International Journal of Horticultural Science 2006, 12 (2): 19–29
Agroinform Publishing House, Budapest, Printed in Hungary
ISSN 1585-0404

A critical evaluation of methods used for *S*-genotyping: from trees to DNA level

Halász, J.[1] & Hegedűs, A.[2]

[1]*Corvinus University of Budapest, Faculty of Horticultural Science, Department of Genetics and Plant Breeding, 1118 Budapest, Ménesi út 44., Hungary;*
[2]*Corvinus University of Budapest, Faculty of Food Science, Department of Applied Chemistry, 1118 Budapest, Villányi út 29., Hungary; E-mail: hegedus.attila@uni-corvinus.hu*

Summary: Fruit setting behaviour of fruit trees remains to be in the focus of plant breeders and growers. Realizing that most species (cherry, apple, pear etc.) are self-incompatible and certain cultivars are cross-incompatible, mutual fertility properties and their reliable determination are of great interest. This review gives a comprehensive description of all known *S*-genotyping procedures, i.e. the classical fruit set analysis after open field test crosses; pollen tube growth monitoring with fluorescent microscopy; stylar ribonuclease electrophoresis (using different types of isoelectric focusing and 2-dimension polyacrilamide gel electrophoresis); as well as the most recent polymerase chain reaction based DNA-level analyses and DNA sequencing. The review presented not only gives a compilation of the bases of the methods described but also provides a critical evaluation and a comparative characterization of their applicability.

Key words: F-box, NEpHGE, PCR, *Rosaceae*, self-incompatibility, *S*-genotype, *S*-ribonuclease

In this review, we describe in detail the advantages and disadvantages of methods used for *S*-genotyping fruit trees. Nowadays, four methods are widely used for the determination of fruit set behaviour and *S*-genotyping of fruit tree cultivars: classical fruit set studies after controlled crosses, monitoring pollen tube growth by means of fluorescence microscopy, *S*-RNase detection (NEpHGE) and *S*-allele-specific PCR. A comparative analysis of the four widely used techniques in an almond breeding programme was performed by *Ortega & Dicenta* (2004). Fruit set studies are easy and cheap, however, environmental effects may circumvent analyses.

Classical methods for *S*-genotyping: fruit set studies at the field

Evaluating fruit set after controlled pollination has been the only way to assign *S*-genotypes to cultivars for so long. The technique itself is simple; however, it has a lot of shortcomings. Before pollination, pollen is collected from all tested cultivars by desiccating the anthers in a Petri dish at ambient temperature. In the orchard, branches must be chosen that seem to have an adequate number of flowers at balloon stage, open flowers must be removed. Flowers are emasculated to prevent self-pollination. Controlled pollinations in the required combinations and directions are carried out by a glass rod, toothpick or merely by fingers when stigmata are receptive and completely covered by an exudate (*Guerriero & Bartolini*, 1995). After about eight weeks, the resulting fruits are counted and the fruit set percentage is determined.

To determine self-compatibility, two methods are used generally (*Nyujtó* et al., 1985; *Burgos* et al., 1993; *Nyéki*, 1996; *Nyéki & Szabó*, 1995). In artificial self-pollination studies, pollen transfer is carried out using pollen of the same cultivar. Autogamy means bagging branches with closed flower buds and the determination of the percentage of fruit set obtained. By this method, the ability of a tree to yield fruits in the absence of pollinizer cultivars and pollinators can be assessed. This is thereby not so informative in cases, when we would like to confirm an *S*-genotype by field crosses. Geitonogamy may result more valuable data. It also means self-pollination, but by directly allocating pollens to the stigmata, thereby failure in fruit set can only be attributed to incompatibility reactions. Bagging can be accomplished by water-proof parchment paper bags. Any open flowers at bagging must be removed, and buds are counted.

Fruit set study is an extremely cheap and not time consuming method; however, for results one must wait 1 or 2 months. Test crosses are generally required for the confirmation of molecular *S*-genotyping studies, and these are really useful for getting information on the function of the described systems, however, they may be hampered by several environmental factors. To eliminate these, crosses can be carried out in the laboratory, and results evaluated by means of fluorescence microscopy.

The greatest disadvantage of these procedures is that *S*-genotyping can only be achieved by a series of crosses, which is not easy to be realized and lasts for years due to the long juvenile phase of the fruit trees. At first, self-incompatibility must be verified by self-pollination. In a species with functional gametophytic self-incompatibility (GSI) system, cross-incompatibility can be found between several cultivars. Cultivars or seedlings belonging to the same inter-incompatibility group are characterized by the same *S*-genotypes.

In a simplified scheme, in case of two inter-incompatible groups, we can assign them the preliminary genotypes S_1S_2 and S_3S_4. However, we must check that these groups do not have a common allele. Fruit set may not reflect a semi-compatible combination, as half of the pollen grains are able to fertilize the ovules, which can result in reasonable yields since many pollen grains may land on a stigma. Therefore, two cultivars representing the two groups are crossed and the progeny is crossed again on to their male parent. If the four alleles are indeed different, four genotypes would be expected (S_1S_3, S_1S_4, S_2S_3 and S_2S_4) with each being equally probable in a non-selective environment. Furthermore, none of them will show incompatibility when crossed on to any of the parents. However, if parents belonging to two different inter-incompatibility groups share one allele, only two *S*-genotypes will occur in the progeny: S_1S_3 and S_2S_3 or S_1S_2 and S_1S_3, according as S_1S_2 was the female or male parent, respectively. This difference occurs because the female parent contributes both *S*-alleles to the progeny, while from the male parent only the allele not common to the female parent alleles is transmitted. These two progenies when crossed on to their female parent will result fully compatible combinations; but when crossed on to their male parents half of the progeny will be compatible and half of them will be incompatible (*Table 1*).

This methodology was used in several fruit tree species to determine their *S*-genotypes. In sweet cherry, *Crane & Lawrence* (1929) tentatively assigned alleles to five incompatibility groups encompassing 19 cultivars. Later, by incorporating the results of the John Innes Institute and other institutes *Matthews & Dow* (1969) published a list, in which six incompatibility alleles were variously assigned to some 140 cultivars in 10 inter-incompatibility groups. This technique was also successfully used for investigating the types of spontaneous and X-ray induced mutations that resulted in self-compatible seedlings (*Lewis & Crowe*, 1954). For almond, *Tufts & Philp* (1922) established two cross-incompatibility groups: Group I (S_cS_d) and Group II (S_aS_b). Later, four additional groups (Group III, S_aS_c; Group IV, S_bS_c; Group V, S_aS_d and Group VI, S_bS_d) resulting from their cross were also identified by fruit set studies (*Kester* et al., 1994).

We must mention that in case of apple or pear cultivars, this method may provide misleading results, since some of the cultivars tend to set fruits by parthenocarpy. In all cases analysis must be complemented by counting the seeds within fruits.

Pollen tube growth analysis with fluorescent microscopy

Fertilization of flowering plants begins when pollens land on the surface of a stigma. Before anthesis, mature pollen grains are dehydrated with water-contents ranging from 6 to 60% because it confers a tolerance to the environmental stresses on them and it may be a necessary prerequisite for pollen viability and subsequent germination (*Lin & Dickinson*, 1984). This process is reversible: reaching a suitable flower, so landing upon an appropriate stigma, pollen hydrates. Pollen hydration is tightly regulated and several molecules are known to be involved in stimulating it. Once they are hydrated, pollen grains attain a distinct polarity and germinate to produce a pollen tube which grows by tip extension (*Franklin-Tong*, 2002). In many plant families where gametophytically controlled self-incompatibility exists, including *Rosaceae*, "wet" stigma type is found, which means that copious secretion fluid accumulates on the surface of stigma and forms a medium for the germination of the captured pollen (*Heslop-Harrison*, 1975).

Pollen tubes' travelling through the stylar tissues (ensuring a proper pathway, nutritive support or guidance to the ovary etc.) as all events of fertilization is a cooperative and highly organized process between the male and female partners. Growing through the style, the pollen tubes push away the mucilaginous cell walls, thus the cells lose their tension and collapse. That is the reason why the intruding pollen tubes do not induce the lateral expansion of styles. Pollen tube growth is heterotrophic at the expenses of the stylar reserves (*Herrero & Hormaza*, 1996), since the reserve substances in pollen grain are not sufficient for the growth of a pollen tube from stigma to ovule. A transmitting tissue-specific glycoprotein was isolated and suggested to have nutritive role, since it was deglycosilated by *in vitro*-growing pollen tubes (*Wu* et al., 1995).

Table 1 A simplified scheme for *S*-genotyping cultivars by evaluating fruit set after controlled crosses. C = compatible; IC = incompatible cross

♀ \ ♂	S_2	S_3
S_1	–	S_1S_3
S_2	–	S_2S_3

♀ \ ♂	S_1	S_2
S_2	S_1S_2	–
S_3	S_1S_3	–

Progeny

Crossed on to the ♀ parent	$S_1S_2 \times S_1S_3$	C	$S_1S_2 \times S_2S_3$	C
Crossed on to the ♀ parent	$S_1S_2 \times S_2S_3$	C	$S_1S_3 \times S_2S_3$	C
Crossed on to the ♂ parent	$S_2S_3 \times S_1S_3$	C	$S_1S_2 \times S_1S_2$	IC
Crossed on to the ♂ parent	$S_2S_3 \times S_2S_3$	IC	$S_1S_2 \times S_1S_3$	C

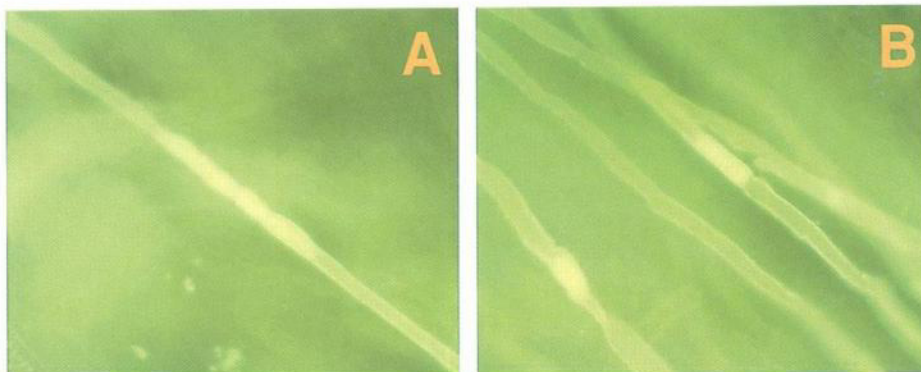

Figure 1. Illustrative micrographs of aniline blue-stained incompatible (A) and compatible (B) pollen tubes growing in the transmitting tissue at the upper part of the style. Incompatible and compatible combinations were obtained after self-pollination of self-incompatible 'Harcot' and self-compatible 'Gönci magyar kajszi' apricot cultivars, respectively (Photo by J. Halász)

Pollen tube is an extremely specialized cell type, including a generative cell, which contains the two sperm cells, and the vegetative nucleus. It has a unique structure: pollen tube is itself haploid and in fact it comprises a cell within a cell. The cytoplasm carrying the vegetative and sperm cells is located toward the growing tip of the front region. In this region there are also several other organelles, like mitochondria, endoplasmic reticulum and Golgi complexes. The cytoplasm is separated from the remainder of the pollen tube by callosic cross walls. These callose plugs are laid down at regular intervals as the tube grows and give the tubes a ladder-like appearance. During growth, the regions behind the callose plugs are vacuolated so the cytoplasm concentrated in the front portion of the tube, regardless of its length (*Franklin-Tong*, 1999).

Pollen tubes' wall consists of two main layers of polysaccharide. The inner callosic wall contains predominantly (1,3)-β-glucan (*Newbigin* et al., 1993). It has been reported by de *Nettancourt* et al. (1973) that there is an increase in the callosic particles in the cytoplasm of pollen tubes after incompatible pollination and the same authors suggested that these callosic particles result from the breakdown of the inner pollen tube cell wall. According to *Cresti & Went* (1976) the increased number of callosic particles in response of an incompatible pollination might be due to a premature degeneration of the cytoplasm as a result of the inhibited growth of pollen tubes caused by the incompatibility reaction.

The fact that callose can be stained selectively with a water-soluble substance aniline blue or similar fluorochromes, was first reported by *Currier* (1957). The stained callose layer in either living or dead tissue will fluoresce intensively in ultraviolet light (*Evans & Hoyne*, 1982). Staining of pollen tubes with aniline blue reveals the presence of callose plugs through the style and provides an advantage over the vital stains previously used, by which the uniformly stained stylar cells and pollen tubes could have only been differentiated by some hardly visible structural properties. However, this outdated technique could also supply some data concerning *S*-genotypes of fruit trees. *Kobel* et al. (1938) had assigned incompatibility alleles to sweet cherry cultivars in Switzerland. They could also distinguish between compatible, semi-compatible and incompatible crosses and assign 11 *S*-alleles to some 20 apple cultivars (*Kobel* et al., 1939).

Martin (1959) described the first appropriate staining technique of pollen tubes in style. His protocol comprised the following steps: styles are fixed in formalin : 80% alcohol : acetic acid for 24 hours or more. After rinsing in tap water, they are treated in an about 8 *N* sodium hydroxide solution for 8 to 24 hours to clear and soften the tissue and to permit adequate penetration of dye. Staining is accomplished in a 0.1% solution of aniline blue dye in 0.1 N K_3PO_4 for 4 hours. For observations the stained styles are put on clean glass slides and are covered with cover slips. The slide must be directly illuminated by ultraviolet light using a conventional microscope in a darkened room.

Under these conditions all the sites along a pollen tube where a callose deposition is present will fluoresce bright yellow to yellow-green, whereas the background tissue will fluoresce pale grey or blue (*Figure 1.*). Varying amounts of callose occur frequently in the sieve tubes and within the epidermal hairs of the style but with some experience pollen tubes can be distinguished from them by their size, shape and distribution in the style. If these difficulties inhibit the clear observation of the tubes, it might be avoided by adequately modifying the above detailed procedure. Prepared slides can be sealed and stored for some months in a refrigerator at 4–5 °C. Success of the technique depends on the sufficient amount of callose. Callose distribution and amount within the pollen tube wall was shown to be variable according to different species as well as several external factors. It sometimes appears through the entire tube; nearly fill its whole length. In other cases, callose is localized as closely spaced plugs. Rarely the amount of callose is too small to make visible the growing tube. It was found in pollen tubes of many self-incompatible species that the amount of callose might be higher after self- than cross-pollination (*Figure 1.*) (*Linskens & Esser*, 1957; *Halász* et al., 2004). In incompatible tubes, the pattern of growth is similar to that initially seen in a compatible pollination, but at some stage, growth becomes irregular, the pollen tube walls become thicker and the tips may burst (*Figure 2A*), while the growth of compatible pollen tubes is unaffected, therefore they can successfully reach the ovary (*Figure 2B*) (*Newbigin* et al., 1993).

Martins's method was rediscovered when self-incompatibility studies became more active at the end of the last century. At this time experiments were extended and species belonging to the *Rosaceae* family were also involved.

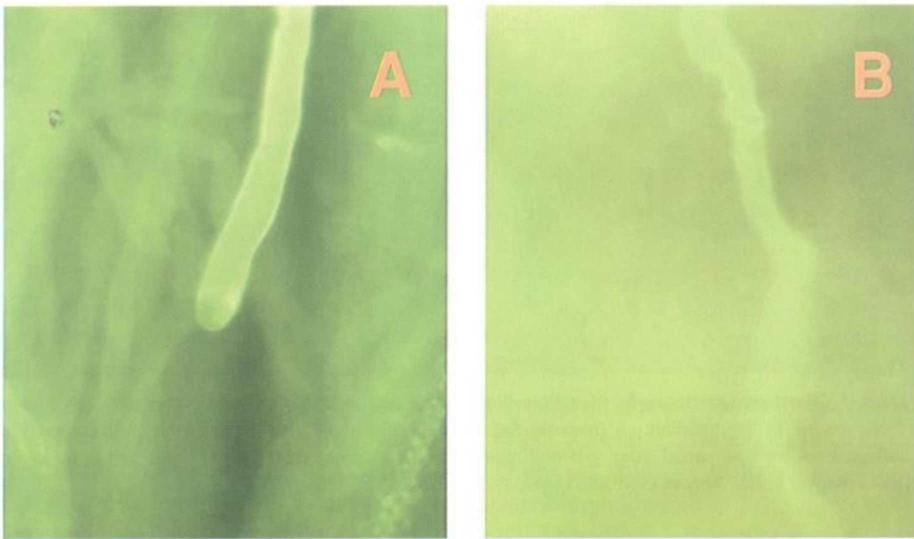

Figure 2. The incompatible (A) pollen tube ceases to grow further at the middle part of the style, it becomes swollen and its tip gets burst; while one of the compatible (B) pollen tubes can reach the basal part of the style and penetrate to the ovary. Incompatible and compatible combinations were obtained after self-pollination of self-incompatible 'Harcot' and self-compatible 'Gönci magyar kajszi' apricot cultivars, respectively (Photo by J. Halász)

Almond was one of the first rosaceous species where this technique was expansively used (*Socias i Company* et al., 1976; *Ben-Njima & Socias i Company*, 1995). *Dicenta & García* (1993) also employed the classical method of Martin with some modifications. Prior to microscopic evaluation the pistils were washed, and treated in a 5% sodium sulphite (Na_2SO_3) in an autoclave at 1–1.2 kg cm^{-2} to soften the tissue and improve staining efficiency (*Jefferies & Belcher*, 1974). A further step was included: the pubescence was removed before pistils were placed on slides and squashing them. This method was also successfully employed by several research groups in case of many almond genotypes (*Bošković* et al., 1999; *Ortega* et al., 2002; *Ortega & Dicenta*, 2003; *Socias i Company & Alonso*, 2004; *Ortega & Dicenta*, 2004).

Apricot styles, just like those of almond, are also hairy; therefore pollen tubes could not be observed surely unless pubescence is completely removed (*Burgos* et al., 1993; *Andrés & Durán*, 1998). First data concerning apricot pollen tube growth was presented by *Egea* et al. (1991). This methodology either by floating flowers on water-filled trays or leaving them on branches was also used in several comprehensive studies (*Egea & Burgos*, 1996; *Burgos* et al., 1997; *Viti* et al., 1997; *Andrés & Durán*, 1998). Fixing pollen tube growth 72 h after pollination leaves enough time for tubes to reach the ovary in case of almond, but it proved to be insufficient for apricot: only 25% of compatible combinations had tubes reaching the ovary during 72 h (*Viti* et al., 1997; *Audergon* et al., 1999). Consequently, authors proposed to extend the time between pollination and fixation to 96 h in case of apricot. The method with slight modifications according to the properties of the adequate plant species was successfully used in case of apple, Japanese pear, European pear (*Sanzol & Herrero*, 2002).

A completely different type of pollen tube analyses, a *semi-vivo* technique was elaborated by a Japanese research team, which was applied by them on several occasions but it has not been taken over by others (*Hiratsuka* et al., 1995; *Zhang & Hiratsuka*, 1999). Calyxes, petals and stamens are removed from flowers, leaving only the pistils intact. After pollinating the stigmas, the styles are excised at the base and imbedded in a medium consisting of agar, sucrose and boric acid. After culturing for 48 h in a dark moist chamber at 25 °C, the basal parts of the styles were stained with cotton blue dye to visualise under a light microscope all pollen tubes protruded from the cut-ends of styles (the basal part).

When an appropriate fluorescent dye was made available, microscopic studies were made easier for the determination of compatibility properties. This needs the longest work time from all four methods described in this review, but in three-four days final results can be obtained. Microscopic observation of pollen tubes is very difficult, even for experienced experts as callose deposits not exclusively occur in pollen tubes to be monitored. Although this technique is burdened with some shortcomings and does not enable direct *S*-genotyping, it remains to be a precious technique to verify molecular results obtained by stylar RNase detection or PCR analysis.

S-genotyping by stylar ribonuclease electrophoresis

The procedure of two-dimensional electrophoresis combines isoelectric focusing in the first dimension with sodium dodecylsulfate electrophoresis as a second-dimensional run (*O'Farrell*, 1975). Isoelectric focusing is carried out in presence of 8–9.5 M urea, a viscous additive. Since basic proteins can not be satisfactorily separated due to the collapse of the pH gradient at the cathodic end, NEpHGE was applied in most *S*-RNase studies rather than IEF. To solve the problem putatively caused by cathodic electro-osmotic migration, immobilized pH gradients may provide a relatively new approach.

The result of 2D-electrophoresis is a pattern of spots with the following standard of presentation: from left to right increasing pI and from top to bottom decreasing molecular weight establishing a coordinate system for evaluation. It offers additional possibilities, as proteins can be electroblotted onto a PVDF (polyvinylidene fluoride) membrane either in order to determine the *N*-terminal amino acid sequence of the target proteins or perform an immunoblot analysis by an antiserum raised against an

S-RNase molecule. This latter is a promising approach as it was evidenced that an antiserum raised against S_4-RNase of Japanese pear also reacted with *S*-RNases of Chinese and European pears, as well as apple (*Tomimoto* et al., 1996). This supported a resemblance among *S*-RNases, nevertheless the differential intensity of the reactions against the antiserum may be due to some difference in recognition sites of the antiserum. In case of almond, $S_{a/5}$-RNase reacted with the anti-S_4-serum prepared from Japanese pear, but no reaction was observed with the $S_{b/1}$-RNase band (*Tao* et al., 1997).

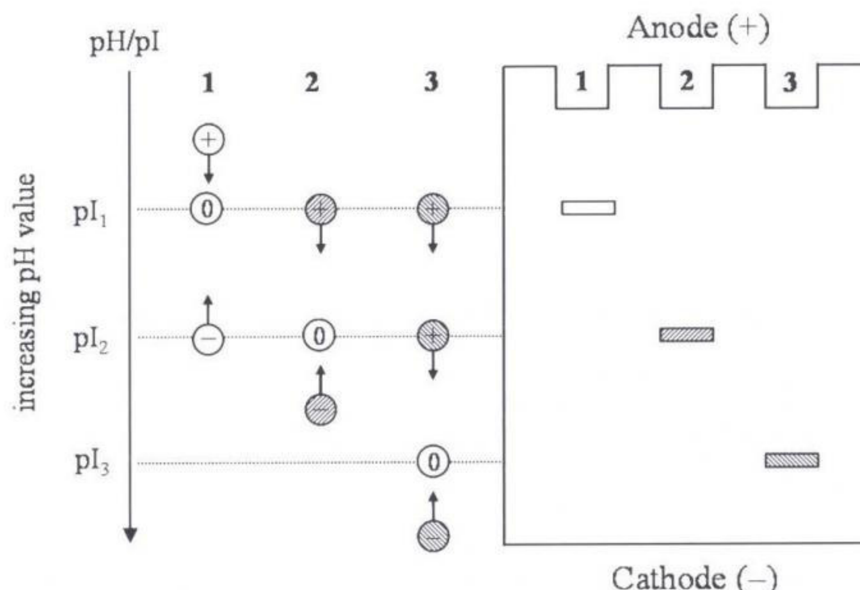

Figure 3. Schematic electrofocusing of three *S*-RNase proteins (designated as 1, 2 and 3) with different isoelectric points (marked as pI_1, pI_2 and pI_3) in a vertical slab gel. Each of them is positively charged above their pI values (as all of them before starting of electrofocusing), and negatively charged beneath. Thus, they will move in an electric field until the position in the gel is reached where the pH of the gradient is the same as pI for that particular protein

As concerns *S*-allele associated protein studies, first 2D results were provided by a Japanese research group (*Sassa* et al., 1993). In this first report, they investigated three *S*-glycoproteins of Japanese pear, which was followed by another study identifying six (S_a–S_f) alleles in the styles of apple (*Sassa* et al., 1994). This study was later extended by detecting S_1–S_7 RNases and characterizing them as being 30–32 kDa basic proteins with pIs of 9.6–10.1 (*Ishimizu* et al., 1996). *Tomimoto* et al. (1996) genotyped Chinese and European pears, in almond, *Tao* et al. (1997) successfully distinguished $S_{a/5}$, $S_{b/1}$, $S_{c/7}$ and $S_{d/8}$-RNases from each other, which was a great advance after he could have only identified two of the four *S*-alleles by means of IEF. In cherry from the six *S*-allelic products tested, only two (S_2- and S_6-RNases) were identified, which was explained by the possibility that some *S*-allelic products overlapped on the gel (*Tao* et al., 1999). Later on, in very similar experiments S_1-, S_7- and S_f-RNases of Japanese apricot were also identified on 2D-electropherograms (*Tao* et al., 2003). Two putative non-*S* RNases were also identified in sweet cherry (*Yamane* et al., 2003b).

The method has several advantages, for example it affords the highest resolution of all the protein electrophoretic methods known at the moment, which is especially important in case of complex mixtures of proteins (*Westermeier*, 1993). Besides it, the physico-chemical properties of proteins, such as the pI and the molecular weight can be read on the 2D-electropherogram as on a coordinate system. At the same time it has a considerable disadvantage as being particularly complicated compared to any other electrophoretic procedure described above.

The discovery that the pistil component of self-incompatibility expresses ribonuclease activity offered promising ways for the investigations, since after the electrophoretic separation proteins could be easily detected by means of an activity staining. As differences among the allelic series of *S*-proteins are sometimes very small and this difference resides rather in the amino acid composition of the proteins than in the number of amino acids composing the *S*-allele encoded RNase enzymes – with molecular weights fall within the range of about 30–40 kDa in case of almond $S_{8/d}$-RNase (*Tao* et al., 1997; *Certal* et al., 2002) –, it seems quite obvious that polyacrilamide gel electrophoresis – elaborated by *Laemmli* (1970) –, where separation is based on a size fractionation of proteins can not be successful. Whereas size of proteins differing in some amino acids is not notably influenced, there dissociation properties, net charge under different pH ranges, their so-called isoelectric points may be rather altered. Consequently, isoelectric focusing of *S*-glycoproteins is a more adequate technique for electrophoretic separation.

"Isoelectric focusing" or "electrofocusing" are names that have been accepted for use since 1967 (*Haglund*, 1971). It covers a high resolution method enabling separation of proteins with as small a difference as 0.02 pH units at the isoelectric point. The isoelectric point (pI) is the pH where the molecule has a net charge of zero, so its electric mobility is also zero. Using mixtures of several ampholyte compounds we can establish a pH gradient between the two poles: the lowest pH value at the anode, the highest at the cathode. When a sample of proteins is added to this pH gradient, the protein molecules will acquire different charges. The net charge of every protein molecule will be determined by the pI for that protein and the pH of the region where the protein is located. Applying an electric potential across the system results in each protein molecule migrating until it has a net charge. When it reaches the pH value, which is identical to its pI value, at the isoelectric point, its net charge will be zero. As the molecule will be ionized in both of the directions from

this position (either toward the cathod or the anode) it will gain negative or positive charges, respectively, so it migrates back to the zone of isoelectric pH. It means that the reciprocal repulsion-attraction effect of the electrodes will be the driving force for focusing all proteins in a narrow band at a point where the pH is equal to their pI (*Figure 3*).

Running is executed from the + pole to the cathode. It means that the acidic reservoir is located on top and the basic reservoir on the bottom. It is inevitable as *S*-RNases are basic proteins and they have only considerable charges (+) under acidic conditions (*Figure 3*). The catalyte and analyte most frequently used are 0.1 M sodium hydroxide and 0.04 M DL-glutamic acid, respectively.

After focusing, gels are specifically stained for ribonuclease activity. *Wilson* (1971) elaborated its original procedure in which *Bošković & Tobutt* (1996) proposed some modifications. The gels are stained with toluidine blue and the ribonuclease izozyme bands appear as achromatic bands on a blue background.

Isoelectric focusing was successfully used for the identification of plant taxons in a range of horticultural crops, including medicinal plants (*Stefanovits-Bányai* et al., 1999), vine cultivars (*Stefanovits-Bányai* et al., 2002) or ornamentals (*Hirsch* et al., 2000) as well as *Prunus* fruit trees (*Messeguer* et al., 1987; *Cerezo* et al., 1989).

First report was written by *Sassa* et al. (1992), who applied a mixture of Ampholine pH 3.5–10 and Ampholine pH 9–11 to separate and detect *S*-ribonucleases from *Pyrus serotina* styles. There is another type of ampholyte mixture sold under the trademark of "Pharmalyte", which is produced as co-polymerisates of glycine, glycylglycine, amines and epichlorhydrin (*Anonymous*, 1998). This proved to be more useful for *S*-RNase separation of many rosaceous species ranging from apple to cherry. "Ampholine" and "Pharmalyte" can also be blended to obtain the most adequate carrier ampholyte mixture as it was successfully used in case of apricot (*Halász* et al., 2005).

Bošković et al. (1997) have found that the electrophoretic separation applied in case of cherry (*Bošković & Tobutt*, 1996) achieved isoelectric focusing, however in almond not all the ribonucleases reached their pI under these conditions. Consequently, this should be regarded as non-equilibrium pH gradient electrofocusing (NEpHGE). This technique was described by *O'Farrell* et al. (1977) as the first part of a high-resolution two-dimensional electrophoresis (2D-PAGE; see below). NEpHGE involves electrophoresis toward the cathode similarly to IEF, but ended before proteins would attain the equilibrium. Its empiric development was fuelled by the collapse of the basic end of the pH gradient emerging for reasons unknown at that time. This phenomenon was extremely serious in case of 2D-PAGE where gels contain urea, in contrast with *S*-RNase detection studies, which are carried out under native conditions, free of any detergents. *O'Farrell* et al. (1977) suggested 1500–2000 Vh for separation of basic proteins. In *S*-RNase studies 1350-2450 Vh values were used according to various species, cultivars and alleles to be separated. As we mentioned focusing run resulting in IEF for a species could only achieve NEpHGE in another species (*Bošković* et al., 1997). Generally higher Vh values are suggested to ensure complete focusing effect during IEF, since lower values (i.e. 950 or 1150 Vh) resulted in insufficient resolution. Sometimes differences in the pI values were observed for the same *S*-RNase protein under identical separation circumstances (*Bošković* et al., 2003), which lead to the conclusion that different sets of marker proteins used in independent experiments may also influence pI determination. Nevertheless, it was not verified, and in general it can be accepted that pI determination by IEF is a precise method, though a cautious evaluation is always required for the correct interpretation.

For composite proteins such as the glyco- or nucleoproteins, the net charge is also influenced by the sugar or the nucleic acid moieties (*Westermeier*, 1993), which should be considered, as *S*-ribonucleases are known to be glycoproteins (*Anderson* et al., 1986). Heterogeneity in the glycan chains attached to the *S*-glycoproteins was first time revealed in *Nicotiana alata* (*Woodward* et al., 1992). In case of a rosaceous species, Japanese pear, *Ishimizu* et al. (1996) proved elegantly by reverse-phase HPLC and mass spectrometry that double spots for S_4-RNase occurring after 2D-electrophoresis are due to microheterogeneity of the sugar moieties. Double spots were also observed for several (S_2–S_5) RNases. *Bošković & Tobutt* (1999) detected that both S_{20}- and S_{24}-RNases also appeared as double bands after IEF and assumed – albeit not verified – that this is because of differences within the glycan side chains of these enzymes. Later *Broothaerts* (2003) attributed the difference between the S_1 and S_{11} alleles of apple to the same phenomenon. An indirect evidence for this type of intraallelic variation was reported by *Van Nerum* et al. (2001): they clarified that S_{22}, S_{23} and S_{25} alleles assigned by *Bošković & Tobutt* (1999) were identical in DNA sequence of the coding region, nevertheless, their pI values were 9.85, 9.80 and 8.50, respectively. The large difference in electrophoretic mobility between S_{25} and the two others with identical amino acid sequence implies that the difference in their glycan chains must also be considerable.

We must highlight the fact that based on these techniques, the discrimination of self-compatibility from self-incompatibility *S*-RNases of Japanese pear and almond could be achieved directly, since these show lower levels of activity or no activity (*Sassa* et al., 1992; *Bošković* et al., 1999). Conflicting results were published for self-compatibility in the sour cherry cultivar 'Érdi bőtermő'. *Yamane* et al. (2003a) reported that this cultivar carries a functional pollen-*S* and a non-functional pistil *S*-allele: expression analyses revealed that S_6-RNase was not transcribed in style. Nevertheless, *Tobutt* et al. (2004) presented NEpHGE and IEF gels of the same cultivar showing four distinct izozyme bands. In other species where loss-of-function mutation affected not the stylar but the pollen component of the *S*-locus, an indirect identification of self-compatible genotypes may be burdened with uncertainty resulting from the same *S*-RNase product of the mutated and non-mutated

polymerase enzyme will replicate the template DNA strand using the previously added four types of deoxyribonucleoside triphosphates.

First report on the PCR amplification of *S*-alleles were described by *Brace* et al. (1993) using the sporophytic self-incompatible *Brassica oleracea*. Primers were constructed from known sequences and allele identification was carried out by digestion with several restriction enzymes. Taking advantage of the abundance of *S*-locus mRNA in stylar tissue, *Solanum carolinense* was the first gametophytic self-incompatible species from which *S*-alleles were amplified using reverse-transcriptase (RT-)PCR (*Richman* et al., 1995). Primer pairs were designed to the conserved regions of the solanaceous *S*-alleles. After the restriction digests in each amplification product two alleles were revealed, this was consistent with the expectation that all individuals are heterozygous under gametophytic self-incompatibility. Homozygocity may only occur if at least one of the alleles has undergone a loss-of-function mutation or in case of selections raised parthenogenetically (*Verdoodt* et al., 1998).

Research on the self-incompatibility of *Rosaceae*, which always lagged behind the studies on *Solanaceae* made up lost ground in 1995, since a very similar report was published in the same year involving cDNA cloning and PCR amplification of two alleles from apple (*Broothaerts* et al., 1995). Furthermore, shortly after this first report a new paper was published to identify three additional *S*-allele cDNAs of apple and develop a molecular technique for the diagnostic discrimination among the five different *S*-alleles of apple (*Janssens* et al., 1995). Many degenerate and allele-specific primers were designed for PCR analyses in several rosaceous fruit species (*Figure 4*) (*Tao* et al., 1999; *Tamura* et al., 2000; *Sonneveld* et al., 2001; 2003; *Sapir* et al., 2004; *Sutherland* et al., 2004; *Vilanova* et al., 2005).

Bošković et al. (1997) found that the band within the ribonuclease zymograms of almond cultivars corresponding to S_1 of the French labelling system was indistinguishable from that corresponding to S_b of the Californian labelling system, and the controlled cross confirmed that these alleles are identical. Nevertheless, *Ma & Oliveira* (2001) observed differences in S_1 and S_b partial sequences at the intron region, which may represent either accidental genetic drift in the absence of genetic pressure or a mechanism for generation of new *S*-alleles.

Pollen component of GSI system was identified in 2003 to be an F-box molecule, which takes part in the ubiquitin mediated proteolysis. First data on an F-box based *S*-genotyping method were provided by *Yamane* et al. (2003c) in Japanese apricot. This method consisted of a *Hind*III digestion of genomic DNA and a subsequent blot analysis with *Pm*SFB$_1$ and *Pm*SFB$_7$ cDNAs under low-stringency conditions. Besides a monomorphic band, one band was recognized for alleles S_1–S_7, while two fragments could be corresponded to allele S_f. The authors discussed this phenomenon as S_f-haplotypes may carry two different genes for SFB leading to the breakdown of SI through competitive interaction. Therefore, additional DNA blot analyses with other restriction endonucleases were conducted, which ruled out this possibility (*Ushijima* et al., 2004). An insertion with several *Hind*III sites was found in the SFB$_f$ sequence (see beforehand), which likely gave the two S_f-haplotype-specific fragments on the *Hind*III blot. Nevertheless, authors are still indebted to a prospective and more comfortable SFB$_f$-specific marker.

As the sequence of the mutated SFB$_4$ allele of sweet cherry has become available, several studies were conducted to develop molecular markers for the S_4'-allele. *Ikeda* et al. (2004) used nested PCR amplifying the SFB$_4$ and SFB$_4$' and two sets of primers creating restriction site specifically in the PCR product of SFB$_4$'. This yielded fragments from SFB$_4$ and SFB$_4$', which were not clearly distinguishable on agarose gels, but they were definitely separated on denaturing 6% polyacrilamide gels. After digestion with the appropriate restriction enzymes, cleaved fragments gave a successful separation even in an agarose gel.

A simpler molecular marker was designed by *Zhu* et al. (2004). They have found not consistent results for the sequence of the 4 bp deletion in SFB$_4$': it was determined to be TTTA or TTAT by BLAST and Clustal W programs, respectively. On the basis of the first sequence deletion they have designed primers, which could specifically amplify the SFB$_4$' allele. The result can be visualized directly on agarose gels, so this primer set can be immediately applied to a marker-assisted cherry breeding program.

Sonneveld et al. (2005) designed allele-specific primers for the S_3- and S_4-SFB sequences, which they have used to check that self-compatibility can not be attributed to a heteroallelic pollen resulting from the duplication of pollen *S*-alleles. *Pa*SFB$_4$ primer pair amplified products of indistinguishable fragment sizes in case of S_4- and S_4'-alleles, while *Pa*SFB$_3$ primer pair could only detect S_3, not the S_3', which indicates a different type of mutation in SFB$_3$ and SFB$_4$.

Consequences

Fruit set studies are inexpensive and easy analyses, however, weather may restrict the possibilities. Monitoring pollen tube growth could make results more independent from environmental conditions and it could also accelerate the analyses. Stylar ribonuclease assay and PCR facilitate direct *S*-genotyping in shorter time compared to the two above mentioned methods; however they also have some shortcomings. They are more expensive and require laboratory skills and molecular biology knowledge for interpreting the results. As compared NEpHGE to PCR, the latter is more expensive regarding both equipments and chemicals needed, but it gives results in a slightly shorter time as concerns the analysis itself. Furthermore, PCR can be used for early selection as does not require flowering material; thereby, cost and time spent on 3–4-year-long cultivation in orchards can be saved. *S*-allele sequencing and the deposition of sequence data in websites

alleles (*Bošković* et al., 2000; *Alburquerque* et al., 2002; *Halász* et al., 2005; *Pedryc* et al., 2005). In these cases molecular methods aimed at pollen part *S*-determinants may yield unequivocal results (see below).

Protein patterns resolved by isoelectric focusing in polyacrilamide gels were not only used for ribonuclease activity staining, but also for detection all stylar proteins by Coomassie brilliant blue or silver staining. *Sassa* et al. (1992) used this method first time to demonstrate that a self-compatible mutant of Japanese pear contains much less of an *S*-RNase protein, which may be the cause for its self-fertility. This method with slight modifications (*Hiratsuka* et al., 1995, 1999; *Zhang & Hiratsuka*, 1999) and by coupling it with SDS-PAGE (*Hiratsuka & Okada*, 1995) was later mainly used by a Japanese research group for expression and inheritance studies on *S*-proteins of Japanese pear cultivars.

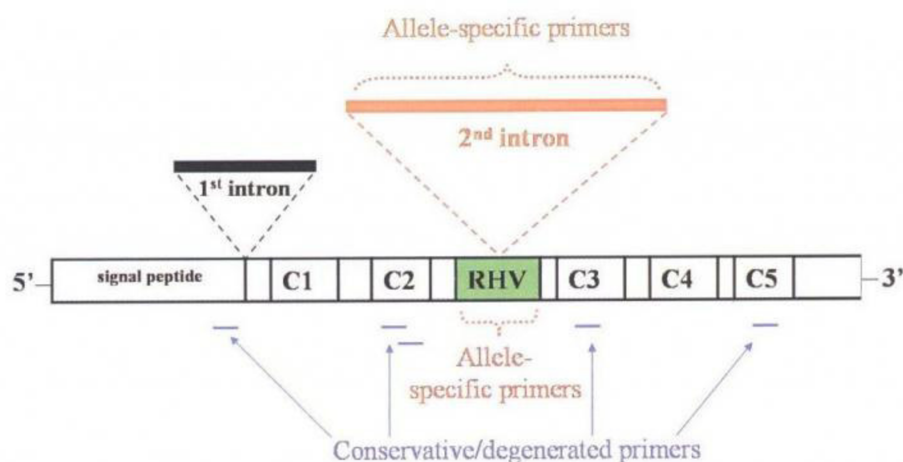

Figure 4. Hybridization sites of conservative and allele-specific primers designed for *S*-RNase based PCR analyses

DNA-based analyses: PCR, sequencing

This section is intended for enumerating techniques based on genomic DNA isolation from plant tissues and polymerase chain reaction (PCR) successfully used for analysing self-incompatibility status. Digestion of the PCR-amplified fragments with restriction endonucleases as well as cloning and sequencing is also mentioned from this aspect.

PCR approaches circumvent the necessity of time-consuming and sometimes equivocal open field crossing experiments to determine the number of mating type alleles in a population, while providing sequence information for evolutionary study at and above the species level (*Richman & Kohn*, 1996). Additional advantages of molecular assays include unambiguous comparisons of alleles described in studies carried out at different times and in different species.

Several DNA extraction protocols are used for the molecular analysis of plants. Usually, for *S*-PCR analysis two main types of DNA isolation methods are utilized. One of them is the protocol based on a detergent, called cetyltrimethylammonium bromide (CTAB), which forms an insoluble complex with nucleic acids (*Doyle & Doyle*, 1987). When CTAB is added to a plant cell extract, the nucleic acid–CTAB complex precipitates, leaving carbohydrate, protein and other contaminants in the supernatant. The precipitate is then collected by centrifugation and resuspended in 1 M NaCl, which causes the complex to break down. Thus the nucleic acids can be concentrated by ethanol precipitation and the RNA removed by ribonuclease treatment. A second method makes use of the fact that nucleic acid molecules, unlike most of the contaminants in a cell extract, have relatively strong negative charges. This means that nucleic acids bind to positively charged surfaces, for instance to the particles in an anion-exchange chromatography resin. The resin is placed in the column and the cell extract added onto it. Nucleic acids are retained in the column, whereas the neutral and positively charged contaminants pass straight through. After washing away the last contaminants, the nucleic acids are recovered by adding a high-salt solution, which destabilizes the electrostatic interactions between the nucleic acid molecules and the resin. This chromatographic method is the base of several kits, which are commercially available (e.g. Qiagen, Germany).

Using the CTAB method for DNA isolation has several advantages and disadvantages involving that it yields a DNA extract of high concentration and it is cheap, however, the obtained DNA solution will be more contaminated and the procedure is time-consuming. In contrast to it, using an extraction kit may provide a less contaminated DNA extract with an overall better quality, but its DNA content will be definitely lower compared to that of the extracts gained by the CTAB method, and these kits are rather expensive.

The method, which has become known as Polymerase Chain Reaction (PCR) was designed by Kary B. Mullis and Fred A. Faloona to obtain many copies of an arbitrary DNA sequence (the template) during a short period of time (*Mullis & Faloona*, 1987). It is necessary that the ends of the sequence be known in sufficient detail that two oligonucleotide primers can be synthetized, which will hybridize to them. The sequence to be synthesized can be present initially as a discrete molecule or it can be part of a larger molecule. In either case, the product of the reaction will be a discrete dsDNA molecule with termini corresponding to the 5' ends of the oligomers employed. A source of DNA including the desired sequence is denatured and the oligonucleotide single-stranded primers hybridize to the edges of the target sequence, then a DNA

(EMBL/GenBank/DDBJ) will help a lot in the harmonization of results from different laboratories working on the self-incompatibility.

References

Alburquerque, N., Egea, J., Pérez-Tornero, O. & Burgos, L. (2002): Genotyping apricot cultivars for self-(in)compatibility by means of RNases associated with *S* alleles. Plant Breed., 121: 343–347.

Anderson, M.A., Cornish, E.C., Mau, S.-L., Williams, E.G., Hoggart, R., Atkinson, A., Bonig, I., Grego, B., Simpson, R., Roche, P.J., Haley, J.D., Penschow, J.D., Niall, H.D., Tregear, G.W., Coghlan, J.P., Crawford, R.J. & Clarke, A.E. (1986): Cloning of cDNA for a stylar glycoprotein associated with expression of self-incompatibility in *Nicotiana alata*. Nature, 321: 38–44.

Andrés, M.V. & Durán, J.M. (1998): Self-incompatibility in Spanish clones of apricot (*Prunus armeniaca* L.) tree. Euphytica, 101: 349–355.

Anonymous (1998): Guide to isoelectric focusing. Amersham Biosciences, APB, RW 5/5/98. http://www.biodesign.dk

Audergon, J.M., Guerriero, R., Monteleone, P. & Viti, R. (1999): Contribution to the study of inheritance of the character self-incompatibility in apricot. Acta Hort., 488: 275–279.

Ben-Njima, N. & Socias i Company, R. (1995): Characterization of some self-compatible almonds. I. Pollen tube growth. HortScience, 30: 318–320.

Bošković, R. & Tobutt, K.R. (1996): Correlation of stylar ribonuclease zymograms with incompatibility alleles in sweet cherry. Euphytica, 90: 245–250.

Bošković, R. & Tobutt, K.R. (1999): Correlation of stylar ribonuclease isoenzymes with incompatibility alleles in apple. Euphytica, 107: 29–43.

Bošković, R., Tobutt, K.R., Batlle, I. & Duval, H. (1997): Correlation of ribonuclease zymograms and incompatibility genotypes in almond. Euphytica, 97: 167–176.

Bošković, R., Tobutt, K.R., Batlle, I., Duval, H., Martinez-Gomez, P. & Gradziel, T.M. (2003): Stylar ribonucleases in almond: correlation with and prediction of incompatibility genotypes. Plant Breed., 122: 70–76.

Bošković, R., Tobutt, K.R., Duval, H., Batlle, I., Dicenta, F. & Vargas, F.J. (1999): A stylar ribonuclease assay to detect self-compatible seedlings in almond progenies. Theor. Appl. Genet., 99: 800–810.

Bošković, R., Tobutt, K.R., Schmidt, H. & Sonneveld, T. (2000): Re-examination of (in)compatibility genotypes of two John Innes self-compatible sweet cherry selections. Theor. Appl. Genet., 101: 234–240.

Brace, J., King, G.J. & Ockendon, D.J. (1993): Development of a method for the identification of *S*-alleles in *Brassica oleracea* based on digestion of PCR-amplified DNA with restriction endonucleases. Sex. Plant Reprod., 6: 133–138.

Broothaerts, W. (2003): New findings in apple *S*-genotype analysis resolve previous confusion and request the re-numbering of some *S*-alleles. Theor. Appl. Genet., 106: 703–714.

Broothaerts, W., Janssens, G.A., Proost, P. & Broekaert, W.F. (1995): cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol. Biol., 27: 499–511.

Burgos, L., Berenguer & T., Egea, J. (1993): Self- and cross-compatibility among apricot cultivars. HortScience, 28: 148–150.

Burgos, L., Ledbetter, C.A., Pérez-Tornero, O., Ortín-Párraga, F. & Egea, J. (1997): Inheritance of sexual incompatibility in apricot. Plant Breed., 116: 383–386.

Burgos, L. & Pérez-Tornero, O. (1999): Review of self-incompatibility in apricot. Acta Hort., 488: 267–273.

Cerezo, M., Socias i Compani, R. & Arús, P. (1989): Identification of almond cultivars by pollen isoenzymes. J. Amer. Soc. Hort. Sci., 114: 164–169.

Certal, A.C., Almeida, R.B., Bošković, R., Oliveira, M.M. & Feijó, J.A. (2002): Structural and molecular analysis of self-incompatibility in almond (*Prunus dulcis*). Sex. Plant Reprod., 15: 13–20.

Crane, M.B. & Lawrence, W.J.C. (1929): Genetical and cytological aspects of incompatibility and sterility in cultivated fruits. J. Pomol. Hort. Sci., 7: 276–301.

Cresti, M. & Went, van J.L. (1976): Callose deposition and plug formation in *Petunia* pollen tubes *in situ*. Planta, 133: 35–40.

Currier, H.B. (1957): Callose substances in plant cells. Am. J. Bot., 44: 478–88.

Dicenta, F. & Garcia, J.E. (1993): Inheritance of self-compatibility in almond. Heredity, 70: 313–317.

Doyle, J.J. & Doyle, J.L. (1987): A rapid DNA isolation procedure from small quantities of fresh leaf tissues. Phytochem. Bull., 19: 11–15.

Egea, J. & Burgos, L. (1996): Detecting cross-incompatibility of three North-American apricot cultivars and establishing the first incompatibility group in apricot. J. Amer. Soc. Hort. Sci., 121: 1002–1005.

Egea, J., Garcia, J.E., Egea, L. & Berenguer, T. (1991): Self-incompatibility in apricot cultivars. Acta Hort., 293: 285-293.

Evans, N.A. & Hoyne, P.A. (1982): A fluorochrome from aniline blue: Structure, synthesis and fluorescence properties. Aust. J. Chem., 35: 2571–2575.

Franklin-Tong, V.E. (1999): Signaling and the modulation of pollen tube growth. Plant Cell, 11: 727–738.

Franklin-Tong, V.E. (2002): The difficult question of sex: the mating game. Current Opinion in Plant Biology, 5: 14–18.

Guerriero, R. & Bartolini, S. (1995): Flower biology in apricot: main aspects and problems. Acta Hort., 384: 261-271.

Haglund, H. (1971): Isoelectric focusing in pH gradients – a technique for fractionation and characterization of ampholytes. In: Glick, D. (Ed.): Methods of biochemical analysis. Vol. 19. pp. 1-104. John Wiley & Sons, New York–London–Sydney–Toronto.

Halász, J., Hegedűs, A., Hermán, R., Stefanovits-Bányai, E. & Pedryc, A. (2005): New self-incompatibility alleles in apricot (*Prunus armeniaca* L.) revealed by stylar ribonuclease assay and *S*-PCR analysis. Euphytica, 145: 57–66.

Halász, J., Ruthner, Sz., Békefi, Zs. & Pedryc, A. (2004): *S*-genotype characterization of several Hungarian apricot varieties. 14th FESPB Congress, Krakkow, Acta Physiol. Plant., 26(3): 168.

Herrero, M. & Hormaza, J.I. (1996): Pistil strategies controlling pollen tube growth. Sex. Plant Reprod., 9: 343–347.

Heslop-Harrison, J. (1975): Incompatibility and the pollen-stigma interaction. Ann. Rev. Plant Physiol. 26: 403–425.

Hiratsuka, S., Nakashima, M., Kamasaki, K., Kubo, T. & Kawai, Y. (1999): Comparison of an *S*-protein expression between

self-compatible and -incompatible Japanese pear cultivars. Sex. Plant Reprod., 12: 88–93.

Hiratsuka, S. & Okada, Y. (1995): Some properties of stylar protein associated with self-incompatibility genotype of Japanese pear. Acta Hort., 392: 257–264.

Hiratsuka, S., Okada, Y., Kawai, Y., Tamura, F. & Tanabe, K. (1995): Expression and inheritance of *S*-proteins in self-compatible and -incompatible Japanese pears. J. Japan. Soc. Hort. Sci., 64: 479–484.

Hirsch, J., Stefanovits-Bányai, É., Schmidt, G., Orlóci, L., Velich, I. & Bisztray, G. (2000): Isoenzyme analysis of Cypress, false Cypress and hybrid Cypress species. Plant Physiol. Biochem., 38: 238.

Ikeda, K., Watari, A., Ushijima, K., Yamane, H., Hauck, N.R., Iezzoni, A.F. & Tao, R. (2004): Molecular markers for the self-compatible S_4'-haplotype, a pollen-part mutant in sweet cherry (*Prunus avium* L.). J. Amer. Soc. Hort. Sci., 129: 724–728.

Ishimizu, T., Sato, Y., Saito, T., Yoshimura, Y., Norioka, S., Nakanishi, T. & Sakiyama, F. (1996): Identification and partial amino acid sequences of seven *S*-RNases associated with self-incompatibility of Japanese pear, *Pyrus pyrifolia* Nakai. J. Biochem., 120: 326–334.

Janssens, G.A., Goderis, I.J., Broekaert, W.F. & Broothaerts, W. (1995): A molecular method for *S*-allele identification in apple based on allele-specific PCR. Theor. Appl. Genet., 91: 691–698.

Jefferies, C.J. & Belcher, A.R. (1974): A fluorescent brightener used for pollen tube identification *in vivo*. Stain. Technol., 49: 199–202.

Kester, D.E., Gradziel, T.M. & Micke, W.C. (1994): Identifying pollen incompatibility groups in Californian almond cultivars. J. Amer. Soc. Hort. Sci., 119: 106–109.

Kobel, F., Steinegger, P. & Anliker, J. (1938): Weitere Untersuchungen über die Befruchtungsverhältnisse der Kirschensorten. Landw. Jahrb. Schweiz, 52: 564–595.

Kobel, F., Steinegger, P. & Anliker, J. (1939): Weitere Untersuchungen über die Befruchtungsverhältnisse der Apfel- und Birnsorten. Landw. Jahrb. Schweiz, 53: 160–191.

Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227: 680–685.

Lewis, D. & Crowe, L.K. (1954): Structure of the incompatibility gene. IV. Types of mutations in *Prunus avium* L. Heredity, 8: 357–363.

Lin, J.J. & Dickinson, D. B. (1984): Ability of pollen to germinate prior to anthesis and affect of desiccation on germination. Plant Physiol., 74: 746–48.

Linskens, H.F. & Esser, K.L. (1957): Über ein specifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepfroplen nach Selbstung und Fremdung. Naturwiss, 44:16.

Ma, R.-C. & Oliveira, M.M. (2001): Molecular cloning of the self-incompatibility genes S_1 and S_3 from almond (*Prunus dulcis* cv. Ferragnès). Sex. Plant Reprod., 14: 163–167.

Martin, F.W. (1959): Staining and observing pollen tubes in the style by means of fluorescence. Stain. Technol., 34: 125–128.

Matthews, P. & Dow, K.P. (1969): Incompatibility groups: sweet cherry (*Prunus avium*). In: R.L. Knight (Ed.) Abstract Bibliography of Fruit Breeding & Genetics to 1965, *Prunus*, pp. 540–544. Commonwealth Agricultural Bureaux, Farnham Royal.

Messeguer, R., Arús, P. & Carrera, M. (1987): Identification of peach cultivars with pollen izozymes. Sci. Hortic. (Amsterdam), 31: 107–117.

Mullis, K. B. & Faloona, F. A. (1987): Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction. Methods Enzymol., 155: 335–350.

Nettancourt, de D., Devreux, M., Bozzini, A., Cresti, M., Pacini, E. & Sarfatti, G. (1973): Ultrastructural aspects of the self-incompatibility mechanism in *Lycopersicon peruvianum* Mill. J. Cell Sci., 12: 403–419.

Newbigin, E., Anderson, M. & Clarke, A.E. (1993): Gametophytic self-incompatibility systems. Plant Cell, 5: 1315–1324.

Nyéki, J. (1996): Pollination and fertilization. In: Nyéki, J., Soltész, M., (Eds.): Floral Biology of Temperate Zone Fruit Trees and Small Fruits. Akadémiai Kiadó, Budapest.

Nyéki, J. & Szabó, Z. (1995): Cross-incompatibility in stone fruits. Horticultural Science, 28: 23–31.

Nyujtó, F., Brózik Jr., S., Brózik, S. & Nyéki, J. (1985): Fruit set in apricot varieties. Acta Agron. Acad. Sci. Hung., 34: 65–72.

O'Farrell, P.H. (1975): High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem., 250: 4007–4021.

O'Farrell, P.Z., Goodman, H.M. & O'Farrell, P.H. (1977): High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell, 12: 1133–1141.

Ortega, E. & Dicenta, F. (2003): Inheritance of self-compatibility in almond: breeding strategies to assure self-compatibility in the progeny. Theor. Appl. Genet., 106: 904–911.

Ortega, E. & Dicenta, F. (2004): Suitability of four different methods to identify self-compatible seedlings in an almond breeding programme. J. Hortic. Sci. Biotech., 79: 747–753.

Ortega, E., Egea, J., Cánovas, J.A. & Dicenta, F. (2002): Pollen tube dynamics following half- and fully-compatible pollinations in self-compatible almond cultivars. Sex. Plant Reprod., 15: 47–51.

Pedryc, A., Halász, J., Hermán, R. & Hegedűs, A. (2005): Genotyping Hungarian apricot cultivars for self-(in)compatibility by isoelectric focusing and PCR analysis. International Journal of Horticultal Science, 11: 69–72.

Richman, A.D., Kao, T-h., Schaeffer, S.W. & Uyenoyama, M.K. (1995): *S*-allele sequence diversity in natural populations of *Solanum carolinense* (Horsenettle). Heredity, 75: 405–415.

Richman, A.D. & Kohn, J.R. (1996): Learning from rejection: the evolutionary biology of single-locus incompatibility. Trend. Ecol. Evol., 11: 497–502.

Sanzol, J. & Herrero, M. (2002): Identification of self-incompatibility alleles in pear cultivars (*Pyrus communis* L.). Euphytica, 128: 325–331.

Sapir, G., Stern, R.A., Eisikowitch, D. & Goldway, M. (2004): Cloning of four new Japanese plum *S*-alleles and determination of the compatibility between cultivars by PCR analysis. J. Hortic. Sci. Biotech., 79: 223-227.

Sassa, H., Hirano, H. & Ikehashi, H. (1992): Self-incompatibility-related RNases in styles of Japanese pear (*Pyrus serotina* Rehd.) Plant Cell Physiol., 33: 811–814.

Sassa, H., Hirano, H. & Ikehashi, H. (1993): Identification and characterization of stylar glycoproteins associated with self-incompatibility genes of Japanese pear, *Pyrus serotina* Rehd. Mol. Gen. Genet., 241: 17–25.

Sassa, H., Mase, N., Hirano, H. & Ikehashi, H. (1994): Identification of self-incompatibility-related glycoproteins in

styles of apple (*Malus* x *domestica*). Theor. Appl. Genet., 89: 201–205.

Socias i Company, R. & Alonso, J.M. (2004): Cross-incompatibility of 'Ferragnés' and 'Ferralise' and pollination efficiency for self-compatibility transmission in almond. Euphytica, 135: 333–338.

Socias i Company, R., Kester, D.E. & Bradley, M.V. (1976): Effects of temperature and genotype on pollen tube growth in some self-incompatible and self-compatible almond cultivars. J. Amer. Soc. Hort. Sci., 101: 490–493.

Sonneveld, T., Robbins, T.P., Bošković, R. & Tobutt, K. R. (2001): Cloning of six cherry sweet self-incompatibility alleles and development of allele-specific PCR detection. Theor. Appl. Genet. 102: 1046–1055.

Sonneveld, T., Tobutt, K.R. & Robbins, T.P. (2003): Allele-specific PCR detection of sweet cherry self-incompatibility (S) alleles S1 to S16 using consensus and allele-specific primers. Theor. Appl. Genet. 107: 1059–1070.

Sonneveld, T., Tobutt, K.R., Vaughan, S.P. & Robbins, T.P. (2005): Loss of pollen-*S* function in two self-compatible selections of *Prunus avium* is associated with deletion/mutation of an *S* haplotype-specific F-box gene. Plant Cell, 17: 37–51.

Stefanovits-Bányai, É., Boross, L., Bernáth, J., Kerepesi, I., Kiss, M. & Lakatos, S. (1999): Identification of plant taxons by isoelectric focusing. International Journal of Horticultural Science, 5: 65–67.

Stefanovits-Bányai, É., Lakatos, S., Hajós-Novák, M., Hajdu, E. & Balogh, I. (2002): Recent development in biochemical characterization of *Vitis vinifera* L. varieties in Hungary. International Journal of Horticultural Science, 8: 257–261.

Sutherland, B.G., Robbins, T.P. & Tobutt, K.R. (2004): Primers amplifying a range of *Prunus S*-alleles. Plant Breed., 123: 582–584.

Tamura, M., Ushijima, K., Sassa, H., Hirano, H., Tao, R., Gradziel, T.M. & Dandekar, A.M. (2000): Identification of self-incompatibility genotypes of almond by allele-specific PCR analysis. Theor. Appl. Genet., 101: 344–349.

Tao, R., Namba, A., Habu, T., Yamane, H. & Sugiura, A. (2003): S_f-RNase and its relation to self-compatibility in Japanese apricot. Acta Hort., 622: 403–409.

Tao, R., Yamane, H., Sassa, H., Mori, H., Gradziel, T.M., Dandekar, A.M. & Sugiura, A. (1997): Identification of stylar RNases associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). Plant Cell Physiol., 38: 304–311.

Tao, R., Yamane, H., Sugiura, A. Murayama, H., Sassa, H. & Mori, H. (1999): Molecular typing of *S*-alleles through identification, chracterization and cDNA cloning for *S*RNases in sweet cherry. J. Amer. Soc. Hort. Sci., 124: 224–233.

Tobutt, K.R., Bošković, R., Cerovi, R., Sonneveld, T. & Rui, D. (2004): Identification of incompatibility alleles in the tetraploid species sour cherry. Theor. Appl. Genet., 108: 775-785.

Tomimoto, Y., Nakazaki, T., Ikehashi, H., Ueno, H. & Hayashi, R. (1996): Analysis of self-incompatibility-related ribonucleases (*S*-RNases) in two species of pears, *Pyrus communis* and *Pyrus ussuriensis*. Sci. Hortic. (Amsterdam), 66: 159–167.

Tufts, W.P. & Philp, G.L. (1922): Almond pollination. California Agricultural Experimental Station Bulletin, 346: 35.

Ushijima, K., Yamane, H., Watari, A., Kakehi, E., Ikeda, K., Hauck, N.R., Iezzoni, A.F. & Tao, R. (2004): The *S* haplotype-specific F-box protein gene, *SFB*, is defective in self-compatible haplotypes of *Prunus avium* and *P. mume*. Plant J., 39: 573–586.

Van Nerum, I., Geerts, M., Van Haute, A., Keulemans, J. & Broothaerts, W. (2001): Re-examination of the self-incompatibility genotype of apple cultivars containing putative "new" *S*-alleles. Theor. Appl. Genet., 103: 584–591.

Verdoodt, L., Van Haute, A., Goderis, I.J., De Witte, K., Keulemans, J. & Broothaerts, W. (1998): Use of the multi-allelic self-incompatibility gene in apple to asses homozygocity in shoots obtained through haploid induction. Theor. Appl. Genet., 96: 294–300.

Vilanova, S., Romero, C., Llácer, G. Badenes, M.L. & Burgos, L. (2005): Identification of self-(in)-compatibility alleles in apricot by PCR and sequence analysis. Journal of the American Society for Horticultural Sciences, 130(6): 893–898.

Viti, R., Monteleone, P., Guerriero, R. (1997): Incompatibility in apricot (*Prunus armeniaca* L.): methodological considerations. J. Hortic. Sci., 72: 961–970.

Westermeier, R. (1993): Electrophoresis in practice. VCH Verlagsgesellschaft, Veinheim.

Wilson, C.M. (1971): Plant nucleases III. Polyacrylamide gel electrophoresis of corn ribonuclease isoenzymes. Plant Physiol., 48: 64–68.

Woodward, J.R., Craik, D., Dell, A., Khoo, K.H., Munroe, S.L.A., Clarke, A.E. & Bacic, A. (1992): Structural analysis of the *N*-linked glycan chains from a stylar glyco-protein associated with expression of self-incompatibility in *Nicotiana alata*. Glycobiology, 2: 241–250.

Wu, H.M., Wang, H. & Cheung, A.Y. (1995): A pollen tube growth stimulatory glycoprotein is deglycosilated by pollen tubes and displays a glycosilation gradient in the flower. Cell, 82: 393–403.

Yamane, H., Ikeda, K., Hauck, N.R., Iezzoni, A.F. & Tao, R. (2003a): Self-incompatibility (*S*) locus region of the mutated S_6-haplotype of sour cherry (*Prunus cerasus*) contains a functional pollen *S* allele and a non-functional pistil *S* allele. J. Exp. Bot., 54: 2431–2437.

Yamane, H., Tao, R., Mori, H. & Sugiura, A. (2003b): Identification of a non-*S* RNase, a possible ancestral form of *S*-RNases, in *Prunus*. Mol. Gen. Genomics, 269: 90–100.

Yamane, H., Ushijima, K., Sassa, H. & Tao, R. (2003c): The use of *S* haplotype-specific F-box protein gene, SFB, as a molecular marker for *S*-haplotypes and self-compatibility in Japanese apricot (*Prunus mume*). Theor. Appl. Genet., 107: 1357–1361.

Zhang, S.-L. & Hiratsuka, S. (1999): Variations in *S*-protein levels in styles of Japanese pears and the expression of self-incompatibility. J. Japan. Soc. Hort. Sci., 68: 911–918.

Zhu, M., Zhang, X., Zhang, K., Jiang, L. & Zhang, L. (2004): Development of a simple molecular marker specific for detecting the self-compatible S_4' haplotype in sweet cherry (*Prunus avium* L.). Plant Mol. Biol. Rep., 22: 387–398.