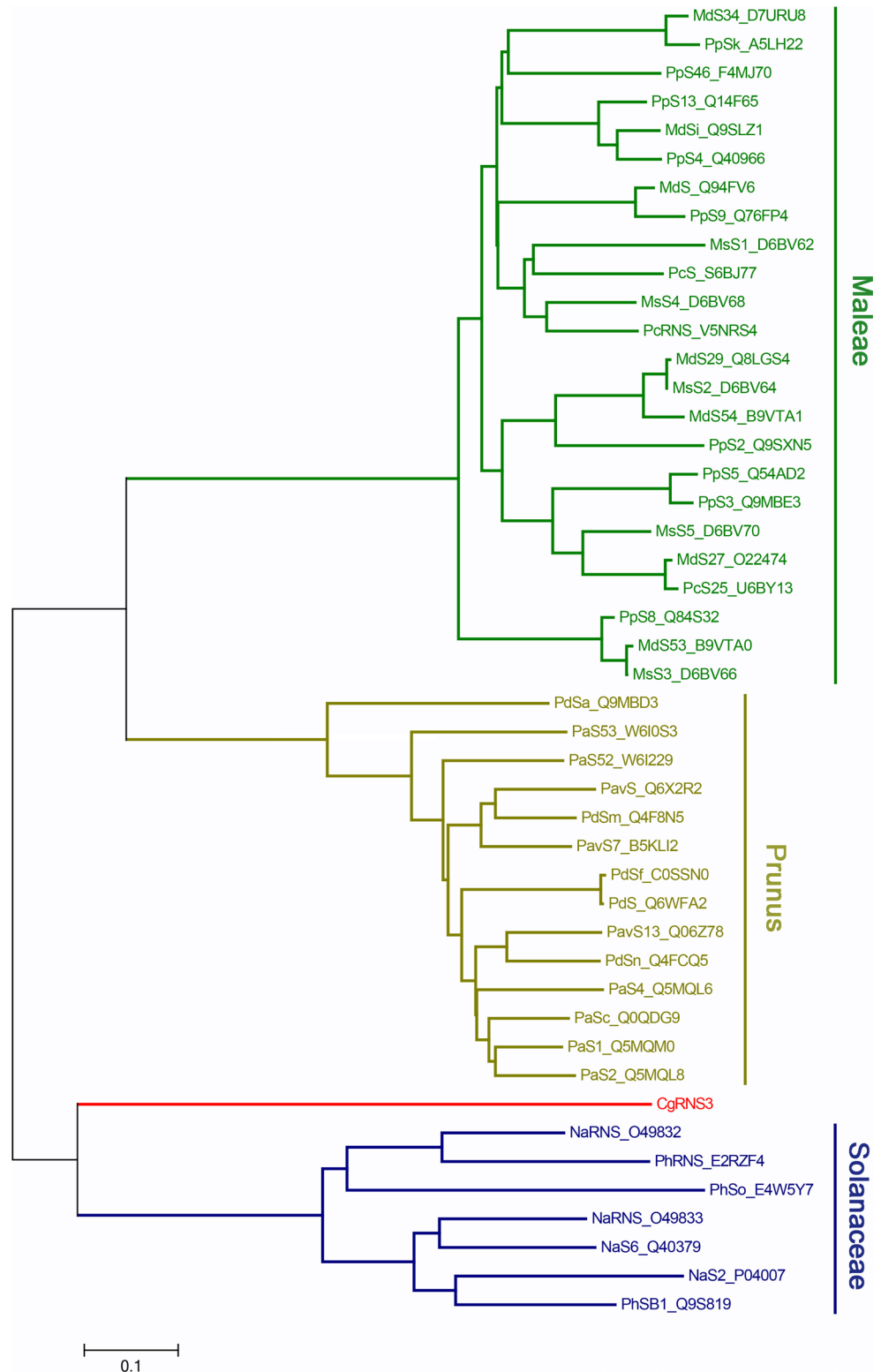
uninhibited to the bottom of style. **b** Self-pollinated (left) and cross-pollinated (right) styles were stained with aniline blue to show the growth of pollen tubes in incompatible and compatible pollinations, respectively. The arrows indicate the position of pollen tubes in style

of CgRNS3 and other reported S-RNases. We found that CgRNS3 fell into a single distance cluster. Thus, we conclude that citrus S-RNases have a considerably distant relationship to the S-RNases from Solanaceae and Rosaceae (Fig. 4). Thus, to some extent, an S-RNase from

citrus, including the conserved sequence motifs, appears distinct from the known S-RNases.

In the past, constructing suppression subtractive hybridization (SSH) cDNA libraries provided an effective method for isolating differentially expressed genes. For this reason,

Fig. 4 The phylogenetic tree between CgRNS3 and other reported S-RNases. The tree was constructed using the neighbor-joining algorithm using the MEGA5.0, and the reliability was inferred from a bootstrap analysis of 1000 replicates. The accession numbers of these reported S-RNases are indicated in the study of Liang et al. (2017). CgRNS3 is indicated with the red color



two SSH libraries of self- and cross-pollinated styles from ‘Shatian’ pummelo were constructed. Among the 30 ESTs obtained from these experiments, one EST was homologous to the S_1 -haplotype sequence from *Ipomoea trifida* and the other was homologous to the S_9 -RNase from *Malus domestica* (Qin et al. 2008). To study the SC ‘Shatangju’ and its SI mutant ‘Wuzishatangju,’ mutant alleles of several genes were discovered using different SSH cDNA libraries (Miao et al. 2011b, 2013b). The expression levels of the gene encoding the S_1 SI locus-linked pollen 3.15 gene ($S_{1-3.15}$) (Miao et al. 2013c) and the gene encoding the ubiquitin-activating enzyme E1 (*UBE1*) (Miao et al. 2013a) in SC ‘Shatangju’ were higher in SC ‘Shatangju’ than in the SI mutant. This is an interesting finding, because male *S*-determinants are F-Box proteins which are involved in ubiquitination. However, further molecular characterization and expression analysis of these two genes provided evidence that they might be associated with the SC response in citrus, rather than serving as the key genes controlling SI (Miao et al. 2013a, c). Moreover, four days after pollination, the gene encoding the S-phase kinase-associated protein 1 (SKP1) was expressed at higher levels in the self-pollinated pistil than in the cross-pollinated pistils. The majority of pollen tubes became twisted after self-pollination in transgenic tobacco, suggesting that high expression of SKP1 has an effect on the growth of pollen tubes and is involved in the SI reaction of ‘Wuzishatangju’ (Li et al. 2015). These data indicate that most of these differentially expressed genes might function downstream of SI or perform functions that are related to the SC response.

After the introduction of next-generation sequencing technologies, RNA-seq became more popular than the construction of SSH cDNA libraries because RNA-seq provides a high-throughput approach for identifying differentially expressed genes. The transcript profiles of the styles from non-, self-, and cross-pollinated ‘Xiangshui’ lemon were determined to find candidate *S* genes using RNA-seq technology. Caruso et al. (2012) identified the differentially expressed genes in laser-microdissected stylar canal cells in ‘Comune’ and the SC mutant ‘Monreal.’ The transcriptomes of another pair of mutants were compared, the SI ‘Wuzishatangju’ and the SC ‘Shatangju’ (Ma et al. 2017). Liang et al. (2015) constructed a transcriptome dataset based from seven tissues from ‘Shatian’ pummelo, which provided a valuable resource for molecular biology studies in citrus. Moreover, the protein expression profiles of three developmental stages of ‘Hyuganatsu’ styles were obtained using two-dimensional gel electrophoresis and MALDI-TOF mass spectrometry (Uchida et al. 2012).

In summary, researchers used mainly two kinds of methods to identify the *S*-determinants of the citrus SI system in the past: homologous amplification and identification of differentially expressed genes. However, to date,

there is no direct proof of a bona fide *S*-RNases in citrus. In xylophyta, SI and SC mutants are valuable to identify the *S*-determinants. The first female factor of Rosaceae SI was identified utilizing a mutated Japanese peer (Nakanishil et al. 1992). Several pairs of SI and SC mutants are also known in citrus, such as ‘Shatian’ pummelo and ‘Zigui shatian’ pummelo (Chai et al. 2011c), ‘Shatangju’ and ‘Wuzishatangju’ (Miao et al. 2011b), and ‘Comune’ and ‘Monreal’ (Caruso et al. 2012). However, a number of distinct mechanisms may lead to the loss of SI function, such as the deletion of a gene, a missense mutation that causes a single amino acid substitution that affects the female determinant (Li et al. 2009; Okada et al. 2008), an insertion in the coding region of the male determinant (Sonneveld et al. 2005), and changes in the non-*S* factors (Li et al. 2016). Thus, to identify *S* factors using SI and SC mutants, researchers should first determine whether the transformation from SI to SC is caused by a mutation that affects the function of the pollen or the pistil. Additionally, in vitro culture systems and antisense oligonucleotide technologies are the most effective means to test the function of candidate genes in xylophyta.

To date, our knowledge of SI in citrus is still less than that in the woody plants of Rosaceae. During the past twenty years, it was confirmed that many accessions of citrus adopt the SI system, especially in pummelo. With a variety of new molecular technologies and advanced sequencing technologies, it has been possible obtain many candidate genes related to SI, but most of these candidates appear to participate in the downstream reactions of SI and do not contribute to the *S*-determinants themselves. The failure of the homologous amplification experiments is consistent with significant differences between the conserved domains of *S*-RNase in citrus and known *S*-RNases. An alternative explanation might be that the *S*-RNase is not the female *S*-determinant. Thus, an important objective is to determine whether citrus is similar to other SI RNase systems in that a stylar RNase activity specifically accumulates to high levels or whether there is an SI mechanism in citrus that is distinct from the *S*-RNase-based mechanism. Franklin-Tong et al. (1991) showed that SI in *Papaver rhoeas* was different than in Solanaceae, because the level of ribonuclease activity in the mature stigmas of poppy is very low. There is no evidence for *S*-RNase activity in citrus yet, but experiments are underway to test this possibility. Although some SI accessions were identified in citrus, none of these accessions were used for reciprocal pollination experiments. This needs to be carried out in order to obtain some clearly specified *S*-haplotype materials to work on. Thus, in many cases, *S*-allele-associated proteins are the female *S*-determinants in styles and are possible to identify and isolate from different materials based on their *S*-haplotypes. These types of experiments will provide information on the processes of

SI in citrus and lay a solid foundation for determining the molecular mechanism of SI in citrus.

Prospect of understanding the special reproductive traits of citrus

To accelerate the breeding efficiency for cultivating more citrus varieties with golden qualities and stable characters, it is essential to understand the mechanism of nucellar embryogenesis and self-incompatibility in citrus by utilizing new methods and technologies when they are developed. After the identification of the candidate gene *CitrRWP*, priorities for the future study of nucellar embryony may include verifying the function of the candidate genes on developmental process and exploring related genes and pathways involved with this process. The study of self-incompatibility in citrus still focuses on the identification of *S* genes. It is essential to isolate the *S*-allele-associated proteins based on the genotypes of the self-incompatible accessions. Altogether, it is foreseeable that the engineering of apomixis and self-incompatibility in a broad range of crops will become a reality in the near future.

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