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Identification of *S* genotypes in loquat (*Eriobotrya japonica* Lindl.) based on allele specific PCR

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ABSTRACT

Loquat (*Eriobotrya japonica* Lindl) is an evergreen fruit tree and belongs to Maloideae in the Rosaceae, which carries the RNase-dependent gametophytic self-incompatibility system (GSI). Use of appropriate pollinator trees is essential for increasing fruit production. *S*-allele specific amplification system is an efficient and rapid method to identify *S*-genotypes. In this research, according to the sequences of *S-RNase* alleles, 9 specific primer pairs for amplifying *S-RNase* alleles (S^2, S^7, S^6, S^8, S^9, S^{10}, S^{11},S^{12} and S^{13}) were designed, which located in the introns or in the areas with high polymorphism. The specificity of the 9 allele specific primer pairs was evaluated by eight loquat cultivars with known *S*-genotypes. After being verified their effectiveness, they were used to *S*-genotype 39 loquat cultivars from Longan and loquat nurseries in Fuzhou national fruit germplasm. The results showed that the 39 loquat cultivars were *S*-genotyped efficiently by allele specific PCR with the 9 primer pairs, in which 28 cultivars were identified for the first time and they were classified into 18 groups according to the genotype. The results also proved that the 9 *S*-allele specific primer pairs can efficiently identified *S*-genotype, which will be very useful for choosing pollinators or crossing parents in loquat production and breeding.

1. Introduction

Loquat (*Eriobotrya japonica* Lindl.) is originated from China (Zhang et al., 1990), which has been widely cultivated for medicinal effect and specialty flavor. Like apple (*Malus* spp.) and pear (*Pyrus* spp.), loquat belongs to the subfamily Maloideae of the Rosaceae and carries the RNase-dependent gametophytic self-incompatibility system. Gametophytic self-incompatibility (GSI) is determined by a single multi-allelic locus, called the *S*-locus, which contained two linked genes: the pistil *S* determinant (*S-RNase*) and the pollen *S* determinant (*SFB/SLF*). In GSI, if the pollen *S* allele matches one of the two pistil alleles, pollen tube growth in the style is arrested and fertilization is prevented (Ten Hoopen et al., 1998). In contrast with apple or pear, loquat was mostly considered a self-compatible species (Rodríguez, 1983; Campbell and Malo, 1986). However, since loquat flowers are so showy and fragrant that strongly attract pollinators, moreover, there are 50–100 flowers aggregated on a panicle, the character of self incompatibility has been neglected. But now, loquat is being increasingly planted in greenhouse or under mesh, where bee activity and pollen transfer from other genotypes are often precluded. The number of loquat fruit setting decreases and the low quality fruit production are common (Cuevas et al., 2003). Therefore, in loquat commercial orchards, setting cross-compatible cultivars that flower simultaneously and harbor different *S*-haplotypes is necessary to ensure successful cross-pollination and result in healthy, attractive fruit.

In order to perform an appropriate selection of the suitable pollinator cultivars and the design of crosses in loquat breeding programs, it is necessary to characterize the *S*-genotype of the main cultivars and the establishment of incompatibility groups in this species. Previously, *S*-genotypes were determined based on crossing experiments, which were sometimes ambiguous because of environmental and physiological effects. According to the sequence characteristic of *S-RNase* gene, DNA sequencing combined with restriction endonuclease(RE) reaction was the common method to identify genotypes such as in apple (Gu et al., 2015), pear (Tan et al., 2008; Sanzol, 2009), apricot (Wu et al., 2009) and sweet cherry (Wunsch and Hormaza, 2004). However, some defects exist when using these methods to identify genotypes. For example, it is difficult to find the suitable RE cutting site which is merely exist in a special *S-RNase* gene, and the amplification will be defeated when the structure of *S-RNase* genes is complex or *S-RNase* gene contains big introns.

As a simple and convenient method, *S*-allele specific PCR

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amplification system has been developed to identify *S*-genotypes in apple (Van Nerum et al., 2001; Broothaerts, 2003), sweet cherry (Sonneveld et al., 2003) and pear (Nashima et al., 2015). In this method, the specificity of the primer pairs is the key to lead efficient amplification. As we known, *S-RNase* genes in subfamily Maloideae of the Rosaceae contain 5 conserved regions (C1, C2, C3, C4 and C5) and a hypervariable region (HV), which is inserted by an intron, locating in between C2 and C3 (Sassa et al., 1996; Ma and Oliveira, 2002). HV is the positive selection area, in which nucleotide substitution, insertion and deletion were often happened (Ishimizu et al., 1998) and intron of *S-RNase* has high sequence polymorphism, thus, the sequences of HV and intron which responsible for *S-RNase* alleles specificity (Matton et al., 1997; Ishimizu et al., 1998) were suitable for designing the primer pairs. Apart from HV and intron, the sequences between C1 and C2 and the sequences in upstream area of C5 also have high polymorphic, which are also the suitable areas to design primers(Kheyrpour et al., 1990; Heng et al., 2008; Verica et al., 1998).

However, few researches on *S*-genotyping of loquat have been done until now. Carrera et al. (2009, 2011) used three loquat-specific *S-RNase* primers (EjC1F, Ej2C2/3R, EjC5R) and three consensus primers (MaC1F1, MaC2/3R1, MaC5R1) to amplify *S-RNase* genes in five loquat cultivars and obtained four *S-RNase* genes (S^1, S^2, S^3, S^4). And then they identified the *S*-genotypes of 31 loquat cultivars and found a new *S-RNase* (S^5-*RNase*) by the method of DNA sequencing and high-performance capillary polyacrylamide electrophoresis. By the same method, Ishimoto et al. (2014) classified 145 loquat cultivars (or lines) into 25 genotypes and identified 5 new *S*- alleles (S^7–S^{11}) and verified that S^5-*RNase*(Carrera et al., 2011) and S^6-*RNase* (Niska et al., 2010) should be the same *S*-haplotype by comparing their cDNA deduced amino acid sequences. Recently, Wang et al. (2017) analyzed *S*-genotypes of 20 loquat cultivars and obtained 2 new *S-RNase* genes (S^{12}-*RNase* and S^{13}-*RNase*) by PCR based methods. Until now, 13 *S-RNase* genes were isolated and sequenced from loquat (Carrera et al., 2009; Carrera et al., 2009, 2010; Ishimoto et al., 2014; Wang et al., 2017).

With more and more loquat *S-RNase* genes were sequenced, developing a fast and easy method rather than DNA sequencing or dCAPs to identify loquat *S*-genotypes is expected. In this article, on the basis of the characteristic of the 9 known *S-RNase* sequences of loquat, 9 pairs of *S*-allele specific primers were designed and effectiveness of the specific primer pairs for *S* genotype identification was evaluated. And the *S*-genotypes in 39 loquat cultivars were identified successfully using the *S*-allele specific PCR.

2. Materials and method

2.1. Materials

Fresh leaves from 39 loquat cultivars (Table 1) were collected from Longan and loquat nurseries in Fuzhou national fruit germplasm, China. All of the plant materials were stored at −80 °C until use.

2.2. Genomic DNA isolation

Genomic DNA was isolated by cetyltrimethy ammonium bromide (CTAB)-based extraction method (Porebski et al., 1997). For loquat leaves were rich in polysaccharide and polyphenol, to obtain high purity of DNA, 2.5 mol/L NaCl was replaced by NaAc when precipitated DNA with ethanol. The extracted DNA was dissolved in 30 μL deionized water and stored at −20 °C until use.

2.3. Designing the S-allele specific primer pairs

Based on the sequences of 9 *S-RNase* alleles previously reported, i.e. S^2-*RNase* (Genbank accession number: GU384665), S^6-*RNase* (KC131132) (Carrera et al., 2009, 2010); S^7-*RNase*(AB872913), S^8-*RNase* (AB872914), S^9-*RNase* (AB872915), S^{10}-*RNase* (AB872916), S^{11}-*RNase* (AB872917) (Ishimoto et al., 2014), S^{12}-*RNase* (KR149297), S^{13}-*RNase* (KR149298) (Wang et al., 2017), 9 *S*-allele specific primer pairs were designed. The length of the primers ranged from 19 bp to 23 bp, GC content was from 35% to 65% and the amplified fragments were longer than 150 bp.

Table 1
The name,origin and phenotype of 39 loquat cultivars.

NO	Cultivars	Origin	Physiological characteristics (Qiao et al., 2011; Qiu and Zhang, 1996)
1	XiazhengNo.1	Fujian	Red pulp, oblate
2	ChanghongNo.3	Fujian	Red pulp, pear-shaped, natural seedling
3	YongchunNo.1	Fujian	Unknown
4	Xialoubaimi	Fujian	White pulp, subrounded
5	Jiajiao	Zhejiang	Red pulp, obovoid
6	Shanliben	Fujian	Red pulp, Subrounded
7	XiazhengNo.2	Fujian	Red pulp, oblate, natural seedling
8	Danbianzhong	Zhejiang	White pulp, subrounded
9	Shiliuben	Fujian	Red pulp, oblate
10	Baili	Fujian	White pulp, subrounded, subtropical plant
11	TaichengNo.4	Fujian	Red pulp, obovoid, seedling progeny
12	Baihua	Anhui	White pulp, subrounded
13	Meihuaxia	Fujian	Red pulp, subrounded, subtropical plant
14	Linmeiwanshou	Fujian	Red pulp, oblate
15	Dafang	Japan	Red pulp, obovoid
16	Mogi	Japan	Red pulp, obovoid
17	Anhuidahongpao	Anhui	Red pulp, subrounded, temperate plants
18	Huangpi	Jiangsu	White pulp, subrounded
19	Wugongbai	Fujian	Red pulp, obovoid, natural seedling
20	Moriowase	Japan	Red pulp, obovoid, branch mutation of 'Mogi'
21	Tangqizhong	Zhejiang	Red pulp, obovoid, natural seedling
22	Zaotao	Fujian	Red pulp, oblate
23	XiabanNo.2	Fujian	White pulp
24	Yunxiaoyuanzhong	Fujian	Unknown
25	Baozhu	Zhejiang	Red pulp
26	Guangrong	Zhejiang	Red pulp, subrounded
27	Zhejiangdahongpao	Zhejiang	Red pulp, temperate plants
28	Tanaka	Japan	red pulp, natural seedling
29	Dazhong	Fujian	Red pulp
30	LongquanNo.1	Sichuan	Red pulp, subrounded
31	PuxuanNo.1	Fujian	Red pulp, obovoid
32	Nagasakiwase	Japan	Red pulp, 'Mogi' × 'Hondawase'
33	Changbingbianhe	Anhui	Red pulp, subrounded
34	Suanpanzhi	Fujian	Red pulp
35	Mitangpipa	Guangxi	Red pulp, natural seedling
36	ZaohongNo.3	Sichuan	Red pulp, subrounded
37	Luoyangqing	Zhejiang	Red pulp, obovoid, natural seedling
38	Duanbingbianhe	Anhui	Red pulp, subrounded
39	Donghuzao	Fujian	Red pulp, subrounded, natural seedling mutant

2.4. S-allele specific amplification system

For PCR amplification, 80 ng/μL DNA was used in 25 μL reaction system containing 1 × PCR buffer, 160 μM dNTPs, 400 nM of each primer and 0.5 μL rTaq. The amplification program was as follows: 1 cycle of 1 min at 94 °C, 35 cycles of 30 s at 94 °C, 30 s at 56 °C to 62 °C depending on different primer pairs, and 1 min at 72 °C, the last cycle of 10 min at 72 °C. 5 μL reaction products and 100 bp DNA ladder (Takara, Dalian, China) were separated by 2% agarose gel and dyed by EB (ethidium bromide) staining under UV light.

2.5. DNA sequencing

Following manufacturer's instructions of the Agarose Gel DNA Purification Kit (TaKaRa, Dalian,China), PCR products were cut from agarose gel and purified. After that the purified products were cloned into pMD19-T and transformed into *Escherichia coli* DH5α. Five positive clones of each allele were selected to sequencing. DNAMAN was used to characterize the sequence and the structure of *S-RNase* genes.

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