Kor. J. Hort. Sci. Technol. 30(6):757-763, 2012 DOI http://dx.doi.org/10.7235/hort.2012.12215

Distribution of S-alleles among Korean Apples by PCR and Cross-pollination

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Abstract. To acquaint correct information about the fertilizability and analyze S-allele based genetic diversity among Korean apples, we investigated self-incompatibility genotypes by PCR and cross-pollination tests in field. As a consequence, S-genotypes of Korean apples were distributed within narrow genetic diversity as S_1S_3 for 'Hongro' and 'Saenara'; S_1S_9 for 'Gamhong' and 'Manbok'; S_3S_5 for 'Seokwang'; S_3S_9 for 'Sunhong', 'Seohong', 'Chukwang', and 'Hwahong'. Coupled with cross-pollination experiments in field, our results provide support for the view that apples are fully compatible when both of their S-loci differ and semi-compatible when they carry one different and one identical S-locus. Furthermore, the results of this study indicate that S-alleles have to be extended to various genotypes for Korean apple breeding.

***Additional key words*:** breeding, compatibility, diversity, *Malus* × *domestica*, S-RNase

Introduction

Apples (*Malus* spp.) have gametophytic self-incompatibility (GSI) which is controlled by a single multi-allelic locus (S-locus), preventing self-fertilization and fertilization between individuals bearing identical S-alleles. After pollination, the grains germinate and produce a tube that enters the stigmatic tissue and elongates through the style towards the ovules where fertilization happens (Broothaerts et al., 2004). However, S-RNases encoded by the S-gene residing at the S-locus in the pistil act on entering pollen tubes, interacting with the F-box protein encoded by the S-gene in the pollen, and degrading the pollen rRNA of elongating tubes. In GSI, when the S haplotype in the pollen matches S-haplotypes in the pistil, the pollen is recognized and rejected by the pistil. This mechanism suggests that successful fruit set are available by different S haplotype (Kim et al., 2006; Sassa et al., 2007). Several considerable researches have been to identify the S-alleles of apple cultivars in recent years, and more than 21 S-alleles have been reported (Broothaerts et al., 2004).

The identification of cross-incompatible cultivars has been based on the results of controlled pollinations in Korea. However, cross-pollination tests between varieties have given variable results, as environmental and physiological factors have an impact on the outcome. Moreover, fruit set is sometimes unreliable because self-incompatibility is not a complete barrier, and some fruits may develop parthenocarpically (Broothaerts, 2003). As an alternative method, microscopic evaluation of pollen tube growth through the pistil has revealed 11 different S-alleles (S_1 to S_{11}) and assignment of alleles for 14 diploid and 12 triploid varieties (Kobel et al., 1939). Likewise, 10 S-alleles were identified among Japanese apple cultivars by Komori et al. (1999, 2000), who assigned a letter symbol S_a to S_i and S_z and reported their correspondence to four of Kobel's S-alleles. As biochemical and molecular methods were developed, Sassa et al. (1996) discriminated S-alleles (S_a to S_f) through characteristic migration patterns of gene products in the alkaline regions of isoelectric focusing (IEF) or 2 dimensional polyacrylamid gel electrophoresis. Using IEF and non-equilibrium pH gel

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※ Received 17 November 2012; Revised 29 November 2012; Accepted 1 December 2012. This work was supported by the research grant of the Chungbuk National University in 2010.

electro focusing and RNase staining, Bošković and Tobutt (1999) identified S_1 to S_{11} and discovered 14 new S-alleles (S_{12} to S_{25}) in European apple cultivars.

Polymerase chain reaction (PCR) methods were also developed and used S-allele specific primers to determine the S-genotype based on nucleotide sequences encoding the S-RNases in apple (Janssens et al., 1995). Since then, the S-alleles in *Malus* have been identified and renumbered using various methods (Broothaerts, 2003; Broothaerts et al., 2004; Kim et al., 2006; Kitahara and Matsumoto, 2002; Matsumoto et al., 2003; Sassa et al., 1996; Van Nerum et al., 2001). Recently, the *SLF* (S locus F-box gene) and *SFB* (S haplotype specific F-box gene) were identified as pollen S-genes, SFBB (S locus F-box brothers) genes were isolated $MdSFBB^{3\text{-}\alpha}$, $MdSFBB^{3\text{-}\beta}$, $MdSFBB^{9\text{-}\alpha}$, and $MdSFBB^{9\text{-}\beta}$ from 'Sekaiichi' (S_3S_9) apple (Sassa et al., 2007).

In Korea, apple growers have grown Korean cultivars with other cultivars or crab apples as pollinizers to produce marketable fruit size and quality since first Korean apple bred and released in 1988. Apple breeders also have gathered S-alleles information of Korean cultivars from cross-pollination tests in the field and made assumptions about the S-alleles from the parents to progress effective and precise breeding program. However, limited knowledge about the S-alleles among Korean apple cultivars makes difficult for the selection of compatible pollinizers, the design of orchards, and improvement of breeding efficiency. And more, it will be a limit for spread of new releasing Korean apples and diversity of apple breeding program. To accomplish these purposes, the S-alleles of thirty apple cultivars including Korean bred apples and their parents were investigated through the PCR method and cross-pollination.

Materials and Methods

Plant Material and DNA Extraction

A total of thirty apple cultivars were used in this study, including fourteen of Korean bred cultivars and twenty six of their parents or important cultivars. The list of 30 tested cultivars and the re-established their S-genotypes as a result of this study and parentage information (if known) are in Table 1. Leaves for genomic DNA extraction were collected during the new growing season at the National Institute of Horticultural & Herbal Science, Suwon, Korea. Collected leaves were pulverized in a tissuelyzer apparatus (QIAGEN, Germany) using DNeasy Plant Mini Kits (QIAGEN, Germany) according to the manufacturer's instructions. The isolated genomic DNA was qualified on agarose gels and quantified by a fluorometer (Model TD360, Turner, USA).

S-allele Specific PCR Analysis

Allele-specific PCR amplification was performed according to Broothaerts (2003) using MJ Research PTC-100 or 200 thermal cyclers. The thermocycler program was 3 min at 94℃, 30 cycles of 15 s at 94℃, 15 s at 60℃, and 30 s at 72℃, and finally 2 min at 72℃, followed by cooling to 4℃. The basic reaction mixture contained 1× PCR buffer (Promega, USA), 1.75 mM of $MgCl_2$, 200 μM dNTPs, 1 μM of each primer, 0.6 unit of *Taq* DNA polymerase (Promega, USA), and roughly 10 ng of genomic DNA template in 20 μL of total volume. The S-allele specific primers for Korean apples in Table 2 were selected by their parentage and designed according to previous reports (Broothaerts et al., 2004; Kim et al, 2006). The annealing temperature or extension time was slightly modified. The PCR products were run on 1.5% agarose gels and were stained with ethidium bromide to genotype the S-alleles.

Artificial Pollination

Self- and cross-pollinations of twenty nine combinations were tested for two years, 2006 and 2007, to confirm the S-alleles of Korean apples and their fertilizability. At the balloon stage of female parent tree, unopened flowers were selected, emasculated, and covered with paper bags for the hand pollination in a few days before the pollinations. Flowers of male parents were collected in just before flower opening and dried pollens were prepared until pollination. The cross pollinated flowers were recovered with paper bags and all other flowers on the same tree were removed. Thirty days after artificial pollinations, all bags were removed from the fruitlets and were evaluated compatibility.

Results and Discussion

S-genotypes Based on PCR Analysis

The PCR based S-genotypes were analyzed from thirty apples including Korean released cultivars, their parents, and some interesting apples (Table 1 and Fig. 1). According to eleven S-allele specific primers, expecting sizes of amplified S-RNase fragments were in the Table 2. To ensure that the primer pairs were selective for a single S-allele, the cultivars were screened against all S-alleles in independent PCR reactions (Broothaerts, 2003; Kim et al, 2006).

Using primer pairs of FTC168 and FTC169, specific amplification products were obtained for all apples that previously were known to bear S_1, including 'Fuji', 'Saenara', 'Hongro', 'Senshu', 'Hwarang', 'Ralls Janet', 'Spur Earliblaze', 'Gamhong', and recently released 'Hongso', 'Hongan', and 'Manbok' (Fig. 1A). The amplification product

Table 1. Parentages and S-genotypes of the thirty apple cultivars including fourteen Korean apples.

Cultivar	Parentage
Hongro (S_1S_3)	Spur Earlyblaze (S_1S_{19}) × Spur Golden Delicious (S_2S_3)
Seokwang (S_3S_5)	Mollie's Dellicious (S_3S_7) × Gala (S_2S_5)
Chukwang (S_3S_9)	Fuji (S_1S_9) × Mollie's Delicious (S_3S_7)
Saenara (S_1S_3)	Spur Earlyblaze (S_1S_{19}) × Spur Golden Delicious (S_2S_3)
Gamhong (S_1S_9)[z]	Spur Earlyblaze (S_1S_{19}) × Spur Golden Delicious (S_2S_3) (?)
Hwahong (S_3S_9)	Fuji (S_1S_9) × Sekaiichi (S_3S_9)[y]
Sunhong (S_3S_9)	Hongro (S_1S_3) × Chukwang (S_3S_9)
Seohong (S_3S_9)	Tsugaru (S_3S_7) × Chukwang (S_3S_9)
Summer Dream (S_7S_9)	Tsugaru (S_3S_7) × Natsumidori (S_3S_9)[y]
Honggeum (S_3S_7)	Senshu (S_1S_7) × Hongro (S_1S_3)
Manbok (S_1S_9)	Earliblaze (S_1S_{19}) × Fuji (S_1S_9)
Hongso (S_1S_3)	Yoko (S_3S_9) × Hongro (S_1S_3)
Hongan (S_1S_3)[z]	Fuji (S_1S_9) × Jonathan (S_7S_9) (?)
Hwarang (S_1S_9)	Sport of 'Fuji'
Tsugaru (S_3S_7)	Golden Delicious (S_2S_3) × ?
Sansa (S_5S_7)	Gala (S_2S_5) ×Akane (S_7S_{24})[y]
Jonagold ($S_2S_3S_9$)	Jonathan (S_7S_9) × Golden Delicious (S_2S_3)
Golden Delicious (S_2S_3)	Grimes Golden (?) × ?
Spur Golden Delicious (S_2S_3)	Sport of 'Golden Delicious'
Red Delicious (S_9S_{28})	Sport of 'Delicious' (S_9S_{28})
Fuji (S_1S_9)	Ralls Janet (S_1S_2) × Delicious (S_9S2_8)
Pink Lady (S_2S2_3)	Golden Delicious (S_2S_3) × Lady Williams (?)
Gala (S_2S_5)	Kidd's Orange Red (S_5S_9)[y] × Golden Delicious (S_2S_3)
Mollie's Delicious (S_3S_7)	(Golden Delicious × Edgewood) × (Red Gravenstein× Close)
Senshu (S_1S_7)	?
Jonathan (S_7S_9)	EsopusSpitzenburg (?) × ?
Kogetsu (S_3S_7)	Golden Delicious (S_2S_3) × Jonathan (S_7S_9)
Ralls Janet (S_1S_2)	?
Yoko (S_3S_9)	Golden Delicious (S_2S_3) × ?
Spur Earliblaze (S_1S_{19})	?

[z]Reported parentages of Korean apples are conflicted depending on S-genotypes from the results.
[y]S-genotypes were reported in Broothaerts et al. (2004).

of S_2 was found in six varieties: 'Jonagold', 'Golden Delicious', 'Pink Lady', 'Gala', 'Spur Golden Delicious', and 'Ralls Janet' (Fig. 1B). The S_3-allele which showed the largest frequency among tested thirty apples was distributed in seventeen cultivars when analyzed with the PCR product was obtained using two sets of specific primers (Figs. 1C and 1D). All cultivars retested to check S_3-allele genotype using primers designed by Kim et al. (2006). $S_{3\text{-}1}$ primer products revealed that 'Hongro', 'Seokwang', 'Sunhong', 'Seohong', 'Honggeum', 'Saenara', 'Golden Delicious', 'Spur Golden Delicious', 'Spur Earliblaze', 'Yoko', and 'Kogetsu'. However, 'Chukwang' was not identified on this PCR reaction but was identified when using $S_{3\text{-}2}$ primer. In contrast, 'Seohong' was not detected on $S_{3\text{-}2}$. Probably, these are caused by SNP in the sequences of cultivars for S_3-allele. To analyze this factor, sequencing for S_3-allele is needed through cloning of 'Chukwang' and 'Seohong'. S_5 allele was genotyped in three cultivars including 'Gala', 'Sansa' ($S_{5\text{-}1}$), and 'Tuscan' ($S_{5\text{-}2}$) but not in Korean apples (Figs. 1E and 1F). The S_7-allele was successfully detected in 'Honggeum', 'Summer Dream', 'Mollie's Delicious', 'Senshu', 'Jonathan', 'Kogetsu', Sansa', and 'Tsugaru' (Fig. 1G). S_9 PCR product was obtained in 'Sunhong', 'Seohong', 'Gamhong', 'Chukwang', 'Hwahong', 'Summer Dream', 'Yoko', 'Jonathan', 'Hwahong', 'Fuji', 'Jonagold', 'Red Delicious', and 'Manbok' (Fig. 1H). The S_3 and S_{10} coding sequences are highly identical (> 96%),

Table 2. S-allele specific primers and their sequences tested in this study.

S-allele	Primer	Sequence (5' to 3')	Fragment size (bp)
S_1[z]	FTC168	ATATTGTAAGGCACCGCCATATCAT	530
	FTC169	GGTTCTGTATTGGGGAAGACGCACAA	
S_2[z]	OWB122	GTTCAAACGTGACTTATGCG	449
	OWB123	GGTTTGGTTCCTTACCATGG	
$S_{3\text{-}1}$[z]	FTC177	CAAACGATAACAAATCTTAC	500
	FTC226	TATATGGAAATCACCATTCG	
$S_{3\text{-}2}$[y]	AS3MRF	GTACCCATTAATTTTCAATTC	1445
	APR3	CAAAGASHGACCTCAACYAATTC	
$S_{5\text{-}1}$[z]	FTC10	CAAACATGGCACCTGTGGGTCTCC	346
	FTC11	TAATAATGGATATCATTGGTAGG	
$S_{5\text{-}2}$[y]	AS5MPF	GAGTCAGTTCCATAATTTC	1380
	APR3	CAAAGASHGACCTCAACYAATTC	
S_7[z]	FTC143	ACTCGAATGGACATGACCCAGT	302
	FTC144	TGTCGTTCATTATTGTGGGATGTC	
S_9[z]	FTC154	CAGCCGGCTGTCTGCCACTT	343
	FTC155	CGGTTCGATCGAGTACGTTG	
S_{10}[z]	FTC12	CCAAACGTACTCAATCGAAG	209
	FTC228	ATGTCGTCCCGTGTCCTGAATC	
$S_{19/28}$[z]	FTC229	TCTGGGAAAGAGAGTGGCTC	304
	FTC230	TTTATGAACTTCGTTAAGTCTC	
S_{23}[z]	FTC222	CAATCGAACCAATCATTTGGT	237
	FTC224	GGTGTCATATTGTTGGTACTAATG	

[z]S-allele specific primers designed according to Broothaerts (2003).
[y]S-allele specific secondary primers designed according to Kim et al. (2006).

showing only single or double nucleotide substitutions dispersed over the sequence, and the corresponding gene products are difficult to distinguish on gels (Broothaerts, 2003). All cultivars that have S_3-allele were detected in S_{10} PCR reaction: 'Hongro', 'Tsugaru', 'Hongan', 'Jonagold', and 'Golden Delicious'. An alternative PCR/digestion method for identification of S_{10} was required (Kitahara and Matsumoto, 2002). Broothaerts (2003) proposed S_{19} was the same as S_{28}, and merged with S_{28}. However, S_{19} is just a rare allele and distinct from S_{28} that was revealed (Broothaerts et al., 2004). 'Spur Earliblaze' was a unique cultivar that has S_{19} allele in this result (Fig. 1I). Because S_{10} from 'Granny Smith' was different from other cultivars bear S_{10}, it was assigned a new number S_{23} allele. 'Pink Lady' that is reported as the progeny of a cross between 'Golden Delicious' (S_2S_3) and 'Lady Williams' was identified as S_{23}. 'Red Delicious' only showed as S_{28}. S_{28} was cloned, sequenced (Matsumoto and Kitahara, 2000) from 'Red Delicious', and then determined by Broothaerts (2003).

In these PCR results, the S-genotypes of Korean cultivars and main apples were determined based on S-allele specific PCR using S-RNase information (Table 1). Depending on S-genotype result of 'Gamhong' (S_1S_9), the S-allele segregation from pollen parent could not be explained by known parentage information that the cross between 'Spur Earliblaze' (S_1S_{19}) and 'Spur Golden Delicious' (S_2S_3). It could be assumed the out crossing by widely used 'Fuji' (S_1S_9) or other S_9 allele containing apples. At the same analysis, 'Hongan' (S_1S_3) could not be derived from the cross 'Fuji' (S_1S_9) × 'Jonathan' (S_7S_9). To identify the genuine pollen parents of apples for ambiguous parentage, further fingerprinting analysis needs using co-dominant marker system.

Fertilizability Assessment by Cross-pollination

Fertilization between apples is controlled by GSI mechanism depending on S-locus. Fertilizability was analyzed through the artificial cross-pollination between tested cultivars for two years to confirm the PCR based S-genotype results (Fig. 2).

The reciprocal cross tested between 'Fuji' (S_1S_9) and 'Gamhong' (S_1S_9) did not set any fruit as same as selfing of 'Fuji'. This result confirmed the PCR based S-genotype of 'Gamhong' is correct despite the difference with known parentage information. The cross-pollinations between apples having the same S-genotype also did not bear the fruit in tested combinations: 'Hongro' (S_1S_3) × 'Saenara'

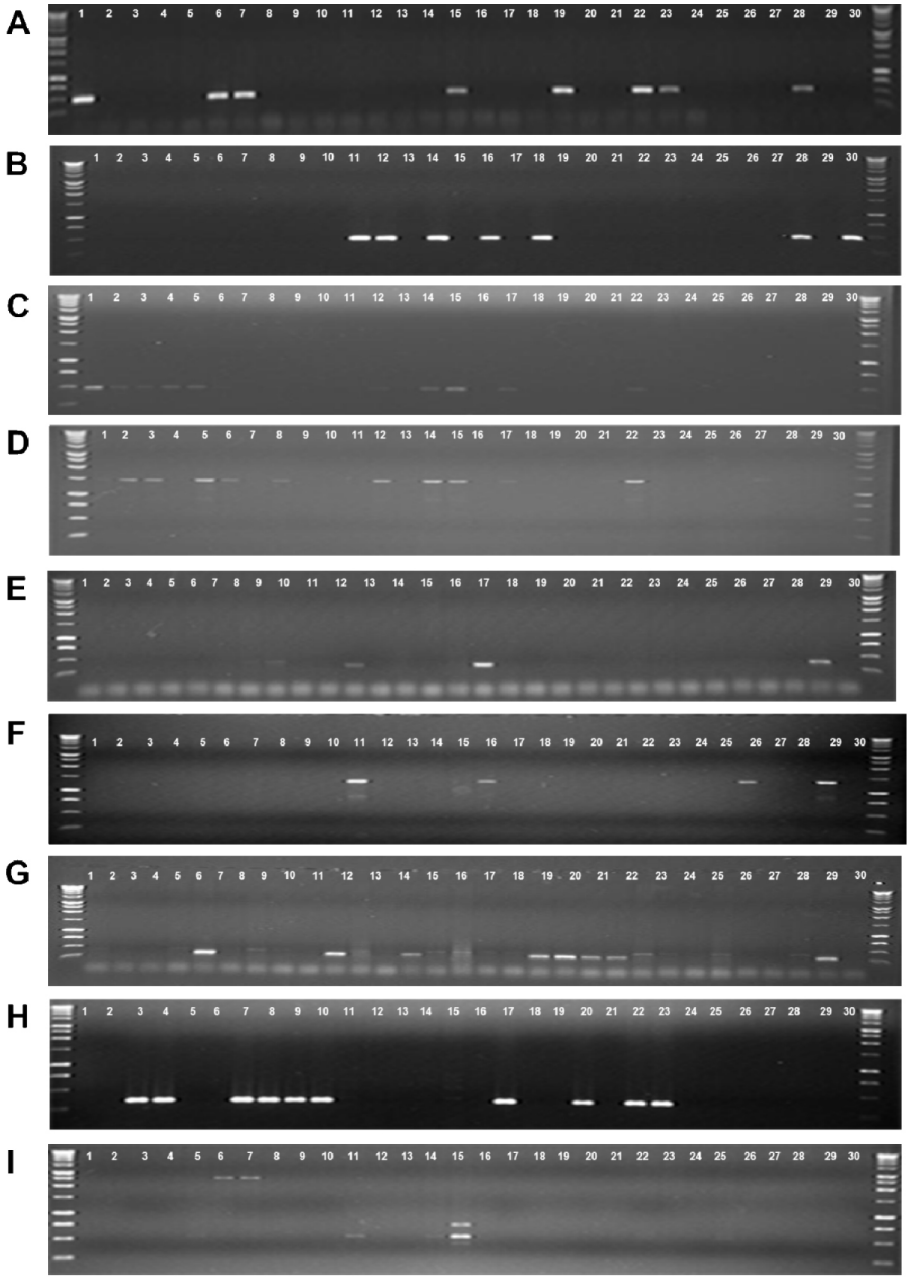

Fig. 1. Electrophoretic separation of S-RNase fragments for each of the S-alleles in thirty apple cultivars. PCR results by using S allele specific primers A, S_1; B, S_2; C, $S_{3\text{-}1}$; D, $S_{3\text{-}2}$; E, $S_{5\text{-}1}$; F, $S_{5\text{-}2}$; G, S_7; H, S_9; I, S_{19}. Lanes 1, 'Hongro'; 2, 'Seokwang'; 3, 'Sunhong'; 4, 'Seohong'; 5, 'Honggeum'; 6, 'Saenara'; 7, 'Gamhong'; 8, 'Chukwang'; 9, 'Hwahong'; 10, 'Summer Dream'; 11, 'Gala'; 12, 'Golden Delicious'; 13, 'Mollie's 'Delicious'; 14, 'Spur Golden Delicious'; 15, 'Spur Earliblaze'; 16, 'Tsugaru'; 17, 'Yoko'; 18, 'Aori #9'; 19, 'Shenshu'; 20, 'Jonathan'; 21, 'Kogetsu'; 22, 'Hwarang'; 23, 'Fuji'; 24, 'McIntosh Wijcik'; 25, 'Maypole'; 26, 'Tuscan'; 27, 'Telamon'; 28, 'Ralls Janet'; 29, 'Sansa'; 30, 'Trajan'.

(S_1S_3), 'Hwahong' (S_3S_9) × 'Chukwang' (S_3S_9), 'Hwahong' × 'Yoko' (S_3S_9), 'Sunhong' (S_3S_9) × 'Yoko', 'Sunhong' × 'Chukwang'. Compare with incompatible combination, semi- and fully compatible combinations of apples were not observed any significant difference. The result of cross-pollinations between 'Fuji' (S_1S_9) × 'Hongro' (S_1S_3, fruit set 87.0%, seed numbers 7.4) and 'Fuji' × 'Chukwang' (S_3S_9, 86.2%, 7.2) were not much difference the combinations between 'Fuji' × 'Seokwang' (S_3S_5, 90.6%, 8.9) and 'Fuji' × 'Tsugaru' (S_3S_7, 89.4%, 8.7). In natural pollination, the pollination rate of the semi-compatible combination is usually lower than that of the fully compatible combination (Sapir et al., 2008). However, artificial hand pollination performed in this study could be a reason of no difference because hand pollination had higher percentage of fertilization than that of natural pollination. As a result, fertilizability test using artificial pollination could identify the combination between same S-allele containing apples but not enough to find out

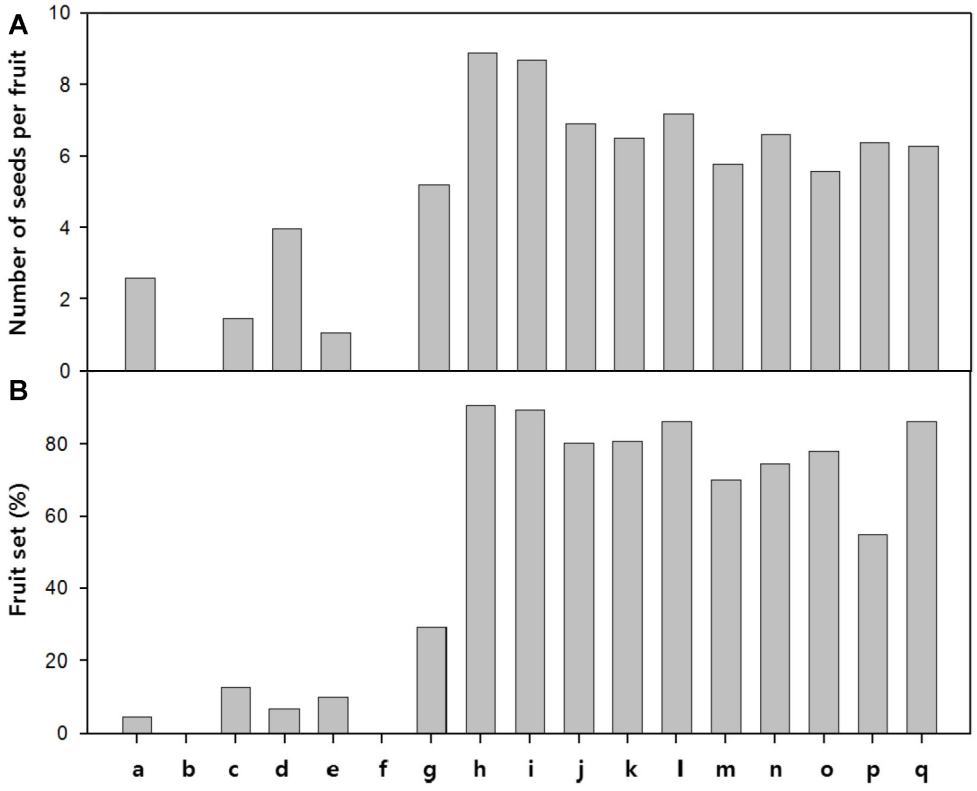

Fig. 2. Percentage of fruit set (A) and number of seeds per fruit (B) following artificial cross-pollination among Korean and major apple cultivars. Cross-combinationa, 'Fuji' × 'Gamhong'; b, 'Gamhong' × 'Fuji'; c, 'Hongro' × 'Saenara'; d, 'Hwahong' × 'Chukwang'; e, 'Hwahong' × 'Yoko'; f, 'Sunhong' × 'Yoko'; g, 'Sunhong' × 'Chukwang'; h, 'Fuji' × 'Seokwang'; i, 'Fuji' × 'Tsugaru'; j, 'Fuji' × 'Hongro'; k, 'Fuji' × 'Hwahong'; l, 'Fuji' × 'Chukwang'; m, 'Hongro' × 'Fuji'; n, 'Hongro' × 'Tsugaru'; o, 'Hwahong' × 'Fuji'; p, 'Hwahong' × 'Hongro'; q, 'Sunhong' × 'Hongro'.

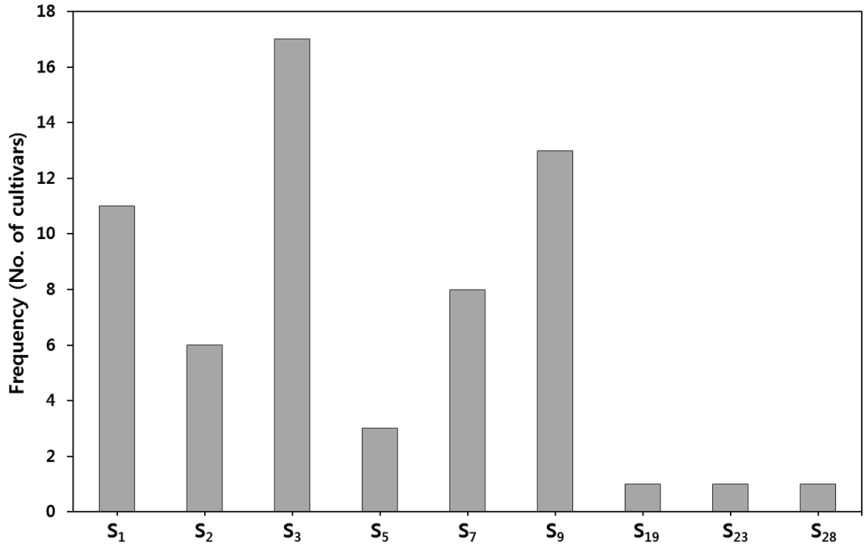

Fig. 3. The distribution of S-alleles among thirty apples including fourteen Korean cultivars.

the difference between semi- and fully combatable combinations. Therefore, it could be used for assistant way of PCR based identification or practical fertilization test for growers.

In conclusion, S-alleles of thirty apple cultivars were distributed through the nine S-alleles (Fig. 3). Five of limited S-alleles including S_1, S_2, S_3, S_7, and S_9 were major alleles among nine of them delivered from main cultivars or widely used apple breeding source in Korea. And more, kinds of S-alleles of fourteen Korean cultivars were more limited to

three alleles: S_1, S_3, and S_9. This reduction of S-allele diversity could be explained by the use of limited numbers of breeding source for quality based hybridization design in the early stage of Korean apple breeding program that commonly used 'Fuji' (S_1S_9) and 'Golden Delicious' (S_2S_3) as parents having bigger sizing and higher sugar containing traits. Following domestication, the genetic variation in crop plants has continued to be reduced by modern intensive plant breeding (Tanksley and McCouch, 1997). Selective breeding within the limited parents containing favorable mutations would have resulted in a progressive narrowing of the genetic base of subsequent populations. This kind of limited genetic diversity of crops renders them more vulnerable to disease and insect epidemics and jeopardizes the potential for sustained genetic improvement over the long term. At the same point of view, none of Korean released apple cultivars has S_2 allele though commonly used S_2 allele containing 'Golden Delicious' for their breeding program. The limited S allele distribution among Korean apple cultivars may indicate the reducing genetic diversity in Korean apples. Diversity of S-genotype could be an important factor has to consider for designing and selection of sustainable apple breeding program based on diversity of genetic background.

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