Theor Appl Genet (1994) 89:201–205

H. Sassa · N. Mase · H. Hirano · H. Ikehashi

Identification of self-incompatibility-related glycoproteins in styles of apple (*Malus* × *domestica*)

Received: 9 October 1993 / Accepted: 10 November 1993

Abstract In this study, stylar proteins of apple (*Malus* × *domestica*) which correlate with known intervarietal incompatibility relationships and have similar characteristics to the *S*-glycoproteins of Japanese pear (*Pyrus serotina*) were surveyed by two-dimensional gel electrophoresis (2D-PAGE). Varietal differences were detected in a group of glycoproteins having Mrs and pIs similar to those of the *S*-glycoproteins of Japanese pear. 2D-PAGE profiles of these glycoproteins were correlated with intervarietal incompatibility relationships. These glycoproteins reacted with antiserum raised against the S^4-glycoprotein of Japanese pear, a result suggesting that they may be the products of *S*-alleles in styles of apple. On the basis of the profiles of the putative *S*-glycoproteins, *S*-genotypes were proposed for each of the apple cultivars examined.

Key words Self-incompatibility · Apple · *Malus* × *domestica* · Two-dimensional gel electrophoresis · *S*-glycoprotein

Introduction

Self-incompatibility is one of the systems to be used by plants which prevent self-fertilization and promote outcrossing. In many cases, self-incompatibility is controlled by a series of codominant alleles at a single locus, the *S* locus (de Nettancourt 1977). In fruit trees with self-incompatibility, the determination of self-incompatibility genotypes (*S*-genotypes) is important for choosing donors of compatible pollen, which are necessary for stable fruit production. However, the determination of *S*-genotypes is more difficult in woody plants than in annual plants because it is time-consuming to conduct genetic analyses. Cultivated apple (*Malus* × *domestica*) exhibits gametophytic self-incompatibility as do other Rosaceae species such as the Japanese pear (*Pyrus serotina*) (Brown 1992). *S*-genotypes have been determined for cultivars of Japanese pear (Terami 1946) but not for major cultivars of apple despite its importance even though some combinations of apple cultivars are known to show cross-incompatibility (Latimer 1937; Yamada et al. 1971; Ishiyama et al. 1993). Manganaris and Alston (1987) reported a marked deficit or absence of the *Got-1* locus in certain genotypes in all backcrosses and in crosses between unrelated apple varieties. They attributed this segregation distortion to the linkage of the *Got-1* locus with the *S*-locus.

S-allele-associated glycoproteins (*S*-glycoproteins) have been identified in the pistils of Solanaceae plants and *Papaver* with gametophytic self-incompatibility and in *Brassica* species with a sporophytic system (Sims 1993). *S*-glycoproteins of Solanaceae plants have high pIs, sugar chains that react with concanavalin A and ribonuclease (RNase) activity. Recently, we reported that basic RNases (*S*-RNases) are associated with *S*-genotypes in Japanese pears (Sassa et al. 1992). The characteristics of the *S*-RNases of Japanese pear, i.e. high pIs, glycoproteins, Mrs of around 30 KDa and style-specific expression, are similar to those of the *S*-RNases (*S*-glycoproteins) of Solanaceae plants (Sassa et al. 1993). In addition, homology of amino acid sequence in the N-terminal region of *S*-glycoproteins was found between Japanese pear and Solanaceae plants. These facts suggest that similar proteins (RNases) are involved in self-incompatibility in other Rosaceae species such as apple.

The identification of *S*-allele-associated proteins for apple cultivars is difficult at present because their *S*-genotypes have not yet been determined. However, it is likely that any proteins which correlate with intervarietal incompatibility relationships in apple and which have similar characteristics to the *S*-glycoproteins of Japanese pear are involved in self-incompatibility in apple.

In the study reported here, we analyzed stylar proteins of apple by two-dimensional gel electrophoresis to identify proteins that satisfy the above criteria. Varietal

Communicated by H. F. Linskens

H. Sassa (✉) · N. Mase · H. Ikehashi
Faculty of Horticulture, Chiba University, Matsudo, Chiba 271, Japan

H. Hirano
Department of Molecular Biology, National Institute of Agrobiological Resources, Kannondai, Tsukuba, Ibaraki 305, Japan

differences in a group of glycoproteins with pIs and Mrs similar to those of the *S*-glycoproteins of Japanese pear were detected and subsequently correlated with intervarietal incompatibility relationships reported earlier (Yamada et al. 1971; Ishiyama et al. 1993). As these glycoproteins reacted with the antiserum raised against the S^4-glycoprotein of Japanese pear, they may be products of *S*-alleles in the styles of apple.

Materials and methods

Plant materials

Four diploid apple cultivars, 'Golden Delicious', 'Natsumidori', 'Fuji' and 'Jonathan', and three triploid cultivars, 'Jonagold', 'Mutsu' and 'Hokuto', were used. Triploid CVS 'Jonagold' and 'Mutsu' are assumed to be derived from the fertilization of unreduced egg cells (2n) by reduced sperm cells (n) (Chyi and Weeden 1984; Harada et al. 1993). 'Jonagold' is a F_1 hybrid of 'Golden Delicious' × 'Jonathan', while 'Hokuto' is a F_1 hybrid of 'Fuji × 'Mutsu'. 'Golden Delicious' is the female parent of 'Mutsu'.

Among these seven cultivars, five cross-combinations, all of which are triploid × diploid combinations, are known to be incompatible, i.e. 'Jonagold' (3x) × 'Golden Delicious' (2x), 'Jonagold' (3x) × 'Natsumidori' (2x), 'Mutsu' (3x) × 'Golden Delicious' (2x), 'Hokuto' (3x) × 'Fuji' (2x) and 'Hokuto' (3x) × 'Jonathan' (2x) (Yamada et al. 1971; Ishiyama et al. 1993). On the basis of these incompatibility relationships, two of the three *S*-alleles of each triploid cultivar are expected to be identical with those of the diploid cultivars, the pollens of which are incompatible to each triploid cultivar.

The flowers of 'Mutsu', 'Jonagold', 'Golden Delicious', 'Jonathan' and 'Fuji' were collected from the Experimental Farm of Chiba University. The flowers of 'Hokuto' and 'Natsumidori' were collected from the Aomori Apple Experiment Station.

Two-dimensional gel electrophoresis

The styles were dissected from flower buds at the balloon stage of development, rapidly frozen in liquid N_2 and stored at $-80°C$ until use. Proteins were extracted from acetone powder prepared from the frozen styles according to the method of Damerval et al. (1986) with lysis buffer (O' Farrell 1975). The supernatant was recovered by centrifugation and subjected to two-dimensional gel electrophoresis (2D-PAGE). Proteins were separated by nonequilibrium pH gradient electrophoresis (NEPHGE) in the first dimension followed by SDS-PAGE in the second dimension, as described previously (Sassa et al. 1993). Proteins in the gels were detected by silver staining using the Sil-Best Stain for Protein/PAGE (Nacalai tesque, Kyoto).

Detection of glycoproteins

The proteins separated by 2D-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane filter (Millipore, Bedford) according to Hirano and Watanabe (1990), reacted with peroxidase-coupled concanavalin A (Honen Oil, Tokyo) and detected by color development of the enzyme reaction as described by Kijimoto-Ochiai et al. (1985).

Immunoblot analysis

Proteins separated by 2D-PAGE were electroblotted onto a PVDF membrane. Free binding sites of the PVDF membrane were blocked with 5% (W/V) non-fat dried milk in TRIS-buffered saline (TBS) containing 20 m*M* TRIS-HCl pH 7.5 and 0.5 *M* NaCl. The blocked membrane was incubated in an antiserum solution diluted to 1:1000 with 3% (W/V) non-fat dried milk in TBS. It was then incubated in a secondary antibody solution containing peroxidase-conjugated goat IgG raised against mouse IgG and stained for enzymatic reaction with 4-chloro-1-naphthol.

Results

Crude preparations of the style extracts were separated by the same NEPHGE/SDS-PAGE procedure as that used in the analysis of the *S*-glycoproteins of Japanese pear (Sassa et al. 1993). A group of proteins with Mrs and pIs similar to those of the *S*-glycoproteins of Japanese pear showed varietal differences. These proteins were tentatively named *S*-proteins with respective superscripts starting from 'a'. 'Golden Delicious' had two *S*-proteins, S^a and S^b (Fig. 1). Two of the triploid varieties, 'Jonagold' and 'Mutsu', both of which are incompatible with the pollen of 'Golden Delicious', had the S^a- and S^b-proteins as well as an additional *S*-protein, S^c or S^d. 'Natsumidori', which is incompatible with 'Jonagold', had S^b- and S^c-proteins. 'Jonathan', which is the pollen parent of 'Jonagold', had the S^c-protein and another *S*-protein, S^e. 'Fuji' had S^c- and S^f-proteins. A triploid variety, 'Hokuto', is incompatible with the pollens of both 'Jonathan' and 'Fuji'. 'Hokuto' had the *S*-proteins S^c, S^e and S^f. As described above, *S*-protein phenotypes were consistent with the reported incompatibility relationships. The Mrs of the S^a, S^b, S^c-, S^d-, S^e- and S^f-proteins were estimated to be 30, 29, 30, 28, 30 and 27 kDa, respectively (Fig. 1).

Stylar proteins of 'Mutsu', 'Jonagold' and 'Hokuto' were separated by 2D-PAGE and examined for glycoproteins by the concanavalin A (Con A)-peroxydase method. It was demonstrated that the *S*-proteins are glycoproteins that react with Con A (Fig. 2).

The reactivity of the antiserum raised against the S^4-glycoprotein of Japanese pear (Sassa et al. 1993) to the *S*-proteins of apple was examined. Stylar proteins of 'Mutsu', 'Jonagold' and 'Hokuto' were separated by 2D-PAGE, blotted onto PVDF membranes and reacted with the antiserum raised against the S^4-glycoprotein of Japanese pear. The apple *S*-proteins were cross-reacted with the antiserum raised against the S^4-glycoprotein of Japanese pear (Fig. 3).

Discussion

To identify the self-incompatibility-related proteins of apple, stylar proteins satisfying the following two criteria were surveyed: i.e. correlation with intervarietal incompatibility and characteristics similar to those of the *S*-glycoproteins of Japanese pear. The stylar proteins of apple were separated by NEPHGE/SDS-PAGE, as in the case of our analysis of the stylar proteins of Japanese pear (Sassa et al. 1993), and varietal differences were observed in a group of proteins having Mrs and pIs similar to those of the *S*-glycoproteins of Japanese pear. The profiles of these proteins were then

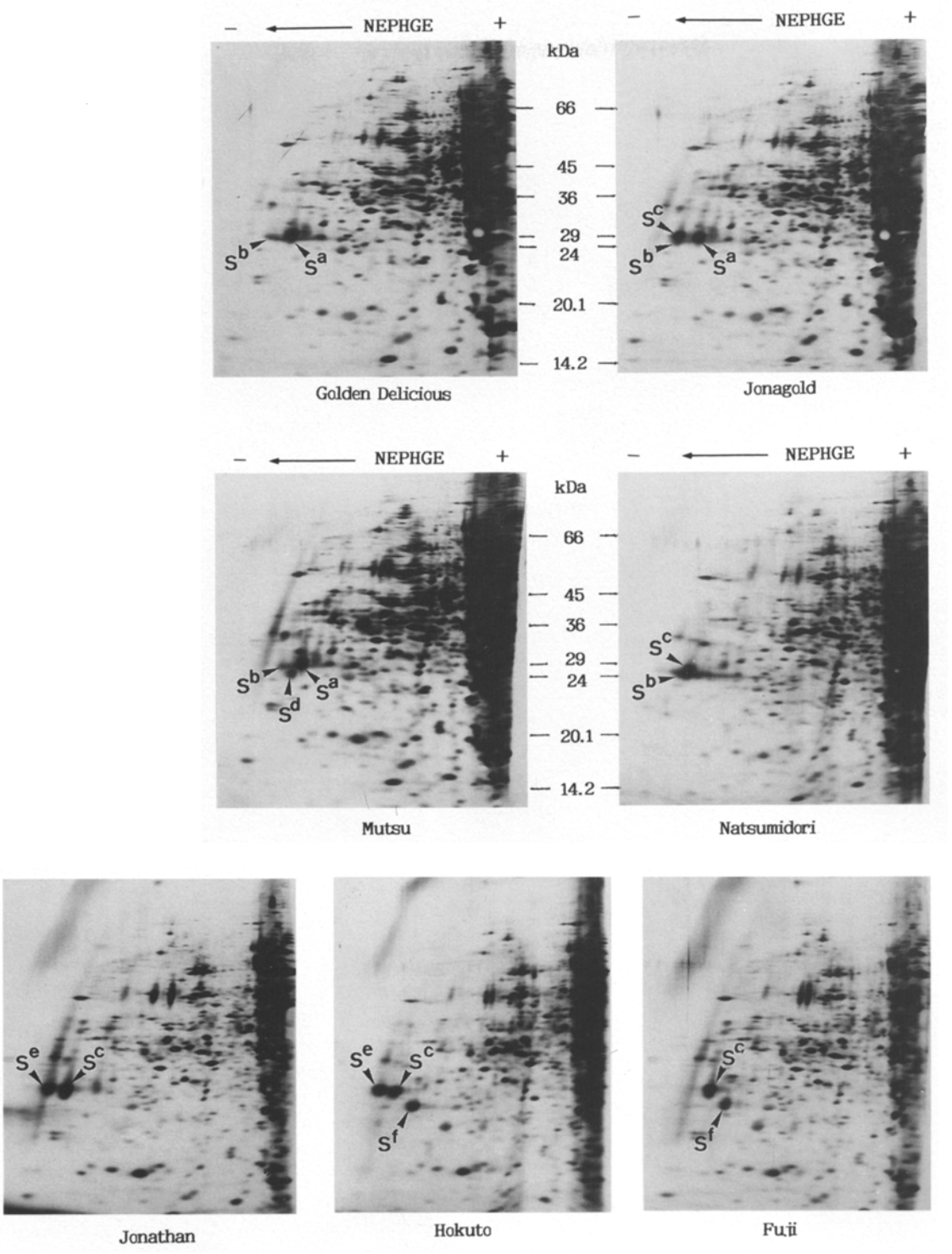

Fig. 1 Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) profiles of style extracts of apple cultivars. Proteins were detected by silver staining. *S*-proteins are marked with *arrowheads*

correlated with intervarietal incompatibility relationships. These proteins were determined to be glycoproteins by their reaction with Con A and thus tentatively labelled as *S*-glycoproteins of apple. Their estimated Mrs of approximately 30 kDa were similar to that of the *S*-glycoproteins of Japanese pear and Solanaceae species. In addition, the *S*-glycoproteins of apple were cross-reacted with antiserum raised against the S^4-glycoprotein of Japanese pear. These facts suggest that the *S*-glycoproteins of apple are proteins similar to those of Japanese pear and that they are products of *S*-alleles found in styles of apple. In Japanese pear, *S*-glycoproteins have RNase activity and characteristics similar to those of *S*-glycoproteins (*S*-RNases) of Solanaceae plants. It would be interesting to examine whether the *S*-glycoproteins of apple have RNase activ-

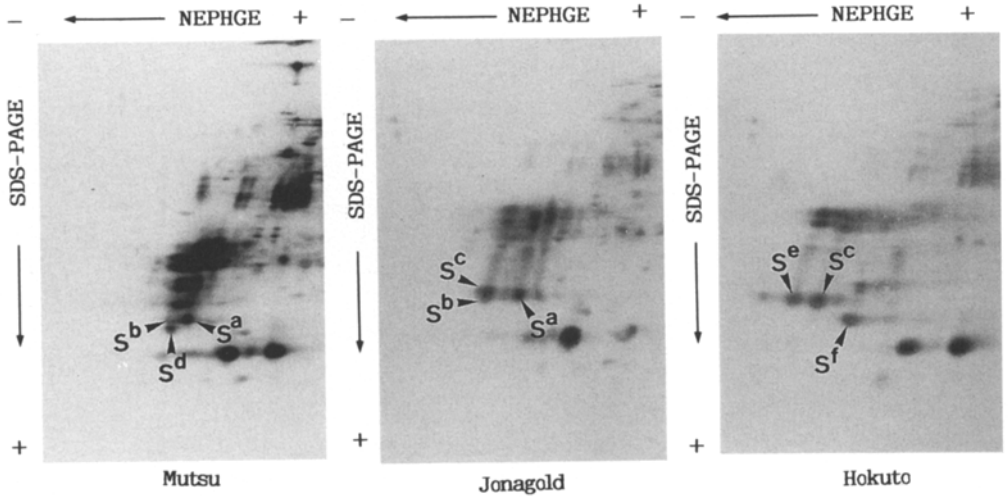

Fig. 2 Identification of glycoproteins with lectin-peroxydase method. Stylar proteins of 'Mutsu', 'Jonagold' and 'Hokuto' were separated by 2D-PAGE, transferred to PVDF membranes and reacted with concanavalin A-peroxydase reagent. *S*-glycoproteins are marked with *arrowheads*

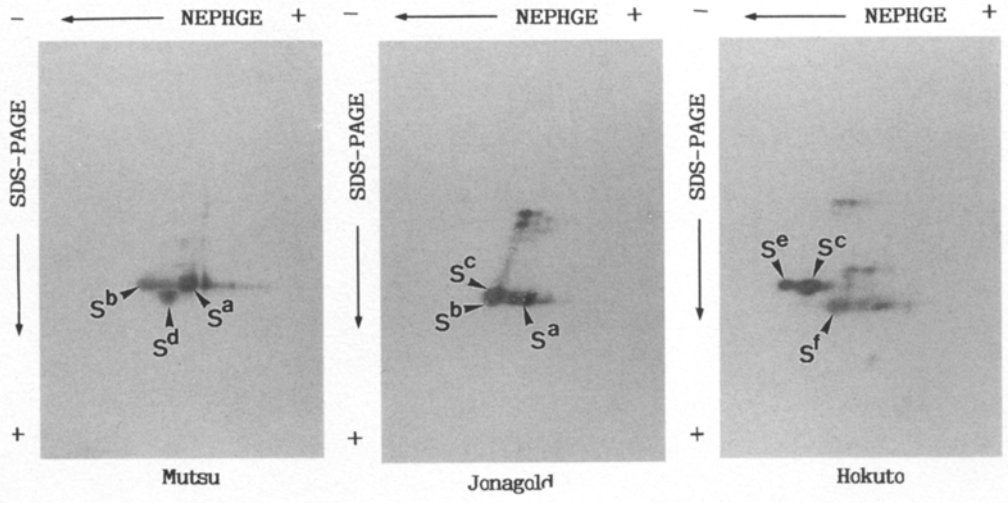

Fig. 3 Reactivity of *S*-glycoproteins of apple with antiserum raised against the S^4-glycoprotein of Japanese pear. Stylar proteins of 'Mutsu', 'Jonagold' and 'Hokuto' were separated by 2D-PAGE and reacted with the antiserum raised against the S^4-glycoprotein of Japanese pear (Sassa et al. 1993). *S*-glycoproteins are marked with *arrowheads*.

Table 1 Intervarietal incompatibility relationships reported earlier and *S*-genotypes proposed in this study in apple cultivars

Female (*S*-genotypes)	×	Male (*S*-genotypes)
Jonagold ($S^aS^bS^c$)	×	Golden Delicious (S^aS^b)
Jonagold ($S^aS^bS^c$)	×	Natsumidori (S^bS^c)
Mutsu ($S^aS^bS^d$)	×	Golden Delicious (S^aS^b)
Hokuto ($S^cS^eS^f$)	×	Fuji (S^cS^f)
Hokuto ($S^cS^eS^f$)	×	Jonathan (S^cS^e)

ity and show structural homology to that of Solanaceae plants.

Based on the profiles of the *S*-glycoproteins, we proposed putative *S*-genotypes of apple cultivars used in this study (Table 1). *S*-genotypes for other cultivars of apple may be estimated by analyzing stylar proteins as performed in this study.

Two triploid cultivars, 'Jonagold' and 'Mutsu', are considered to have received 2n gametes from a common maternal parent, 'Golden Delicious' (Chyi and Weeden 1984; Harada et al. 1993). Our data also support this conclusion. In the case of 'Hokuto', our data suggest that the maternal parent, 'Fuji', contributed the 2n gamete. The *S*-genotypes proposed by us are consistent with the parental relationships examined. The only exception is 'Mutsu', which has been reported to be the male parent of 'Hokuto'. Since the proposed *S*-genotypes of 'Hokuto' and its female parent 'Fuji' are $S^cS^eS^f$ and S^cS^f, respectively, and are consistent with both parental and incompatible relationships, the male parent of 'Hokuto' is expected to have the S^e-gene. However, the *S*-genotype of 'Mutsu' is $S^aS^bS^d$ according to the profile of the *S*-glycoproteins. Therefore, there is a possibility that another cultivar carrying S^e, such as 'Jonathan', is the true male parent of 'Hokuto'. Fingerprinting by restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) analysis may give critical information on the parental relationships of 'Hokuto' and 'Mutsu'.

Acknowledgments The authors are grateful to Prof. N. Hirata and Prof. E. Takahashi of Chiba University for their valuable advice in preparing the plant materials. We acknowledge the collaboration of Mr. T. Kudo of the Aomori Apple Experiment Station and Mr. S. Kaneko of the Experimental Farm of Chiba University in collecting the flowers of the apple varieties.

References

Brown SK (1992) Genetics of apple. In: Janick J (ed) Plant breeding reviews vol 9. John Wiley & Sons, New York, pp 333–366

Chyi YS, Weeden NF (1984) Relative isozyme band intensities permit the identification of the 2n gamete parent of triploid apple cultivars. Hort Science 19:818–819

Damerval C, de Vienne D, Zivy M, Thiellement H (1986) Technical improvements in two-dimensional electrophoresis increase the level of genetic variation detected in wheat-seedling proteins. Electrophoresis 7:52–54

de Nettancourt D (1977) Incompatibility in angiosperms. In: Frankel R, Gall GAE, Linskens HF (eds) Monographs in theoretical and applied genetics vol 3. Springer, Berlin Heidelberg New York

Harada, T, Matsukawa K, Sato T, Ishikawa R, Niizeki M, Saito K (1993) DNA-RAPDs detect genetic variation and paternity in *Malus*. Euphytica 65:87–91

Hirano H, Watanabe T (1990) Microsequencing of proteins electroblotted onto immobilizing matrices from polyacrylamide gel electrophoresis: application to an insoluble protein. Electrophoresis 11:573–580

Ishiyama M, Kitayama H, Sato T (1993) Cross-incompatibility in apple cultivars. Abstr Jpn Soc Hortic Sci Autumn Meet. p. 162 (in Japanese)

Kijimoto-Ochiai S, Katagiri YU, Ochiai H (1985) Analysis of N-linked oligosaccharide chains of glycoproteins on nitrocellulose sheets using lectin-peroxidase reagents. Anal Biochem 147: 222–229

Latimer LP (1937) Self- and cross-pollination in the McIntosh apple and some of its hybrids. Proc Am Soc Hortic Sci 34:19–21

Manganaris AG, Alston FH (1987) Inheritance and linkage relationships of glutamate oxaloacetate transaminase isoenzymes in apple. 1. The gene *Got-1*, a marker for the *S*-incompatibility locus. Theor Appl Genet 74:154–161

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

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

Sims TL (1993) Genetic regulation of self-incompatibility. Crit Rev Plant Sci 12:129–167

Terami H, Torikata H, Shimazu Y (1946) Analysis of the sterility factors existing in varieties of the Japanese pear (*Pyrus serotina* Rehd.). Studies Hortic Inst Kyoto Imp Univ 3:267–271 (in Japanese)

Yamada M, Suzuki C, Ishiyama M, Sato T, Nakamura Y, Ishizawa K (1971) Fruit set by cross- or self-pollination in apple. Tohoku Agric Res 12:282–285 (in Japanese)