Review of the self-incompatibility in apple (*Malus* × *domestica* Borkh., syn.: *Malus pumila* Mill.)

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Summary: Apple (*Malus* × *domestica* Borkh.) is one of the most important fruit crops showing ribonuclease-mediated self-incompatibility, and no self-compatible apple cultivars are known. Twenty-nine *S*-alleles were identified in apple and many more incompatibility groups are present compared to sweet cherry. Results from a Belgian, English and a Japanese research group are combined and the *S*-genotypes of the most important world cultivars are collected. Two different allele labelling system are reconciled and detection methods used in case of the specific alleles are shown. Effects of the resistance breeding programmes are discussed; and scientific efforts involving transgenic technology to create self-compatible genotypes are shown. This review covers the most interesting issues regarding self-incompatibility in apple.

Key words: apple, *Malus* spp., self-incompatibility, *S*-genotype, transgenic plants

Importance of fertility relationships among apple cultivars

In 2005 apple production in the world totalled at a level of 63,488,907 Mt (*Faostat*, 2005). The great majority of apple cultivars are self-incompatible (*Broothaerts & Van Nerum*, 2003). Because of self-incompatibility, fruit production is dependent on the activity of insects, which is strongly influenced by weather conditions; therefore, irregular fruit production is a continuous threat.

The fruit set behaviour is controlled gametophytically by a locus, which is a multigene complex: an *S*-RNase gene is expressed in the pistils and an *S*-haplotype-specific F-box gene in the pollen tubes. Self/nonself recognition process and the consequent acceptance or rejection takes place between the protein products of these genes. Cultivars sharing common *S*-genotypes are mutually self-incompatible, their mating will not result a progeny. Knowledge on the *S*-genotypes of cultivars can be used to improve cross-pollination efficiency for commercial fruit production. It is extremely important in case of species, for which self-fruitful cultivars are not available. Semi-compatible cultivars that share one of their *S*-alleles have been widely grown together within the same orchards, which is not always optimal. *Schneider* et al. (2001) reported that the apple cultivar 'Jonathan' (*S₇S₉*) was a low potency pollinizer for 'Topred' (*S₉S₂₈*) trees in Israel. Information regarding *S*-allele composition of cultivars may also give support to design controlled hybridization for breeding or research purposes. In Hungary, also several studies published evaluating the flower biology and the compatibility relationships as well as the association of cultivars in apple orchards (*Nyéki* et al., 1982; *Soltész*, 1996a,b).

It must be highlighted that a definitive discrimination between several apple species and the domestic apple cannot be achieved, as domestic apple itself has been regarded as an interspecific hybrid of several species for so long. However, DNA-based evidence has been found recently supporting that only one wild species is involved (*M. pumila*), and fieldwork in Central Asia has revealed a large range of variation in wild populations, with some wild trees bearing fruit almost identical to most older name orchard cultivars (*Hogan & Rodd*, 2006). Anyhow, scientific literature regarding apple self-incompatibility presents an allele series irrespective of which allele originates from actually which species. We discuss all known alleles accordingly, because the possibility that other wild species might have contributed to the evolution of this fruit crop can not be excluded as these species can freely hybridize.

S-allele pool of apple and techniques used for *S*-genotyping

Some apple cultivars are cross incompatible, and some triploid × diploid combinations fail whereas the reciprocals succeed. At the beginning of the twentieth century some self-fertile cultivars were reported (*Soltész*, 2003), however, *Crane & Lawrence* (1929) brought a new aspect to this question by checking the seed content of fruits. Isolated flowers of some cultivars produced nearly 5% set but their seed content ranged between 0–0.9 indicating a quite

decisive influence of parthenocarpy which is a cultivar linked property. One of the first reports on the fertilization of apple cultivars was written by *Kobel* et al. (1939), who, by monitoring pollen tube growth and distinguishing between compatible, semi-compatible and incompatible crosses, assigned 11 alleles to some 20 local cultivars and labelled them in a numerical system S_1 to S_{11}, which was later continued by European research groups. Nevertheless, molecular analyses involving separation of stylar S-RNases by two-dimensional gel electrophoresis (2D-PAGE), isoelectric focusing (IEF) or non-equilibrium pH-gradient electrophocusing (NEpHGE) and detection by antibodies or ribonuclease activity staining, as well as the cloning and sequencing of S-allele cDNAs and genomic DNAs were only started in the last decade of the 20th century. In an early study, glycoprotein fractions were partially purified from apple virgin styles, and their biological activity was tested on *in vitro* pollen tube growth (*Speranza & Calzoni*, 1990). First molecular S-genotyping of apple cultivars was carried out by *Sassa* et al. (1994), who conducted 2D-PAGE analyses and described 6 alleles, S_a-S_f in four diploid and three triploid cultivars. This was later extended by three other alleles (S_g-S_i) (*Komori* et al., 2000). *Tobutt* et al. (2000) showed that linkage of the self-incompatibility locus with the locus of the resistance to woolly apple aphid suggested by *Knight* et al. (1962) could not be substantiated.

Besides the 11 alleles described by *Kobel* et al. (1939), 14 putative S-ribonucleases (S_{12}-S_{25}) were shown in NEpHGE gels by *Bošković & Tobutt* (1999). Overall 15 S-RNase encoding DNA fragments were sequenced during eight years by a Belgian research group (*Broothaerts* et al., 1995; *Janssens* et al., 1995; *Van Nerum* et al., 2001; *Broothaerts*, 2003). Similarly, 8 alleles were sequenced in Japan (*Sassa* et al., 1996; *Kitahara* et al., 1999, 2000; *Matsumoto & Kitahara*, 2000; *Kitahara & Matsumoto*, 2002a,b). Some results based on NEpHGE were re-evaluated after sequence analyses: for example S-genotype of 'Granny Smith' was corrected from S_3S_{10} to S_3S_{23} (*Broothaerts*, 2003). *Van Nerum* et al. (2001) confirmed the existence of the S_{20}, S_{22} and S_{24} alleles, while S_{23} was found to be identical with S_{22} (see below) and S_{25} with S_{10}. Realizing that the same alleles described in several versions under distinct names create a considerable confusion in apple S-genotyping, *Broothaerts & Van Nerum* (2003) collected all data from the available publications obtained by different analyses and re-examined and modified the annotation of S-alleles in apple (*Table 1*). Furthermore, *Broothaerts & Van Nerum* (2003) after reconciling the contradictory results, carried out a comprehensive and extensive work to classify cultivars into incompatibility groups (*Table 2*).

Two S-alleles that were cloned from the red-leaved crab-apple (*Malus floribunda* Sieb. ex Van Houtte) cultivar 'Baskatong' were denoted by *Verdoodt* et al. (1998) as S_{26} and S_{27}, because it was unclear at that moment to which of the 25 S-RNases determined by *Bošković & Tobutt* (1999) they corresponded. Later, two similar nucleotide sequences were recognized for S_{27}, which differed at only four

Table 1 Correspondences between European and Japanese S-allele nomenclature in apple, and methods for S-genotyping (Modified after *Broothaerts & Van Nerum* (2003) encompassing information from the following papers: *Bošković & Tobutt* (1999); *Broothaerts* (2003); *Broothaerts* et al. (1995); *Janssens* et al. (1995); *Kitahara* et al. (2000); *Kitahara & Matsumoto* (2002a,b); *Kobel* et al. (1939); *Komori* et al. (2000); *Matityahu* et al. (2005); *Sassa* et al. (1994); *Sassa* et al. (1996); *Schneider* et al. (2001); *Matsumoto* et al. (1999); *Matsumoto* et al. (2003); *Matsumoto & Kitahara* (2000); *Van Nerum* et al. (2001) and *Verdoodt* et al. (1998))

European labelling	Japanese labelling	Methods for S-genotyping*	European labelling	Japanese labelling	Methods for S-genotyping*
S_1	S_f	C, R, S	S_{15}		R
S_2	S_a	C, R, S	S_{16}		R, S
S_3	S_b	C, R, S	S_{17}		R
S_4		C, R, S	S_{18}		R
S_5		C, R, S	S_{19}		R
S_6		C, R	S_{20}	S_g	C, R, S
S_7	S_d	C, R, S	S_{21}		R
S_8		C, R	S_{22}		R, S
S_9	S_c	C, R, S	S_{23}		S
S_{10}	S_i	C, R, S	S_{24}	S_h	C, R, S
S_{11}		C, R	S_{25}	S_z	C, R, S
S_{12}		R	S_{26}		S
S_{13}		R	S_{27}		
S_{14}		R	$S_{30/28}$	S	C, R, S
S_{15}		R	S_{29}		S

*C = test cross, R = S-RNase detection, S = DNA sequencing

nucleotides within the intron region, and thus, encoding the same protein (*Van Nerum* et al., 2001). They were discriminated by an endonuclease digestion procedure and labelled as S_{27a} for the 'Baskatong' allele and S_{27b} for the one isolated from cultivars 'Alkmene', 'Merlijn' and 'Delbard Jubilé'. These were previously genotyped as S_5S_{22} and S_2S_{23}, respectively. Because S_{22}, S_{23} and S_{27b} referred to identical sequences, *Broothaerts* (2003) suggested using S_{22}. The related S_{27a}-allele has been detected in 'Bohnapfel' and seemed to correspond with the S_{16}-RNase described for this cultivar. This, however, still waits for confirmation. S_{16} or S_{27a} was only detected in another crab-apple cultivar 'Maypole' ($S_{10}S_{16/27a}$), which has 'Baskatong' as one of its parents.

The second allele of 'Baskatong' (S_{26}) is also extremely rare, it was only found in two crab-apple species, despite the widespread use of these crab-apples in breeding programs (*Broothaerts & Van Nerum*, 2003). It was detected in *Malus floribunda* 821, a common source of scab resistance genes, and could therefore have been introgressed in domestic apples (*Broothaerts*, 2003). Another species, *M. simcoe*, is $S_3S_{16/27a}$, and has apparently transmitted the $S_{16/27a}$-allele to 'Baskatong' ($S_{16/27a}S_{26}$). S_t-RNase was cloned from a wild apple species, *M. transitoria* for which allele-specific primers amplified a fragment in 'Ribston Pippin' and 'Jacques Lebel'. The S_t-RNase is highly homologous to apple and particularly to pear S-alleles (*Broothaerts & Van Nerum*, 2003). Investigation on these alleles may provide further interesting implications.

Table 2 Main incompatibility groups in apple (Slightly modified after Broothaerts & Van Nerum (2003) and supplemented by the information specified by Kitahara et al. (2005)

	S_2	S_3	S_4	S_5	S_6	S_7	S_8
S_1	Berner Rosen Parker's Pippin Rall's Janet *Kanada R*^{S3} *Spigold*^{S3}	J Musch *Adam's Pearmain*^{S10}*?* King of Pippins Northern Spy North Queen Sauergrauech *Kanada R*^{S1} *Spigold*^{S1} *Blenheim Orange*^{S14} *Hacnine*^{S9} *Jacques Lebel*^{S14}		White Transparent	Amanishiki	Ontario Senshu *Hokuto*^{S9}	
S_2		Golden Delicious Jester Rubin Transparente de Croncels Chüsenrainer *Kanada R*^{S1} *Spigold*^{S1} *Jonagold*^{S9} *Boskoop*^{S5} *Mutsu*^{S20} *Shizuka*^{S20}	Champagne Reinette	Falstaff Gala Greensleeves Topaz *Boskoop*^{S3}		Arlet Danziger Kantapfel Orin	
S_3				Elstar Fiesta Rubinette Sampion Shinsei Winter Banana *Boskoop*^{S2} *Citron d'Hiver*^{S12}*?* *Liberty*^{S10} *Menzauer Jägerapfel*^{S18}	Oberrieder Glanzrienette Oetwiler	Idared Kogetsu Princess Redfree Spijon Tsugaru *Stajn Ros*^{S8}	*Stafnel Rosen*^{S7}
S_5						Sansa Vanda *Brünn*^{S10} *Karmijn S*^{S9}	J Grieve
S_7							*Stafnel Rosen*^{S3}

Incompatibility groups among apple cultivars

A large number of apple cultivars have been *S*-genotyped during the recent years. In *Table 2* triploid cultivars are written in italics in both groups characterized by the given three alleles. Triploid cultivars will show cross-incompatibility when pollinated by any of the diploid cultivars of the same group. Triploid cultivars are themselves bad pollinators producing merely sterile pollen grains. *Table 2* consists of 171 cultivars.

Only those *S*-alleles are shown that occurred in more than one domestic apple cultivar (omitting the crab-apple species), altogether 16 different alleles. From the remaining 11 alleles, 10 (S_{11}-S_{19}, S_{21}) have been detected in the cultivars 'Adam's Pearmain', 'Citron d'Hiver', 'Gravenstein', 'Blenheim Orange', 'Kaiserapfel', 'Bohnapfel', 'Jacques Lebel', 'Menzauer Jägerapfel',

'Bohnapfel', 'Ribston Pippin', respectively, by *Bošković & Tobutt* (1999) and one, the 'Baskatong' (S_{26}) by *Verdoodt* et al. (1998).

In apple, many more incompatibility groups are present compared to sweet cherry (*Broothaerts & Van Nerum*, 2003). If we consider the most common 16 *S*-alleles, 120 diploid combinations are possible: n(n-1)/2, although members have been found in only 57 of the 120 incompatibility groups. The high number of alleles operating in the population explains why full incompatibility between two cultivars is rarely observed in apple crosses. Within the cultivars ranked in the top-20 according to the world production, 19 are included in *Table 2*; and only one incompatibility group (S_2S_3) is represented by more than one cultivar encompassing 'Golden Delicious' and two triploids 'Jonagold' and 'Boskoop'. Furthermore, most known scab resistant cultivars carry the S_2- or the S_3-allele, as a consequence of the use of common

Continue Table 2

	S_9	S_{10}	S_{20}	S_{22}	S_{23}	S_{24}	S_{25}	S_{28}
S_1	Fuji, Shinko, Spencer Seedless, Ranzan, *Hacnine*[S3], *Hokuto*[S7], *Ribston Pippin*[S21]	*Kaiserapfel*[S15]	'American Summer Pearmain'				Milton	
S_2	Ambitious, Kinsei, MegumiRedgold, Summerred, *Jonagold*[S3]	Prima, Spencer	*Mutsu*[S3], *Shizuka*[S3]	Delbard Jubilé	Pink Lady	Honeycrisp, Worc Pearm	Trajan, Shamrock, Summerland	Cameo, Goldrush, Orei, Aori 3
S_3	Florina, Kent, La Paix, Natsumidori, Nebuta, Priscilla, Rewena, *Hacnine*[S1], *Jonagold*[S2]	Ahrista, Delcorf, Ecolette, Shinano Red, Telamon, Puritan	Meku 10, *Mutsu*[S2], *Shizuka*[S2]	Merlijn	Granny Smith		Macoun, Patricia, Victory	Ging Gold, Gold Supr, Korei
S_4			*Gravenst*[S13]			Noblow		Gloster
S_5	Clivia, Cox's Orange Peppin, Kidd's OR, *Karmijn*[S7]	Charlotte, Tuscan, *Brünn*[S7]		Alkmene			Cortland, Early McIntosh, Niagara	Toyo
S_7	Himekami, Jonathan, *Hokuto*[S1], *Karmijn*[S5]	*Brünn*[S5]	Indo, Trezeke M			Akane, Beni No Mai, Jonamac	Aori 1	Rero 11
S_8	Wellington							
S_9		Spartan				Braeburn	Kitakami	Delicious, Holly, Jonadel, Melrose, Murasaki
S_{10}				Lobo		Discovery, Vista Bella	McIntosh, Wijcik Empire	
S_{20}						Rome Beauty		
S_{24}							Tydeman's Early Worcester, Tohoku2	

breeding parents (*Broothaerts & Van Nerum*, 2003; *Tóth*, 2005; *Tóth & Pedryc*, 2005), which may also pose incompatibility problems in the future. All other most popular cultivars are at least semi-compatible among each other.

The widespread use of 'Golden Delicious', 'Delicious', 'Jonathan', 'McIntosh' and 'Cox' Orange Pippin' in breeding programs has resulted in the accumulation of their S-alleles (S_2, S_3, S_5, S_7, S_9, S_{10} and S_{28}) in the domestic apple cultivars. The incompatibility groups constituted by these 7 alleles are amongst the ones that have the highest number of entries. Particularly the S_1S_3, S_2S_3, S_3S_9 and S_3S_5 groups.

We mention as a matter of curiosity that in contrast to heterozygosity at the S-locus being a prevailing condition under GSI, as it was previously described, *Verdoodt* et al. (1998) reported that some selections raised parthenogenetically from 'Idared' are homozygous S_3S_3 or S_7S_7, and others raised by androgenesis from 'Braeburn', are S_9S_9 or $S_{24}S_{24}$.

Tobutt et al. (2000) presumed that 'M4' and 'Irish Peach' might have S_3S_3 and S_1S_1 genotypes, respectively.

Will self-compatibility in apple remain a vain hope?

No fully self-compatible apple cultivar is known all over the world. Maybe this is the reason why apple was the first and up to now the only rosaceous species for which transgenic technology was exploited to introduce self-compatibility. To obtain self-fertile apple trees, a strongly self-incompatible apple cultivar, 'Elstar' (S_3S_5) was transformed with either a sense or an antisense copy of the endogenous S_3-allele (*Van Nerum* et al., 2000). Transformation was also carried out with only a 3' or 5' part of the *S*-gene in antisense direction, since *Van der Krol* et al. (1990) reported that in some cases a partial antisense sequence could express stronger inhibition on endogenous gene expression compared to full-length antisense sequences. All four constructs were controlled by the CaMV35S constitutive promoter. Such types of experiments have the inconvenience that due to the long juvenile phase of plants, one can wait several years for the results to be evaluated. For each tree part of the flowers was self-pollinated and part was cross-pollinated with pollen of the cross-compatible apple cultivar 'Delbard Jubilé' (S_2S_4). Analysis of fruit and seed set revealed that in some lines the self-incompatibility mechanism was switched off. Fruit and seed set and pollen tubes in the ovule after self-pollination in these lines were similar to that obtained after cross-pollination. In contrast to the lines with partial antisense sequences, none of the lines with full-length antisense S_3 gave a high fruit-set after self-pollination, and this seems to confirm the establishments of *Van der Krol* et al. (1990).

The transformation techniques of apple were further improved, when *Broothaerts* et al. (2001) described a multiplex PCR technique for apple, which simultaneously demonstrated the presence of both a transgene sequence within a transformation vector and an endogenous (e.g. *S*-like or *S*-RNase) gene. Successful amplification with *S*-allele-specific primers could also confirm the identity of the cultivar used in the experiment, which is useful to avoid mislabelling of lines during subculturing and other operations.

In the latest study of the same research group, the employed gene silencing constructs were evaluated to be outdated (*Broothaerts* et al., 2004). This is because the long period between the establishment of transgenic plants and the time, when enough data could be collected after a long juvenile phase and a 3-year-period of fruit-set study. This outdated technology to achieve *S*-RNase gene silencing was a co-suppression approach. A very similar phenomenon is seen in case of the polyploid plants: in the heteroallelic diploid pollen the two *S*-alleles may interact competitively, so that the pollen is rejected in styles having one or both of the same *S*-alleles. In some cases this seems to be the reason for self-compatibility in the tetraploid sour cherry cultivars (*Tobutt* et al., 2004), and presumably in some hexaploid plum cultivars. As it was shown by Western blot analysis, the *S*-RNase signal was completely absent from the transgenic pistils. It indicates that the transgene has resulted in a complete or at least significant downregulation of not only the targeted S_3-allele, but also the other S_5-allele.

The accelerated development of molecular techniques in the future will allow us to make alterations in the plant genome in a more specifically engineered way. Experiments for regeneration and transformation of fruit species are quite intensive, and therefore, in case of acceptance, scientists may be ready to establish self-compatible cultivars of nearly all species, for which a reliable regeneration-transformation system can be elaborated in the future. This is, however, not such a simple question. Even if health concerns can be reconciled, we must not forget about the potential long-term ecological hazards. Transgenic alleles for self-compatibility can easily escape and extensively distributed by pollinating insects. This may cause the accumulation of SC alleles in related wild species, a process, which is absolutely controversial to the original "purpose" of plants to remain self-incompatible, and avoid inbreeding. Transgenic self-fertile trees may also be used as a tool to study inbreeding depression or for the development of homozygous breeding lines (*Broothaerts* et al., 2004).

I am highly convinced that a more comprehensive *S*-genotyping study concentrating on the available species which could contribute to the evolution of the domestic apple, will reveal deeper correspondences and also may provide valuable information on the origin of some apple cultivars.

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