Theor Appl Genet (1999) 98: 541–550

V. Ruffio-Châble · J. P. Le Saint · T. Gaude

Distribution of *S*-haplotypes and relationship with self-incompatibility in *Brassica oleracea*. 2. In varieties of broccoli and romanesco

Received: 27 July 1998 / Accepted: 5 August 1998

Abstract The self-incompatibility (SI) character in *Brassica* is controlled by the *S* locus which contains several genes. One of them, the *SLG* (*S* Locus Glycoprotein) gene encodes a soluble glycoprotein expressed in the stigma. We used antibodies directed against SLGs and a combination of isoelectric focusing (IEF) and immunoblotting methods to identify *S* haplotypes, the allelic forms of the *S* locus, in commercial and open-pollinated varieties of broccoli and romanesco. We found 23 class-I and three class-II *S* haplotypes among the 199 plants analysed. Nevertheless, for a few plants, SLGs were not detected by the antibodies and these plants, designated Hw for "white pattern" haplotypes, were apparently homozygous at the *S* locus. Diallel crosses between Hw plants revealed the existence of four different Hw haplotypes. Several hypotheses are discussed to explain the non-recognition of the SLG products in these Hw haplotypes. The data of the present study were compared with those obtained in a previous investigation carried out on cauliflower. As in cauliflower, we observed a high frequency of the sx haplotype and a great variability in the strength of the SI phenotype for sx plants (in the homozygous or heterozygous state). For both broccoli and romanesco, about 50% of the plants presented a SI phenotype strong enough to be exploited for hybrid production.

Key words Self-incompatibility · *S*-haplotype · *S*-Locus glycoprotein · Immunochemical analysis · *Brassica oleracea*

Communicated by H. F. Linskens

V. Ruffio-Chable (✉)
INRA, Station d'Amélioration des Plantes, BP 29,
35653 Le Rheu Cedex, France
Fax: +33 299 28 5120
E-mail: vruffio@rennes.inra.fr

J. P. Le Saint
INRA, Laboratoire d'Amélioration des Plantes Maraichères,
Kerdévez, 29250 Plougoulm, France

T. Gaude
Reproduction et Développement des Plantes,
UMR 9938 CNRS-INRA-ENSL, Ecole Normale Supérieure
de Lyon, 46 Allée d'Italie, 69364 Lyon Cedex 07, France

Introduction

The self-incompatibility mechanism prevents self-fertilization and promotes outcrossing, i.e. a plant will reject its own pollen or pollen from other plants carrying the same *S* haplotype. In *Brassica* species, self-incompatibility (SI) is thought to be controlled by a single locus, the *S*-locus. The SI system has been described as being under sporophytic control, the pollen behaviour being determined by the diploid genotype of the mother plant. Several genes have been described at the *S* locus. Two of them are better known and are probably involved in the SI reaction: the *S* Locus Glycoprotein (*SLG*) gene which encodes a glycoprotein secreted in the stigma papillae (Nasrallah et al. 1985), and the *S* Locus Receptor Kinase (*SRK*) gene which encodes a transmembrane protein (Stein et al. 1991; Nasrallah et al. 1994; Giranton et al. 1995). A third *S* gene, the *S* Locus Anther (*SLA*) has been detected for the S2 haplotype of *Brassica oleracea* (Boyes and Nasrallah 1995), but does not seem to be required for the SI response (Pastuglia et al. 1997). Several other *S* family sequences have been found in the genome, not linked to the *S*-locus: three *SLR* (*S*-Locus Related) genes have been described, *SLR1* (Trick and Flavell 1989), *SLR2* (Boyes et al. 1991) and *SLR3* (Cock et al. 1995). Their role has not been established. They are probably not involved in the SI reaction and have shown little polymorphism. *SLG* and *SRK* genes belong to a gene complex participating in the recognition reaction. The allelic forms are henceforth designated as *S*-haplotypes

and are classified as either class I or class II (Nasrallah et al. 1991) according to sequence similarity, immunoreaction and heredity (dominance/recessivity) considerations.

Self-incompatibility has long been exploited to produce F_1 hybrids in cole crops. Pearson (1932) was the first author to propose the use of self-incompatibility to multiply F_1 hybrids of broccoli before the genetic control of this biological phenomenon was understood. Nowadays, most cole crop varieties are F_1 hybrids. Several hybridization systems (cytoplasmic male sterility, nuclear male sterility and self-incompatibility) are now available for *Brassica* crops; the advantages and the limits of each of them can be discussed in terms of their reliability, the ease of their introduction into inbred lines, and the propagation of the female lines. Self-incompatibility, because of its presence in all cole crops with various degrees of expression, remains of interest in many cases. Even if it is not used as a hybridization system, breeders always need to know about the self-incompatibility among their breeding material in order to perform self-pollination and back-crosses, and for the control of cross compatibility between the inbred lines of a breeding programme.

The cauliflowers, the broccoli and the romanesco are all flower-heading forms of *B. oleracea*. However, two groups are to be distinguished based on the relative ontology of the curd, i.e. the floral bud differentiation at marketable maturity. The curd of cauliflower and romanesco is formed by the proliferation of meritems, 90% of which abort, whereas broccoli curd surface is constituted by the floral bud (Gray 1989; Malatesta and Davey 1997). Differentiation has probably been progressive between each type. Ancient authors referred to sprouting forms of cabbage; the first distinction between heading and sprouting was made in the 12th century. The question as to when and how-often cauliflower and broccolis diverged into separate crops remains open (Crisp 1982).

An *S* haplotype survey was previously performed among cauliflower inbred lines belonging to a breeding programme started 25 years ago (Ruffio-Châble et al. 1997). Broccoli and romanesco have been recently introduced in Brittany and are the subject of a new breeding programme, with the aim of producing varieties adapted to prevailing agronomic conditions.

In the present study, we have investigated the variability for the SI phenotypes in order to create lines homozygous at the *S*-locus with the strongest form of SI reaction. We have also tried to preserve the greatest variability at the *S*-locus among lines in order to ensure intercompatibility between lines as frequently as possible.

As with cauliflower (Ruffio-Châble et al. 1997), *S*-haplotypes were identified on stigma extracts after isoelectric focalisation (IEF) and immunostaining using two anti-SLG antibodies. Seed set and pollen-tube counts were both used to estimate the self-incompatibility reaction. The *S*-haplotype distribution will be discussed in the light of the previous investigation of cauliflower and other groups of *B. oleracea* studied in the laboratory. The particular case of the *S*-haplotype sx, also described in cauliflower (Ruffio-Châble et al. 1997; Ruffio-Châble 1998), is emphasized because of its frequency and variability for the level of self-incompatibility encountered in plants bearing this haplotype. We also show that two antibodies (anti-class I and anti-class II) are not sufficient to detect all the *S*-haplotypes belonging to broccoli and romanesco.

Materials and methods

Plant material

The *S* haplotype survey was carried out on 23 cultivars of broccoli (*B. oleracea* L. var. *italica* Plenck) and ten of romanesco (*B. oleracea* L var. *botrytis* L), (Table 1 a, b). For broccoli, two groups were analysed: (1) the green-heading form, also named calabrese, and (2) the purple-heading form. The *S*-haplotypes and the level of self-incompatibility were determined for 2–12 plants per cultivar. Thus 119 plants of broccoli and 80 plants of romanesco have been studied. The cultivars were chosen to represent a wide range of variability available to the breeder: commercial cultivars as well as open-pollinated varieties from the Gene Bank of Wellesbourne (UK) were analysed for broccoli, but only open-pollinated varieties from the Gene Bank of Wellesbourne for romanesco. The broccolis were sown in January and cultivated in insect-proof polythene tunnels. Flowering occurred between May and August. The romanescos were sown in November in a greenhouse and flowered from July to October. The fertility of broccoli and romanesco plants was estimated by crossing with SC-haplotype cauliflower plants to ensure cross compatibility.

Phenotype determination

Phenotype determination was performed as previously described for cauliflower (Ruffio-Châble et al. 1997) by both pollen-tube counts and seed set. Briefly, pollen-tube counts were repeated twice on three flowers, and seed set was evaluated on six flowers repeated three times. Fertility was controlled by cross pollinations with cauliflower having the SC-haplotype to ensure cross-compatibility between the studied plants and the testers (Ruffio-Châble et al. 1997).

Protein extraction and immunodetection

As previously described for *B. oleracea* (Gaude et al. 1993, 1995), stigma proteins were extracted, separated on isoelectric-focusing (IEF) polyacrylamide gels, and electrotransferred onto nitrocellulose membranes. Class-I and class-II haplotypes were immunodetected by using two types of antibody: a polyclonal anti-class-I SLG antibody and a monoclonal antibody specific for class-II SLG, both of which were previously described (Gaude et al. 1993, 1995). SLR1 proteins were detected by an anti-SLR1 antibody, already described for the *S*-haplotype survey in cauliflower (Ruffio-Châble et al. 1997). SDS-PAGE electrophoresis was performed according to Gaude et al. (1991).

Results

S-haplotype survey

Of the 199 plant extracts analysed, immunoblot analysis revealed 26 patterns. According to the antibody specificities, 23 haplotypes belong to class I, only three to class II. Among class-I haplotypes, six have already been detected in cauliflower (SA, SD, SE, SF, SG, SH) whereas the other 17 were previously unknown (H1–H17). The designation of the haplotype (Hn) was chosen to avoid confusion with the *S*-haplotype collection of Ockendon (1975). Determining the correspondence between both lists of names is currently in

Table 1a Origin of the broccoli plants tested

Cultivar code	Designation	Description[a]	Origin (company or country)
Hybrids from private companies			
V01			Vilmorin
V02			Vilmorin
SHO	Shogun		Northrup King
SKI	Skiff		Royal Sluis
FUT	Futura		Asgrow
GEM	Gem		Asgrow
Open-pollinated varieties from private companies			
I01	Ramoso calabrese tardivo		Scaravatti
I02	Ramoso calabrese		Scaravatti
ML	Medium late		Asgrow
RG	Royal green		Hung Nong
SCO	Scorpio		Asgrow
TOR	Toro		Asgrow
Open-pollinated varieties from the gene bank of Wellesbourne			
W05	Maialora	Broccoli	Italy
W08	Broccoli precocissima	Calabrese	Italy
W12	Broccolo di minestra spicata	Calabrese	Italy
W14	Broccolo innorota/gennorota	Calabrese	Italy
W19	Cavolfiore di sicilia precoce	Purple heading broccoli	Italy (Sicily)
W21	Cavolo broccoli di Lavagna	Black broccoli	Italy
W23	Cavolo broccoli agostina	Broccoli	Italy
W28	Couve broculo roxo	Portuguese broccoli[b]	Portugal
W31	Frivalora	Broccoli	Italy
W32	Green heading brocolo	Broccoli	Great Britain
W40	Ramoso calabria tardivo	Broccoli	Italy

[a] Description registered at the Gene Bank of Wellesbourne
[b] Was classified as purple-heading broccoli (Gray 1989)

Table 1b Origin of the romanesco plants tested

Cultivar code	Designation (gene bank HRI Wellesbourne)
C19	Cavolbroccolo Romanesco
C21	Cavolo broccolo Romanesco
C25	Cavolo broccolo Romanesco Precoce
C26	Cavolo broccolo Verde Romanesco
C27	Cavolo broccolo Verde Romanesco Medio Precoce
C28	Cavolo broccolo Verde Romanesco Type
C29	Romanesco
C34	Romanesco Natalino
C36	Romanesco San Guiseppe
C37	Romanesco Tardivo

progress. Class II was represented by only three haplotypes (sx, sy and sz). Figures 1 a and b show the specific patterns observed in broccoli and romanesco. They covered a large range of pI, from 4.8 to 9.3. Common bands not associated with the *S*-locus were detected by the anticlass-I antibody as was observed in cauliflower extracts. The band detected at pI = 8.4 had no known specificity. The anti-SLR1 antibody identified three allelic forms of *SLR1* (Fig. 1c). Several bands identified each allele (*SLR1b*, *SLR1c*, *SLR1d*). The main bands for each of them were respectively at pI 7.9, 6.8 and 7.0.

The distributions among the groups are indicated in Fig. 1 d. The romanesco, the green and the purple forms of broccoli presented respectively 9, 13 and 10 *S*-haplotypes of class-I. For the class-II S-haplotypes, sx and sy were present in all the groups but sz was found only in the green forms of broccoli. Of the 199 plants, a majority (51%) were apparently homozygous at the *S*-locus. We have examined the homozygosity state of the S-locus by producing progenies by selfing or crossing with a known homozygous plant. Among the 37 progenies analysed, and chosen to be representative of the material, 13 were homozygous, the others were heterozygous as indicated by the polymorphism shown from selfing (Fig. 2a) or from crossing (Fig. 2b). These plant extracts could not be recognized by either antibody. They were identified as Hw (for "white pattern"). Moreover, about 10% among all the tested plants presented the "white pattern", 13/119 for broccoli and 7/80 for romanesco.

Table 2 a and b compiles the data of the immunoblotting and the progeny analysis. The hybrid (V01) presented two *S*-haplotypes, as was expected for a F_1 hybrid. If we consider the distribution among the other hybrids and open pollinated varieties from the private companies, there is no major difference: they show two or three *S*-haplotypes. Four of the varieties of the Gene

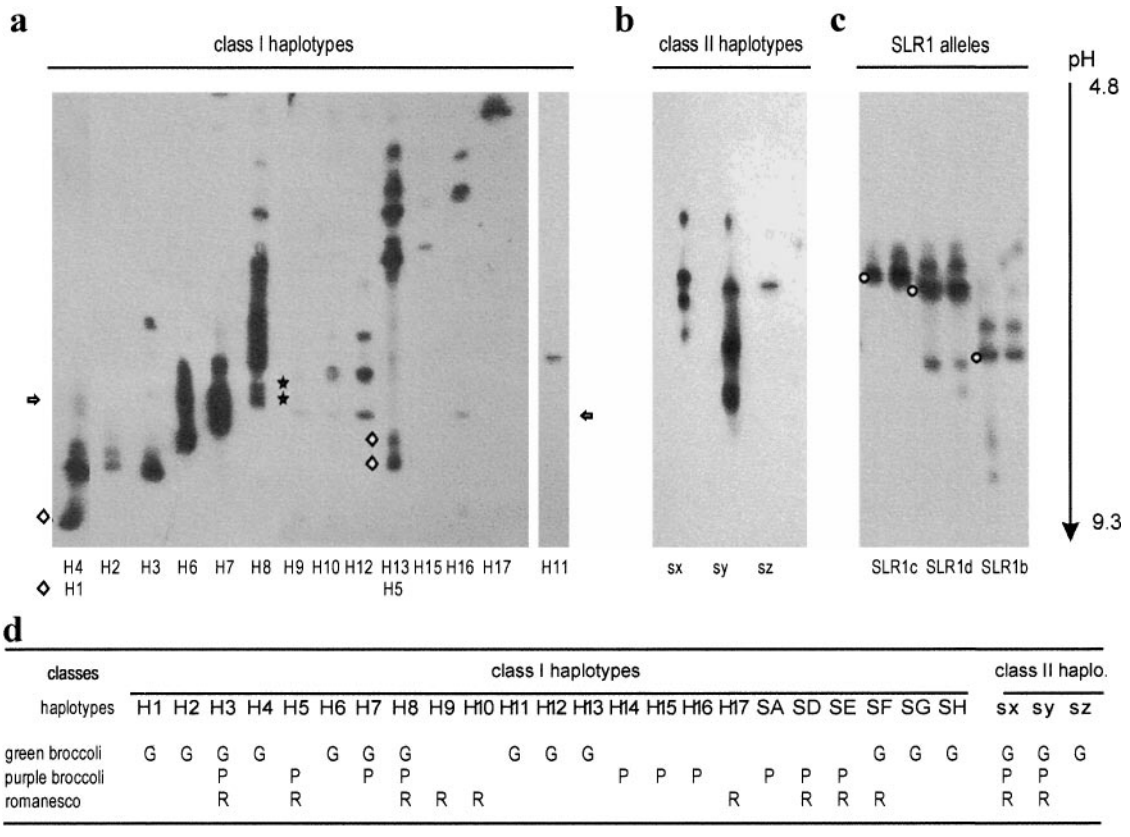

Fig. 1a–d IEF immunodetection patterns of *S*-haplotypes identified in broccoli and romanesco. Each sample corresponds to about 5 μg of stigma proteins extracted from one plant. Proteins were separated on a 3.5–9.5 pH-gradient IEF gel and electrotransferred onto nitrocellulose for immunodetection. The blot was successively treated by anticlass-I antibody (**a**), anticlass II antibody (**b**) and anti-SLR1 antibody (**c**). The class-I haplotypes already identified in cauliflower are not illustrated (Ruffio-Châble et al. 1997), but are shown in part (**d**), where the distribution between green-heading broccoli (*G*), purple broccoli (*P*) and romanesco (*R*) is given. **a** Two patterns were found only in the heterozygous state and the specific bands of one of both haplotypes are indicated by a "◇". The H9 haplotype was very faintly marked and was difficult to illustrate, the bands are indicated by a star "★". The level where the non-specific band could be detected by the anti-class I antibody is indicated by a "⇒". **c** Each SLR1 allele is represented by two lanes, the main band is indicated by "○"

Bank of Wellesbourne (W05, W08, W12, W31) had the same varietal structure. These varieties looked like hybrids of non-fixed lines (one line at least was heterozygous at the *S*-locus) and were sometimes mixed with selfed parental lines. The purple-heading forms and the romanesco varieties were very variable, as might be expected in open-pollinated varieties. One variety (W32) from the Gene Bank appeared homozygous for the sx haplotype. We also found that all its sxsx plants were self-compatible, so W32 could be a self-fertile line variety.

Table 2 shows the high frequency of the sx-haplotype which as in cauliflower, was the most frequent. Among the populations of the Gene Bank of Wellesbourne, sx was detected in 9/11 of the broccoli varieties and in 9/10 of the romanesco varieties. Among individuals of broccoli and romanesco, this haplotype represented 37% and 46% of the plants respectively, in either a homozygous or heterozygous state.

To determine whether the Hw haplotype corresponded to a single haplotype or existed under different allelic forms, we cross-pollinated several plants of the Hw phenotype. Table 3 shows the results of the cross pollinations according to a diallel scheme. Four groups can be identified which we numbered Hw1–Hw4. One of them, Hw3, was found both in broccoli and romanesco. The case of the hybrid 'Shogun' was interesting in that a study of its progeny revealed that two S-haplotypes, Hw1 and Hw3, were actually present in the parental plant.

In an attempt to go further with the identification of the SLGs of the Hw haplotypes, we undertook an SDS-PAGE analysis of total stigma proteins, followed by an immunodetection of blots with the anti-class I and anti-class II antibodies. Such an alternative approach has been shown to facilitate identification of the *S* locus and *S* locus-related proteins in *Brassica* (Gaude et al. 1993; Giranton et al. 1995). However, even with this approach no SLGs were detected (data not shown). These results suggest that either the two types of

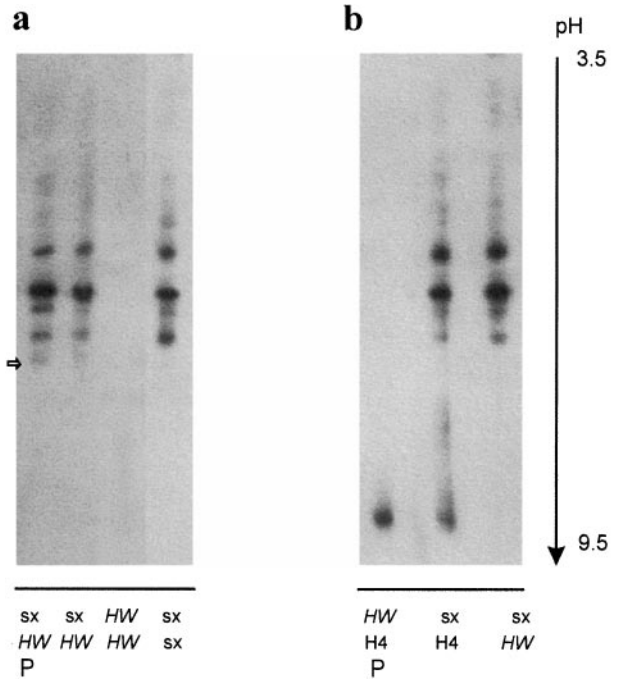

Fig. 2a, b Analysis of the progenies of heterozygous plants with Hw haplotypes. **a** The patterns observed in the self-progeny of a sxHw plant. The mother plant is shown on the left. The heterozygosity was detected by an extra band in the sx pattern, indicated by an *arrow*. In the selfed progeny, three patterns are described corresponding to the three genetic states (sxHw, HwHw and sxsx). **b** The patterns observed in a top-cross progeny. The mother plant was apparently homozygous for the H4 haplotype. In the progeny of the cross with a sx haplotype, two types of patterns were observed: H4sx and Hwsx. In this Hw haplotype, the heterozygous Hwsx was not characterised by an extra band in its pattern

antibodies we used were not capable of recognizing the N-terminus of SLG-Hw proteins, or else that no SLG or only very minute amounts of SLGs were present in the stigmas of Hw plants.

Variability for the level of the self-incompatibility phenotype

The *S*-haplotype survey accounted for the variability of one aspect of the self-incompatibility phenotype: the system of recognition between the stigma and the pollen. We will now consider a second aspect of the phenotype: the level of self-incompatibility, i.e. the strength of the rejection of the pollen grain on the stigma. The results of two methods, pollen-tube counting and seed-set observation, are relevant. The analysis was undertaken with plants for which the fertility controls ensured neither a female nor a male deficiency: 183 out of 199 plants were thus considered in the analysis. Table 4 shows the distribution of the level of self-incompatibility according to both techniques. Four groups of phenotypes could be defined, i.e. strictly self-incompatible (ABF score = 0.0, SCI = 0%), strongly self-incompatible ($0.1 < \text{ABF score} < 1.0$, $1\% < \text{SCI} < 5\%$), partially self-incompatible ($1.1 < \text{ABF score} < 4.0$, $6\% < \text{SCI} < 50\%$), and self-compatible ($4.1 < \text{ABF score} < 8.0$, $51\% < \text{SCI} < 100\%$). By observing the pollen tubes 24 h after pollination (ABF method), 73% of the plants were strongly or strictly self-incompatible. Only 50% remained in these classes according to the SCI values, which reflected the expression of the duration of the rejection phenomenon during the life of the flower. Among 81 plants classified as strictly self-incompatible by the ABF method, only 31 (38%) remained in this category according to their SCI values. The same observation can be made for the strongly self-incompatible plants, 18 out of 53 (34%) remained strongly self-incompatible. The evolution of the self-incompatible barrier during the life of the flower justifies the distribution of the plants above the diagonal in the table: the number of pollen tubes has been calculated 24 h after pollination and so could increase after this time in cases of partial self-incompatibility. Nevertheless, the presence of plants under this diagonal was not expected. The detection of pollen tubes in the style should be associated with the production of seeds, particularly when all the control crosses confirmed the good fertility of the flowers. The unreliability of the observations or/and the non-strict correspondence between ABF classes and SCI classes could explain the observed cases of partially self-incompatible plants and plants self-compatible by the ABF method, which were finally revealed to be strongly self-incompatible and partially self-incompatible respectively, by the SCI evaluation. However, the occurrence of a plant with numerous pollen tubes observed in the style 24 h after pollination but with no seeds harvested on self-pollinated siliquae (where the fertilily controls are good) is more difficult to understand under the hypothesis of a sporophytic control of self-incompatibility.

The analysis of the level of self-incompatibility underlines the particular behaviour of plants with an sx-haplotype (in the homozygous or heterozygous state). They were equally represented in the four groups of phenotype identified by the ABF method (Fig. 3). With other haplotypes, plants were mainly strictly or strongly self-incompatible.

Discussion

S-haplotype distribution

Two studies have previously estimated the variability of the *S*-haplotype in the 'italica' group: one on Cape broccoli (Ockendon 1980) and another on the green-heading form of broccoli (Voss Stern et al. 1982).

Table 2a *S*-haplotypes of individual broccoli plants for the different cultivars studied. Hw indicates a pattern without any immunostained bands. When self or cross progeny were observed or when both patterns were immunodetected, both *S*-haplotypes are indicated. When the locus state was not checked, only one *S*-haplotype is shown

Cultivar	Plants								
	1	2	3	4	5	6	7	8	9
Hybrids from private companies									
V01	H4 sx	H4 sx	H4 sx	H4 sx	H4 sx				
V02	H4 H1	H4 H1	H4 H1	H4 Hw	H4 SF				
SHO	Hw Hw	Hw Hw	Hw Hw	Hw Hw	Hw Hw	Hw Hw			
SKI	SF H7	SF H7							
FUT	sx[a]Hw	sx[a] Hw	sx[a]	sx[a] Hw	sx[a] Hw				
GEM	H2	Hw Hw	Hw Hw	Hw Hw	H2 Hw				
Open-pollinated varieties from private companies									
I01	sz	sz	H11 sz	sz	H2 sz				
I02	sx Hw	sx sx	Hw Hw	sx sx	sx sx				
ML	H11 Hw	sx sx	sx Hw	sx Hw	sx Hw				
RG	H4 Hw	H4 Hw	H4	H4	H7 H4				
SCO	H7 H4	H7 H4	H4 SF	H4 SF	H7 H4				
TOR	sz Hw	H11sz	H11 sz	H11 sz	sz Hw				
Open-pollinated varieties from the gene bank of Wellesbourne									
W05	sx Hw	Hw	sx[a]Hw	Hw Hw	sx Hw				
W08	H2	sx	H2 Hw	sx sx	sx sx				
W12	sx	sx	H12 H12	H13 H12	sx[a]				
W14	SG	–[b]	H7	–[b]	SG				
W19	SD	sx	SF	SD	SD H7	SE H14	SD	SA H16	Hw
W21	H8 sx	sx	H6						
W23	bl	H7 sy	sx						
W28	sx[a]	H3 H7	sy Hw	sx Hw	H13 H5	H3 sy	H7 sy	H15	H15 H3
W31	H3	sx[a] SH	sx[a] SH	sx H3	sx[a] SH	sx[a] SH			
W32	sx	sx sx	sx	sx sx	sx	sx			
W40	H2 sy	Hw	sz	sz					

[a] Bands only detected in cases of heterozygosity between one class-I S-haplotype and the sx haplotype.
[b] Not observed

Table 2b *S*-haplotypes of individual romanesco plants for the different cultivars studied

Cultivar	Plant											
	1	2	3	4	5	6	7	8	9	10	11	12
C19	H2 Hw	sx	sx	H2	Hw	H17						
C21	Hw	H5	sx	sx	H17 H17	H17	H17	SF H17	SE	sy		
C25	H5 SE	sx sx	sx H10	sx H10	sy	SD sy	H5	sx H5	sx	Hw	sx	sx SE
C26	sx	SF	sx									
C27	sx SE	H5 H5	sx[a]H10	H5	sx sy	sx[a]H10	SD	SE	SD	Hw	H5 sy	sx
C28	sx[a]H8	sx	SE H17	sx	H5 Hw	Hw	H17 H10	H17				
C29	sx	SF	sx[a]H10	sx SE	sx	sx sx	sx H2	H5	sx sy	sx sx	sx Hw	sx H10
C34	sx	Hw	sy H5	Hw	sy	sy	H2					
C36	SD	SE	sx SE	sx Hw	sx[a]H10	SD	sx	sx				
C37	SD	Hw										

[a] As in **a**

'Futura' and 'Gem' were two varieties common to our study and that of Voss Stern et al. (1982). In these varieties, the latter authors only detected S18 and S51 haplotypes which were identified by crossing onto the *S*-haplotype reference collection maintained at HRI, Wellesbourne, UK (Ockendon 1975). In 'Futura', by using our immunodetection approach, we only observed the sx haplotype. This sx haplotype seems to correspond to S15 in Ockendon's nomenclature as sx plants and S15 plants are cross-incompatible (data not shown). The heterozygosity of plants was detected by the appearance of new bands in the sx protein pattern, as previously observed in cauliflower (Ruffio-Châble et al. 1997). Here, we have reached the limit of both techniques. Firstly, S15 was known to be one of the most recessive haplotypes (Thompson and Taylor

Table 3 Results of the diallel pollination between plants for which no protein pattern was detected by anti-SLG antibodies. *S*-haplotypes were distinguished and identified as Hw1 to Hw4. They were different to the SB-haplotype previously detected in cauliflower. The results of the cross are noted: "–", mean score < 1; " + -", $1 <$ mean score < 4; " + " mean score > 4 (ABF method)

Female	Male								
	ML 06	SHO 02	GEM 04	FUT 02	W28 02	C21 01	C37 05	C19 09	Cauliflower SB
ML 06 (SP)	–	–	+	+	+	+ –	+	+	+
SHO 02 (SP)	–	–	+	+	+	+	+	+	+
GEM 04 (SP)	+	+	–		+	+	+	+	+
FUT 02 (SP)	+	+	–	–	+		+		
W28 02 (SP)	+	+	+	+	–	–	+	+	+
C21 01	+	+	+		–	–	+	+	+
C37 05	+	+	+	+	+	+	–	–	+
C19 09	+	+	+		+	+	–	–	+
Cauliflower SB	+	+	+		+	+	+	+	–
S-haplotype detected	Hw1	Hw1	Hw2	Hw2	Hw3	Hw3	Hw4	Hw4	SB

SP = 2 plants from the self-progeny were tested

Table 4 Distribution of broccoli (B) and romanesco (R) plants according to two measures of the level of self-incompatibility; relationship between both measures: pollen-tube counts and the calculation of the self-compatibility index (SCI[a])

Classes estimated by pollen-tube counts	SCI[a] classes							
	0%	1–5%	6–50%	51–100%	Not evaluated[b]	Sum of plants		
						R	B	Total
0.0	31	29	15		6	39	42	81
0.1–1.0	6	18	18	2	9	24	29	53
1.1–4.0	4	2	16	8	1	10	21	31
4.1–8.0	1		3	14		5	13	18
Romanesco	18	26	16	8	10			
Broccoli	24	23	36	16	6			
Sum of plants	42	49	52	24	16			

[a] SCI = no. of seeds after self-pollination/no. of seeds after cross pollination
[b] The fertility was too low to calculate an SCI index

1966 a, b, 1971; Ockendon 1982), so it was difficult to detect by testing against the *S*-haplotype collection. We have confirmed the recessive state for sx in both stigma and pollen in the Futura plants (data not shown), so it was really impossible to be detected by crossing. Secondly, in 'Gem' and 'Futura', besides sx we have also observed the Hw pattern in our study. Moreover, we found one Hw haplotype in common to both varieties in the diallel study between plants without SLG detected by the antibodies. Therefore, the Hw haplotype could be S18 or S51. Thus, there is no inconsistency between the study of Voss Stern and his colleagues and our results; rather the differences can be explained by the fact that the limits of both techniques have been reached. For the other published study on Cape broccoli (Ockendon 1980), a comparison was possible for the class-II *S*-haplotypes S2 and S15. By the similarity of protein pattern and cross-incompatibility, S2 is the same as sz. S2 was present in all the varieties of Cape broccoli and with a greater frequency. We have detected it in only two varieties of broccoli. In our investigation, sz = S2 is far less frequent than sx = S15, which was found in four out of seven varieties of Cape broccoli where, however, it was probably under-estimated due to its recessivity. If we consider the data of another investigation (currently in progress) using the same immunodetection approach on open-pollinated varieties of kale and cabbage of the west of France, we have found that sx was the only haplotype common to all the groups of *B. oleracea*.

The morphological type does not seem to be linked to the distribution of *S*-haplotypes in Brassica. However, some haplotypes are only found in one group; for example, the SC haplotype was only detected in summer and autumn cauliflower and was specific for their self-fertility, elsewhere SJ seems to be specific to winter cauliflower. An analysis of the genetic diversity of cauliflowers and broccolis by RAPD markers and morphological characters has shown that the populations clustered according to their geographical origins and

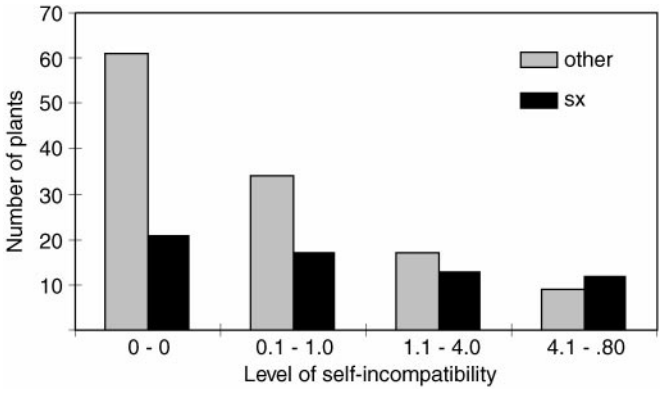

Fig. 3 The distribution of broccoli and romanesco plants according to the level of self-incompatibility measured by the number of pollen tubes 24 h after pollination. The plants of sx haplotype (in a homozygous or heterozygous state) were distinguished from the others. Self-incompatibility is expressed by classes of mean scores: mean score = 0, no pollen tube was detected (strict self-incompatibility); mean score $I \in [0.1, 1.0]$, at the most only one pollen tube was detected (strong self-incompatibility); mean score $I \in [1.1, 4.0]$, 1–15 pollen tubes were counted (partial self-incompatibility); mean score > 4.1, more than 15 pollen tubes were counted (self-compatibility)

not in terms of their agronomical group (Massie et al. 1997). Thus, in the present study, the *S*-haplotype distribution among cultivars could be better compared according to their geographical origin than to their within-crop group. Moreover, Giles (1941, in Gray 1989) described the case of broccolis and cauliflowers which were found growing in close proximity, in Italy and Sicily, with an opportunity for cross-pollination, thus enhancing the likelihood of *S*-haplotype exchange. We can extend these observations to all *Brassica* in many areas of traditional cole-crop cultivation. Considering *SLG* sequence similarity and divergence between species, the *S*-haplotype polymorphism can be considered ancient and was likely to have pre-dated speciation in the genus *Brassica* (Boyes et al. 1997; Kusaba et al. 1997; Dwyer et al. 1989) and, *a fortiori*, in the species *B. oleracea*. Nevertheless, this comparison could be of interest in order to trace the historical evolution of the crop group. For this purpose, the *SLR1* alleles are perhaps also of interest as markers. The *SLR1* gene segregates independently of the *S*-gene and is highly conserved among cruciferae species (Lalonde et al. 1989). Within each crop group studied, we have observed only two alleles per group. The exception was the Portuguese variety which presented a particular *SLR1*-allele (*SLR1d*). Geographic specificity is strengthened by this example. We confirmed the sequence conservation of the *SLR1* gene compared to the *S*-locus. It is noteworthy that broccoli and romanesco present the same *SLR1*-alleles (*SLR1b* and *SLR1c*) while cauliflower shares only one allele with the two other groups (*SLR1b*). Consequently, in terms of the *SLR1* alleles and *S*-haplotypes, romanesco and broccoli seemed closer to each other than to cauliflower. However, if we consider curd characters, romanescos belong to the cauliflower type. Thus, the differentiation of *Brassica* crops appears to be independent for these characters.

Haplotype classes

The anticlass I antibody recognised 23 haplotypes and the anticlass II antibody three haplotypes among broccoli and romanesco plants. Nevertheless, they did not react with at least four haplotypes. Consequently, we have some difficulty in classifying these four haplotypes according to the two classes. We can imagine three possibilities: (1) there could be more than two classes, (2) the available antibodies are not able to recognize all the haplotypes of each classes, or (3) these haplotypes have no SLG products. To discuss the first two hypotheses, we come back to the definition of the classes. The two classes have been defined according to the strength of the self-incompatibility reaction and their genetic behaviour relative to other haplotypes in the stigma and the pollen. The affinity for antibodies initially confirmed the distinction between two groups of *S*-haplotypes (Kandasamy et al. 1989; Nasrallah et al. 1991). The proximity of the genomic sequences in one class was then identified (Gaude et al. 1995; Kusaba et al. 1997). Antibodies were raised against one representative haplotype: anticlass I antibody was produced using the S6-haplotype by Kandasamy et al. (1989) and the S9 by Gaude et al. (1993), and anticlass II antibody using P57Sc (= S15) by Gaude et al. (1993). Recently, in a sequence analysis of 31 *S*-haplotypes in *B. oleracea* and *Brassica campestris*, the similarity between class-I SLG sequences ranged from 73% to 97.5% (in terms of the amino-acid sequence of the putative mature protein). Thus, the less-similar class-I haplotype (73%) is not much less divergent than a class-I and a class-II haplotype (64–69% similarity between class-I and class-II) (Kusaba et al. 1997). At the *S*-locus, continuity for the genetic variability is more probable, so that the affinity of the *S*-haplotype products with our anticlass-I and anticlass-II antibodies should be more-or-less strong. Nevertheless, in a third hypothesis, we cannot exclude the lack of, or else the presence of only a minute quantity of, the SLG product. One Hw haplotype belonging to the variety 'Futura' could be S18 or S51 according to previous observations (Voss Stern et al 1982). S18 was known in our laboratory to be one haplotype not detected by anti-SLG antibodies or any other staining method (ConA peroxidase, Coomassie blue, silver staining). This observation raises the question as whether any SLG product of the S18-haplotype is necessary for self-rejection of the pollen. The case of the S2 haplotype has already been pointed out in terms of the low

Table 5 Percentage of plants for each level of self-incompatibility, evaluated by the SCI index, for cauliflower, broccoli and romanesco

SCI	Level of self-incompatibility	Cauliflower[a]			Broccoli	Romanesco
		Autumn	Winter	Total		
0%	Strict	10	7	8	24	26
1–5%	Strong	12	12	12	23	38
6–50%	Partial	23	46	36	36	24
51–100%	Compatibility	55	35	44	16	12
No. of tested plants		49	74	123	99	68

[a] Ruffio-Châble et al. 1997

amount of S2-glycoprotein (Gaude et al. 1995) identified by immunodetection and immunolabelling of the stigma. Moreover, this low amount was correlated with the low expression of SLG S2-transcripts. The expression of genes located at the *S*-locus seems complex. In fact, the SLG product, the putative protein marked by immunoblotting, is not a single product of the *S*-locus at anthesis, *SRK* is simultaneously transcribed (Stein et al. 1996). Other products have also been detected: a truncated form of SRK (eSRK) for the S3-haplotype of *B. oleracea* (Giranton et al. 1995), and a membrane-anchored form of SLG for the S2-haplotype of *B. oleracea* (Tantikanjana et al. 1993). A SLG-like protein was also detected in a transgenic tobacco transformed by SRK9 (Suzuki et al. 1996). Consequently, the complexity of the products enlarges the possible candidates for pollen-stigma recognition. As yet, we cannot say which stigma product interacts with the yet-unknown pollen product.

Level of the self-incompatibility reaction and breeding application

As we discovered in cauliflower, the sx-haplotype could not be associated with only one level of the self-incompatibility reaction (Ruffio-Châble et al. 1997; Ruffio-Châble 1998). Hodgkin (1980) had previously described this large degree of variation, from self-compatibility to self-incompatibility, for S15 genotypes of Brussels sprouts. Its great frequency in all the studied crops in the laboratory and the variability of the strength of the rejection phenotype in the plant, give it a particular interest.

When we compare the three studied cole crops for their self-incompatibility expression, our conclusions confirm what was already known: namely, the generally weak level of self-incompatibility in cauliflower (Watts 1963; Nieuwhof 1974). In Table 5, for each of the cole crops studied, we have indicated the frequency of the four levels of self-incompatibility (based on the calculation of the SCI index). Autumn cauliflowers were mainly self-compatible while winter cauliflowers presented a partial self-incompatibility; for both types of cauliflower, about 20% of the plants are highly or strongly self-incompatible. For broccoli and romanesco, most of the plants showed levels of self-incompatibility strong enough (respectively 50% and 54%) to envisage the exploitation of self-incompatibility as a hybridization system.

To conclude, this study has revealed the limitation of immunostaining. *S*-haplotypes are probably more variable than we first expected. Nevertheless, we can distinguish 30 *S*-haplotypes among our heading forms of *B. oleracea*. Immunostaining remains an important technique to rapidly identify the *S* products. A molecular method of haplotype-specific PCR identification has also recently been developed. Brace et al. (1994) have demonstrated that a PCR technique can be used to identify *S*-haplotypes in *S*-homozygous plants for different genetic backgrounds in *Brassica*. This technique is less suitable for *S*-heterozygotes since both *S*-haplotypes are not amplified in the same way, indeed often only one is identified. Elsewhere, SSCP analyses (Delorme et al. 1995) or RFLP analyses (Nasrallah et al. 1985) have been used to identify all the *S*-haplotypes, but the two techniques require considerable time and means, and could not easily be used routinely in a breeding program. To-date, no single technique is able to recognise all *S*-haplotypes in both homozygous and heterozygous states. The present study with essentially practical aims, has high-lighted two fundamental questions: whether the *S*-haplotypes could be classified in two classes and whether the SLG products are necessary for the SI reaction.

Acknowledgements Thanks to Dave Astley and Angela Pinnegar for providing the material from the Gene Bank of HRI Wellesbourne (UK). VRC would like to thank Christian Dumas for the welcome in his laboratory. We also thank Anne-Marie Thierry for her technical assistance, and Bernard Le Jeune, Nadine Morvan, Nathalie Diare, Manuella Genouel, Stéphanie Demaure and other students for their help with phenotype determination. TG is a member of the Centre

National de la Recherche Scientifique. The broccoli breeding programme is supported by CERAFEL (Comité Economique Régional Agricole Fruits et Légumes) of Brittany.

References

Boyes DC, Nasrallah JB (1995) An anther-specific gene encoded by an *S* locus haplotype of *Brassica* produces complementary and differentially regulated transcripts. Plant Cell 7 : 1283–1294

Boyes DC, Chen CH, Tantikanjana T, Esch JJ, Nasrallah JB (1991) Isolation of a second *S*-Locus-Related cDNA from *Brassica oleracea*: genetic relationship between the *S* locus and two related loci. Genetics 127 : 221–228

Boyes DC, Nasrallah ME, Vrebalov J, Nasrallah JB (1997) The self-incompatibility (*S*) haplotypes of *Brassica* contain highly divergent and rearranged sequences of ancient origin. Plant Cell 9 : 237–247

Brace J, Rydes CD, Ockendon DJ (1994) Identification of *S*-alleles in *Brassica oleracea*. Euphytica 80 : 229–234

Cock JM, Stanchev B, Delorme V, Croy RRD, Dumas C (1995) *SLR3*: a modified receptor kinase gene that has been adapted to encode a putative secreted glycoprotein similar to the *S* locus glycoprotein. Mol Gen Genet 248 : 151–161

Crisp P (1982) The use of an evolutionary scheme for cauliflowers in the screening of genetic resources. Euphytica 3 : 725–734

Delorme V, Gaude T, Heizmann P, Dumas C (1995) Use of immunochemical and SSCP analyses to test homozygosity at the *S* locus of *Brassica oleracea* genotypes. Mol Breed 1 : 237–244

Dwyer KG, Chao A, Cheng B, Chen CH, Nasrallah JB (1989) The *Brassica* self-incompatibility multigene family. Genome 31 : 969–972

Gaude T, Denoroy L, Dumas C (1991) Use of a fast protein-elec trophoretic purification procedure for N-terminal sequence analysis to identify *S*-locus-related proteins in stigmas of *Brassica oleracea*. Electrophoresis 12 : 646–653

Gaude T, Friry A, Heizman P, Mariac C, Rougier M, Fobis I, Dumas C (1993) Expression of a self-incompatibility gene in a self-compatible line of *Brassica oleracea*. Plant Cell 5 : 75–86

Gaude T, Rougier M, Heizman P, Ockendon DJ, Dumas C (1995) Expression level of the *SLG* gene is not correlated with the self-incompatibility phenotype in the class-II S-haplotypes of *Brassica oleracea*. Plant Mol Biol 27 : 1003–1014

Giranton JL, Ariza MJ, Dumas C, Cock JM, Gaude T (1995) The *S* locus receptor kinase gene encodes a soluble glycoprotein corresponding to the SRK extracellular domain in *Brassica oleracea*. Plant J 8 : 827–834

Gray AR (1989) Taxonomy and evolution of broccolis and cauliflowers. Baileya 25 : 28–46

Hodgkin T (1980) The inheritance of partial self-compatibility in *Brassica oleracea* L. Inbreds homozygous for different *S*-alleles. Theor Appl Genet 58 : 101–106

Kandasamy MK, Paolillo DJ, Faraday CD, Nasrallah JB, Nasrallah ME (1989) The *S*-locus-specific glycoproteins of *Brassica* accumulate in the cell wall of developing stigma papillae. Dev Biol 134 : 462–472

Kusaba M, Nishio T, Satta Y, Hinata K, Ockendon D (1997) Striking sequence similarity in inter- and intra-specific comparisons of class-I *SLG* alleles from *Brassica oleracea* and *Brassica campestris*: implications for the evolution and recognition mechanism. Proc Natl Acad Sci USA 94 : 7673–7678

Lalonde BA, Nasrallah ME, Dwyer KG, Chen CH, Barlow B, Nasrallah JB (1989) A highly conserved *Brassica* gene with homology to the S-locus-specific glycoprotein structural gene. Plant Cell 1 : 249–258

Malatesta M, Davey J (1997) Cultivar identification within Broccoli, *Brassica oleracea* L. (*italica* group), and cauliflower, *Brassica oleracea* L. (*botrytis* group). ISHS Symposium on Brassicas. Acta Hortic 407 : 109–113

Massie IH, Astley D, King GJ (1997) Patterns of genetic diversity and relationships between regional groups and populations of Italian landrace cauliflower and broccoli. ISHS Symposium on Brassicas. Acta Hortic 407 : 45–53

Nasrallah JB, Kao TH, Goldberg ML, Nasrallah ME (1985) A cDNA clone encoding an *S*-locus specific glycoprotein from *Brassica oleracea*. Nature 318 : 263–267

Nasrallah JB, Nishio T, Nasrallah ME (1991) The self-incompatibility genes of *Brassica*: expression and their use in the genetic ablation of floral tissue. Annu Rev Plant Physiol Plant Mol Biol 42 : 393–422

Nasrallah JB, Rundle SJ, Nasrallah EM (1994) Genetic evidence for the requirement of *Brassica oleracea S*-locus receptor kinase in the self-incompatibility response. Plant J 5 : 373–384

Nieuwhof M (1974) The occurrence of self-incompatibility in cauliflower (*Brassica oleracea* var. *botrytis* L. subvar. *cauliflora* DC), and the possibility to produce uniform varieties. Euphytica 23 : 473–478

Ockendon DJ (1975) The *S*-allele collection of *Brassica oleracea*. Incompatibility Newslett 5 : 82–84

Ockendon DJ (1980) Distribution of *S*-alleles and breeding structure of Cape broccoli (*Brassica oleracea* var. '*italica*'). Theor Appl Genet 58 : 11–15

Ockendon DJ (1982) An *S*-allele survey of cabbage (*Brassica oleracea* var. *capitata*). Euphytica 31 : 325–331

Pastuglia M, Ruffio-Châble V, Delorme V, Gaude T, Dumas C, Cock JM (1997) A functional *S*-locus anther gene is not required for the self-incompatibility response in *Brassica oleracea*. Plant Cell 9 : 2065–2076

Pearson OH (1932) Incompatibility in broccoli and the production of seed under cages. Proc Am Soc Hort Sci 29 : 468–471

Ruffio-Châble V (1998) Complexity of self-incompatibility phenotype in *Brassica* and some thoughts about its genetic control. Acta Hortic 459 : 281–288

Ruffio-Châble V, Hervé Y, Dumas C, Gaude T (1997) Distribution of *S*-haplotypes and its relationship with self-incompatibility in *Brassica oleracea*. 1. In inbred lines of cauliflower (*B. oleracea* var 'botrytis'). Theor Appl Genet 94 : 338–346

Stein JC, Howlett S, Boyes DC, Nasrallah ME, Nasrallah JB (1991) Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc Natl Acad Sci USA 88 : 8816–8820

Stein JC, Dixit R, Nasrallah ME, Nasrallah JB (1996) *SRK*, the stigma-specific *S*-locus Receptor Kinase of *Brassica*, is targeted to the plasma membrane in transgenic tobacco. Plant Cell 8 : 429–445

Suzuki G, Watanabe M, Toriyama K, Isogai A, Hinata K (1996) Expression of *SLG9* and *SRK9* genes in transgenic tobacco. Plant Cell Physiol 37 : 866–869

Tantikanjana T, Nasrallah ME, Stein JC, Chen CH, Nasrallah JB (1993) An alternative transcript of the *S*-locus glycoprotein gene in a class-II pollen-recessive self-incompatibility haplotype of *Brassica oleracea* encodes a membrane–anchored protein. Plant Cell 5, 657–666

Thompson KF, Taylor JP (1966 a) Non-linear dominance relationship between *S*-alleles. Heredity 21 : 345–362

Thompson KF, Taylor JP (1966 b) The breakdown of self-incompatibility in a cultivar of *Brassica oleracea*. Heredity 21 : 637–648

Thompson KF, Taylor JP (1971) Self-incompatibility in Kale. Heredity 27 : 457–471

Trick M, Flavell RB (1989) A homozygous *S* genotype of *Brassica oleracea* expresses two *S*-like genes. Mol Gen Genet 218 : 112–117

Voss Stern GA, Ockendon DJ, Gabrielson RL, Maguire JD (1982) Self-incompatibility alleles in broccoli. Hortscience 17 : 748–749

Watts LE (1963) Investigations into the breeding system of cauliflower *Brassica oleracea* var. *botrytis* (L.). Euphytica 12 : 323–340