Mechanisms of self-incompatibility and unilateral incompatibility in diploid potato (*Solanum tuberosum* L.)

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Mechanisms of self-incompatibility and unilateral incompatibility in diploid potato (*Solanum tuberosum* L.)

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This thesis encompasses a part of the scientific research carried out on diploid potato, at the former department of Plant Breeding, Wageningen Agricultural University. An international cooperation on self-incompatibility (SI) in potato between partners in Italy (University of Siena), Germany (Max Planck Institute, Cologne) and The Netherlands (KUN, Nijmegen and WAU, Wageningen) was focused on various aspects of SI. This joint project was supported by the European Community 'Bridge Programme' (BIOT-CT-900172).

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Key words: *Solanum tuberosum*, *Solanum verrucosum*, self-incompatibility, self-compatibility, unilateral incompatibility, unilateral incongruity, *S*-glycoprotein, *S-RNase*, sense, antisense, overexpression.

Bibliographic Abstract: This thesis describes the creation and selection of diploid potato genotypes with well defined self-incompatibility (SI) reactions. The contribution of the stylar products of the incompatibility alleles, the *S*-glycoproteins, is described for both the gametophytic self-incompatibility reaction and the incomplete interspecific crossing barrier that exists between diploid potato and its self-compatible relative, *S.verrucosum*. This barrier is called unilateral incompatibility or unilateral incongruity (UI). Complex interactions between incompatibility determining genes are described.

Stellingen

1 De aanscherping van de unilaterale incongruentie-hypothese door de *S*-locus uit te sluiten van de bijdrage aan interspecifieke incompatibiliteit (Hogenboom, 1973) is onterecht. De UI hypothese heeft in deze vorm derhalve zijn tijd gehad (dit proefschrift).

2 De RNase-activiteit van *S*-glycoproteïnen speelt in het unilaterale incompatibiliteitssysteem bestaande tussen *Lycopersicon peruvianum* en *L.esculentum* een ondergeschikte rol (Rick, 1986; Kowyama et al., 1994; Royo et al., 1994; dit proefschrift).

3 Het "allele-specific dominant negative effect" van een RNase-defecte kopie van een werkzaam incompatibiliteitsallel zoals beschreven door Mc Cubbin et al (1997) is op zijn best co-dominantie.

4 Alle verwijzingen door de in dit proefschrift genoemde auteurs naar oudere literatuur m.b.t. de uitdrukking "wederzijdse afzwakking (mutual weakening)" betreffen in tegenstelling tot wat wordt gesuggereerd niet de uitdrukking maar slechts het verschijnsel.

5 De "two-power competition" hypothese van Abdalla (1970) die beschrijft hoe *S.verrucosum* en *S.tuberosum* kruisingstechnisch van elkaar gescheiden zijn, heeft een hoog antropopatisch gehalte (= het vertonen van menselijke gevoelens).

6 Cytoplasmatische Mannelijke Steriliteit zoals die optreedt in het systeem *S.verrucosum* x *S.tuberosum* (Abdalla &Hermsen, 1971b) is geen probleem voor de veredeling maar een oplossing.

7 Het gebruik van incompatibiliteit in de hybride-rassen-productie van zelfcompatibele Solanaceae is een achterhaald idee.

8 De toekenning van de soortstatus aan *S.sucrense* door Hawkes wordt ontkracht door de beschrijving van Hawkes (1989). (Hawkes &Hjertig, The potatoes of Bolivia. 1989).

9 De detectie van 9 (negen!) verschillende incompatibiliteitsallelen in een diploïde F1 van *Nicotiana glauca* (Pandey, 1981) is een sterke aanwijzing voor slaperigheid bij de referenten of hoge activiteit van specifieke paramutatie (sic). Dit pleit voor het vermelden van de referenten bij gerefereerde artikelen.

10 De mogelijkheid van xenotransplantatie zou voor het individu niveau zeer aantrekkelijk zijn, maar ongewenst voor de gemeenschap.

11 Het model zoals dat door de theoretisch natuurkunde Gerard 't Hooft werd gepresenteerd in het programma Noorderlicht ("Kosmische anarchie",VPRO, 28-12-1997, 20:05 - 20:28) impliceert niet alleen zoals hij zelf al aangaf, predestinatie, maar ook dat de geest een functie is van de materie.

12 De "Pensee" die bekend staat als "Het Godsbewijs van Pascal" bewijst alleen Pascal's opportunisme en zijn feilbaarheid in de kansrekening.

13 Slimheid verhoudt zich tot A.I.O.-schap als *S*-homozygotie tot incompatibiliteit.

Stellingen behorende bij het proefschrift "Mechanisms of self-incompatibility and unilateral incompatibility in diploid potato (*Solanum tuberosum* L)"

Ronald Eijlander
Wageningen, 14 september 1998

Voorwoord

In de jaren zestig en zeventig werd er aan het toenmalige Instituut voor Plantenveredeling van de Landbouw Hogeschool Wageningen veel onderzoek gedaan aan aardappel en aan aardappel verwante soorten, en dit gaat in feite tot op de huidige dag verder. In die tijd is er veel bijzonder materiaal ontwikkeld. Een deel van dit materiaal heeft direct of indirect zijn weg gevonden naar aardappelveredelingsbedrijven, terwijl ander materiaal verder gebruikt werd op tal van andere onderzoeksinstellingen. Nakomelingen van drie heel bijzondere aarappelplanten vonden zo hun weg naar het Max Planck Instituut te Keulen, alwaar Dr Richard Thompson en medewerkers opnieuw een aantal eigenaardigheden onder de loupe namen, maar dit keer met modernere, moleculaire technieken. De complicaties in de analyses waren aanleiding om de hulp in te roepen van zowel Prof. Dr Ir Jacobsen's onderzoeksgroep "Genetische variatie en reproductie" van de vakgroep Plantenveredeling als van de emeritus hoogleraar Prof. Dr Ir Hermsen, die nog steeds actief was op de vakgroep en bereid gevonden werd zijn kennis omtrent dit materiaal met anderen te delen. Deze samenwerking resulteerde uiteindelijk in een bij de vakgroep geplaatste positie voor een A.I.O. binnen een E.E.G. gefinancieerd internationaal project. Deze positie werd door mij ingevuld en hier heb ik dan ook de afgelopen jaren met veel plezier aan gewerkt.

Dit proefschrift is een weergave van slechts een deel van al het werk wat ik samen met vele anderen aan dit aardappelmateriaal heb verricht. Het is voor sommigen, niet in de laatste plaats voor mijzelf, misschien dan ook frustrerend te moeten zien dat zoveel werk niet in publicatievorm het daglicht zal zien. De tegenslagen en achteraf onjuist gebleken werkhypothesen hebben mij en mijn studenten dan ook een zekere "faam" opgeleverd. Ik herinner mij nog de uitspraak naar een student toe, toen een experiment wat normaal gesproken nooit mis gaat, maar nu jammerlijk de mist in ging, die luidde: " ja, wat had je anders verwacht, je werkt immers bij Ronald". Het heeft deze student er echter niet van weerhouden om ook zelf A.I.O. te worden. Maar het proefschrift is er dan toch gekomen.

Ik wil hierbij mijn promotor prof. dr ir. Evert Jacobsen bedanken. Evert, je hebt mij in de gelegenheid gesteld dit toch wel erg leuke onderzoek te verrichten. Je hebt voortdurend de grote lijnen in het oog gehouden en je hebt er voor gezorgd dat e.e.a. hopenlijk toch nog enigszins begrijpelijk op papier is gezet. Ons hemelsbrede verschil in stijl heeft er voor gezorgd dat we het nodige geduld met elkaar moesten hebben, maar we zijn er samen toch uitgekomen. Ikzelf ben in ieder geval tevreden met het uiteindelijke resultaat. Ik hoop dat jij en de lezers dat ook zijn.

Verder wil ik mijn co-promotor Dr Ramanna bedanken voor zijn, zoals dat heet, dagelijkse begeleiding. Ik heb u, zoals al vele promovendi voor mij, leren kennen als aimabel, geduldig en onbaatzuchtig. U vond altijd tijd om mijn uiteenzettingen aan te horen en vanuit uw grote kennis mee te denken. Ik heb in u niet alleen een kundige collega gevonden, maar ook een vriend.

Prof. Hermsen heb ik reeds in mijn studententijd en ook nu tijdens mijn A.I.O.-schap leren waarderen als docent en als mens. Ik heb met u vele, vele uren aan tafel doorgebracht, gebogen over papieren vol met afstammingen en genetische modellen, speculerend over wat er nu weer aan de hand kon zijn. Uw kennis van het materiaal en het verschijnsel "incompatibiliteit" zijn voor mij van onschatbare waarde geweest. Uw bijdragen aan mijn onderzoek hebben dan ook geresulteerd in een co-auteurschap, waar u mij een groot plezier mee heeft gedaan.

Dirk-Jan Huigen wil ik bedanken voor al het werk dat hij gemerkt en ongemerkt voor mij heeft verricht. Jij coördineerde, samen met de mensen van Unifarm, het werk in de kassen. Toen ik op de vakgroep kwam, had jij het voorwerk al gedaan en kon ik zo aan de slag. Je hebt me veel organisatorische taken uit handen genomen en waarschuwde wanneer er weer eens wat hooi van de overvolle vork afviel. En tot de promotie aan toe blijf je betrokken bij wat er moet gebeuren. Veertien september is waarschijnlijk de laatste dag dat je nog voor me in de weer bent. Bedankt.

Marja Schippers en Anja Posthuma, jullie hebben voor mij het nodige in-vitro werk verricht en veel werk uit handen genomen. Dit betrof een aantal transformatie-experimenten, maar de meeste tijd is denk ik toch wel gaan zitten in de instandhouding van de gigantische hoeveelheid basisklonen en transformanten. Hoewel niet alles heeft opgeleverd wat er van werd verwacht, zijn er toch leuke dingen uitgekomen. Een deel hiervan vinden jullie in dit proefschrift terug.

Voorts wil ik de mensen van TUPEA bedanken. Het lijkt erop dat ik magische vingertjes heb om PC's vast te laten lopen. Het inloggen onder mijn eigen naam was vaak al voldoende om een PC op tilt te krijgen. Een mailtje aan jullie via een niet-recalcitrante computer was meestal voldoende om jullie te doen uitrukken. Jullie hebben me altijd naar tevredenheid uit de brand geholpen.

Dan heb ik nog met een groot aantal studenten samengewerkt. Hoewel de meeste van hun werk weinig terug zullen vinden in dit proefschrift, wil dit niet zeggen dat zij niet een belangrijke bijdrage

hebben geleverd. Monique Mouwen was reeds bezig met diverse analyses toen ik op de vakgroep binnenkwam. Zij werd gevolgd door Mohammed Sohani, Bart Bronnenberg, Marlijn Vos, Jaap Kooyman, Ester Abad I Cantero en Wendy ter Laak. Het was prettig om met jullie samen te werken. Ik hoop dat dit wederzijds is. Een deel van de beloning zit in ieder geval in dit proefschrift.

Voorts zijn daar de vele collega's van de laboratoria die ik wil bedanken. Om er een paar te noemen: Luuk Suurs, Elly Jansen, Irma Straatman en Marjan Bergervoet. Ik heb jullie allemaal wel voor de voeten gelopen en met vragen en problemen lastig gevallen. Als goede collega's hebben jullie meegedacht en meegeholpen waar dat nodig was. Ik heb er veel van opgestoken.

Binnen de vakgroep heb ik met vele mensen een prettig contact gehad. Dit betreft niet alleen de mensen waarmee ik op de kamer heb gezeten of gedurende mijn schrijffase op de Terp lief en leed heb gedeeld, maar ook vele andere mensen. De lijst is lang, en om het risico van mensen vergeten te vermijden, wil ik jullie zonder verder namen te noemen hierbij bedanken voor de gezellige tijd.

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Mij kinderen weten haast niet beter dan dat werken inhoudt dat pappa of "weg" is, of op zolder achter de computer zit, of dat het ervoor heeft gezorgd dat pappa weer "heel erg moe" is. Jullie moeten me maar geloven, het is meestal heel erg leuk, tenminste als je het werk doet waar je, zoals ik, zelf voor hebt kunnen kiezen. Ik hoop dat ik er in mijn volgende baan meer voor jullie kan zijn.
En ten slotte mijn partner, Leontine. Je weet wat het is om te promoveren, maar ook bij jou zal de vertwijfeling wel eens hebben toegeslagen, al heb je dat nooit zo laten blijken. Je kwam wel eens met de vraag "moet je nu echt weer naar het lab/overwerken/achter de computer/vannacht doorwerken/humeurig zijn/etc", maar over het algemeen heb je met een bewonderenswaardige tolerantie mijn A.I.O.-trekjes geaccepteerd. Ik hoop dat ik deze trekjes nu achter me gelaten heb. Bedankt.

Contents

Chapter 1
General introduction.

Flowering plants can propagate themselves in two ways: vegetatively and sexually. Vegetative propagation leads normally to individuals that are genetically identical to the original plant. Well-known examples of vegetative propagation are the runners produced by strawberry (*Fragaria*) and the tubers produced by potato. Variation in nature on vegetative propagation is endless, various plant organs can be used to produce clonal offspring. Even seeds, normally the result of a sexual process, can produce clonal offspring: for instance, twin-embryos (polyembryony) in seeds of citrus often contain clones of the mother plant (e.g. Webber, 1948) and apomixis in blue grasses (*Poa* sp) is another good example of bypassing syngamy. Vegetative propagation is attractive when important characteristics have to be kept together, but this limits the response of the plant to changes in the environment: response depends completely on the genetic information present in the genotype dealing with.

Sexual propagation, involving fertilisation and genetic recombination, provides plant species not only another mechanism of spreading in the environment, but also the possibility to adapt to changes in the environment or to invade different environments. Self-fertilisation or hybridisation with a close relative (inbreeding) limits the variation in the offspring and thus limits adaptiveness. Due to inbreeding, accumulated fitness-negative characteristics which might be recessive, have a higher chance of becoming homozygous, thus reducing the fitness of those plants. Another effect of inbreeding, that can be advantageous, is that it also purifies species from a part of those fitness negative traits and can fix positive gene combinations. Combined with a certain amount of outcrossing, which ascertains also the adaptiveness, inbreeding proved to be a succeesful strategy for some species, and can be found in many important cultivated crops, such as wheat, barley, peas and beans, which are called self-pollinators. Inbreeding is, however, for many plant species a risky strategy to rely on.

There are many mechanisms to prevent or limit selfing. Some are based on floral morphology, others on difference of maturation time of male and female reproductive organs within individual flowers. A number of these self-fertilisation impeding mechanisms are well visible and recognisable, based on temporal or spatial separation of male and female reproductive organs. Dioecy, which means that plants carry either male or female flowers, is such a mechanism, well known from, for instance, willow (*Salix*). Separation mechanisms, such as protogyny (temporal separation, e.g. *Victoria amazonica,* anonymus), monoecy (male and female flowers on the same plant, e.g.in *Zea mais*), and hermaphrodity (flowers are male and female at the same time, e.g. potato or cabbage) do exist that are less strict then dioecy.

Self-incompatibility systems

Some of the variations on the separation mechanisms as described above are effective, but cannot always avoid high levels of self-fertilization or cross-hybridization with closely related genotypes, thus leading to inbreeding.

In many cases, undesired selfing does not lead to fertilization, though, due to specific pre-

fertilization barriers. Barriers against self-fertilisation can be found at many places, e.g., on the stigmatic surface (e.g., Cruciferea, like *Brassica oleracea*), at various places in the transmitting tract in the style (e.g., various Solanaceae, like *Petunia hybrida* and *Solanum tuberosum*), in the ovary (e.g., *Beta vulgaris*) or even in the ovule just before syngamy (*e.g., Theobroma cacao*). Mango (*Magnifera indica*) shows fertilization even after selfing, but the resulting zygotes die approximately two weeks post-fertilization (Sharma and Singh, 1970), although it can be disputed whether or not this phenomenon really belongs to incompatibility. Some of those incompatibility characteristics appear to be correlated with aspects such as pollen being bi- or trinucleate, dry or wet stigma surface, etc. These aspects are extensively reviewed and exemplified in the monograph of De Nettancourt (1977) and recently by, for instance, De Nettancourt (1997), Kao and McCubbin (1997) and Nasrallah (1997). It is known that the sporophytic incompatibility system (to be explained later) contains both diallellic and poly-allelic systems. The heteromorphic system appears to coincide with diallelic systems, which means that differences in flower morphology reflect which types are intercompatible and which are not. The homomorphic incompatibility systems (sporophytic and gametophytic) do not betray their intercrossability by their morphology. The barriers that are active in plants with dry stigmata are usually on or directly under the stigma, whereas plant species with wet stigmata usually display the barriers in the style or , less frequently, even in the ovaries. Most gametophytic SI systems display wet stigmata and bi-nucleate pollen. Dry stimata and tri-nucleate pollen are usually found in sporophytic systems.

Sporophytic Self-Incompatibility.

In the sporophytic self-incompatibility system (SSI) the genotypes of both the pollen parent and the pollen recipient (the sporophytes) determine whether a combination is compatible or not. The pollen (the gametophyte) reflects the genotype of the pollen donor but not the actual genotype of the pollen itself.

Sporophytic heteromorphic systems.

In the heteromorphic self-incompatibility systems there exists an association between incompatibility groups and floral morphology: incompatibility behaviour depends on the phenotype. This is believed to be always sporophytic (Pandey, 1970). Distylic and tristylic systems have been described for various plant species. A heterodistylic self-incompatibility system has been described, for instance, for primrose (*Primula*) or *Hypericum* . Flowers with long anthers and short styles (Pin), can only fertilize plants with long styles and short anthers (Thrum), and vice versa. Offspring will segregate in a 1: 1 ratio of Pin and Thrum. Here selfing is excluded, but full sib mating is possible in 50 % of the cases.

It has been reported for several primrose species that linkage between *S*-morphology and actual crossability can be broken or disrupted (Ernst, 1932, 1936, reviewed by De Nettancourt, 1977; Sharma and Boyes, 1961). Some of the biochemical aspects of the system have been characterised by Heslop-Harrison et al (1981) and Shivanna et al (1981).

Sporophytic homomorphic systems

Sporophytic homomorphic self-incompatibility has been described for the Cruciferae and at least five other families (Bateman, 1955; Charlesworth, 1988), but it has become clear that sporophytic systems can display characteristics that are normally found in gametophytic self-incompatibility systems and vice versa (see also review by De Nettancourt, 1997). SSI is, as mentioned before, characterised by the fact that the interaction between pollen and stigma/style depends on the dominance relationships between the *S*-alleles of both parental plants (the sporophytes), the pollen (the gametophyte) carrying the information of the pollen donor in the pollen coating (Stephenson et al., 1997). The genotype of the pollen grain itself is of no importance for the reaction in SSI (Fig. 1a), this in contrast with gametophytic self-incompatibility (GSI), where the genotypes of the style and that of the pollen grain itself determine whether a combination is compatible or not (Fig. 1b). In such a sporophytic system the fraction of compatible pollen in a population, is a function of the number of alleles present, as well as dominance relationships between *S*-alleleles in pollen and style. Polyallely results then in numerous compatible combinations, which is increased when *S*-alleles can be dominant over other *S*-alleles (e.g., *S1* over *S2* in the pollen phenotype, as in Fig. 1, most right combination).

Most of the fundamental research on the one-locus multi-allelic sporophytic self-incompatibility system is done within the Brassicaceae (reviewed by Nasrallah, 1997), that belong to the Cruciferae. *Brassica* displays a one-locus, multi-allelic system, the genetics of it being elucidated by Bateman (1955). He described a single *S*-locus that segregated Mendelian. Due to the incompatibility mechanism, *S*-homozygotes are possible but will be very rare: plants are in general heterozygous. Recom- bination between different alleles would in theory lead to a rapid increase

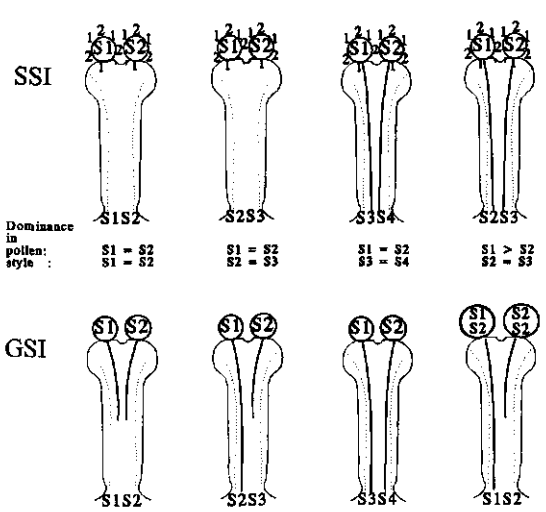

Fig. 1. Compatible and incompatible combinations in a sporophytic one-locus self-incompatibility (SSI) system (top) and a one-locus gametophytic self-incompatibility (GSI) system (bottom). Some dominance relationships between *S*-alleles combinations are shown (SSI, top). The pollen grains are genetically of the genotype *S1* or *S2*. The pollen grains in the sporophytic system are coated with both *S1* and *S2* determinants. The pollen recipients are, from left to right: *S1S2*, *S2S3*, *S3S4* and *S2S3* (top) or *S1S2* (bottom).The gametophytic incompatibility shows the effect of two different alleles present *in* the same pollen grain, as produced by polyploids (bottom right). The interaction between different *S*-alleles (known as competitive action or mutual eakening) causes self-compatibility, a phenomenon not known from the sporophytic system, but here - (inversed) dominance relationships between the *S*-alleles can also bring about (self-) compatibility (top right).

of the number of alleles and perhaps even to a break-down of the SI system. Although a multitude of *S*-alleles has been found (Nou et al., 1993; Brace et al., 1994), frequent recombination on the *S*-locus seems not to be the case. The reason is that the *S*-locus complex (also called *S*-haplotype) has been shown to contain a subset of genes in close linkage (*S*-locus complex, Fig. 2 top), that maintain functional specificities and do function as a set (see also review by Nasrallah, 1997). Two genes, important for the stylar SI reaction, have been investigated extensively, and also other genes linked to those two genes are investigated on their contribution to the pollen-style interaction, leading to either a compatible or an incompatible reaction.

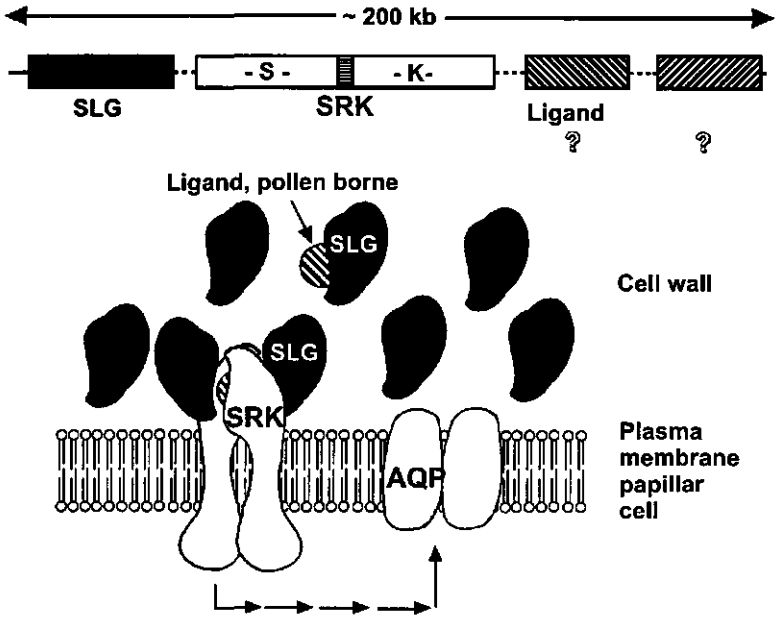

Fig. 2 The *S*-locus and a model for self-incompatibility in *Brassica*. Top: a diagram showing the genes that code for the stigmatic receptor molecules *SLG* (*S*-locus glycoprotein) and *SRK* (S-locus receptor kinase). Linked to this is a hypothetical pollen ligand-encoding gene. The putative SI involved *SLL* (*S*-locus pollen ligand) genes are located between *SLG* and *SRK* (*SLL*and linked genes are not shown). The unlabeled box to the right represents one of several genes that map to the *S*-locus complex but whose contribution to the SI response is unclear yet. For simplicity, introns in *SRK* are not depicted, but the extracellular (S) and the linked kinase (K) domain are presented seperately. The size of the locus spans approximately 200 kb, but can vary with the *S* haplotype.

Bottom: Model for the hypothesized interactions between *SLG*, *SRK* and pollen ligands at the surface of a stigmatic epidermal cell. *SRK* spans the membrane. When SLG, SRK and the pollen ligand bind, a complex intracellular signal transduction cascade is initiated, finally resulting in the arrest of pollen tube germination and growth. The arrows indicate a Ca^{2+} and phosphorylation dependent signal transduction pathway and is acting on a membrane protein related to water-transporting aquaporins (AQP). With minor modifications, from Nasrallah (1997).

The two stigma expressed *S*-locus genes that are required for an inhibition of self-pollen, are both highly polymorphic. One of these genes codes for the so-called *S*-locus glycoprotein (*SLG*), which is a soluble cell wall-localized glycoprotein with a molecular weigth of 55-65 kD (Nasrallah and Nasrallah, 1984; Nasrallah et al., 1985, 1987), highly polymorphic and useful for *S*-phenotype identification. The second gene codes for an *S*-locus receptor kinase (*SRK*), which is a receptor-like kinase that spans the membrane (Stein et al.,1991, 1996). *SLG* sequences are higly homologous with sequences of the extracellular (S) domain of *SRK*. It is speculated that early in the evolution of the *S*-haplotypes *SLG* arose from *SRK* by duplication (Tantikanjana et al., 1993). Quite some (pseudo-) genes belonging to this family have been found to be clustered and linked to the *S*-locus (Suzuki et al., 1997), although also unlinked related genes were detected that might play a role in the SI process (Luu et al., 1997). Other features of *SLG* and *SRK* led to the belief that the hypothesized *S*-locus encoded pollen SI-determinant is a ligand for the receptor. *SRK* and *SLG* should bind to different sites of the same pollen ligand, thus precipitating an intracellular (Ca^{2+} dependent) phosphorylation cascade that results in the arrest of self-type pollen (Fig. 2, bottom). The sense and anti-sense approach for elucidating more of the functions of *SLG* and *SRK* is seriously hampered by the high sequence homology between those two genes. Sense and antisense inhibition will in most cases affect both genes, whereas only one effect was hoped for (Conner et al., 1997). That both genes play a key role in SI, is undisputed, however.

Although the pollen component is still unknown, some candidate genes and products have been found. Yu et al (1996) found in *B. napus* two genes located in between *SLG* and *SRK*, one of them (SLL_1 , S-locus pollen ligand 1) being *S*-locus and SI specific. Its expression was only detectable in anthers. It was deduced that the SLL_1 protein was 2 or 3 kDa, but no related sequences could be found in the databases. Stephenson et al (1997) analysed protein fractions from SI pollen from *B. oleracea* and detected water soluble components, with a $M_r \leq 10$ kDa. From this, a basic, cysteine-rich protein could be isolated that belongs to the family of Pollen Coat Proteins class A (PCP-A), one of which is known to bind to stigmatically expressed components of the *S*-locus in *Brassica*. PCP-A1 is regarded as a candidate for playing a role in SI and perhaps also in a specific type of interspecific incompatibility: unilateral incompatibility. Unilateral incompatibility in *Brassica* might be related to self-incompatibility (Hiscock and Dickinson, 1993).

Gametophytic Self-Incompatibility

In the gametophytic self-incompatibility system (GSI) the genotypes of the pollen (gametophyte) and the pollen recipient (the sporophyte) determine whether or not a combination is compatible (Fig. 1b). In the SSI system the genotype of the pollen itself is of no importance for SI, but the information about the pollen donor, carried at the outside of the pollen grain, is (fig. 1a). This difference in information supply by the pollen forms the basis of distinguishing GSI and SSI. Nevertheless, classifications are not always clear-cut and GSI species can have other features that are more common in the SSI group (e.g. rye, Wehling et al, 1994) or vice versa. Thus, also within the gametophytic self-incompatibilty system several distinct groups can be recognized. Based on

the number of loci involved, the number of plant families showing one-locus GSI, is presumably larger than those found for multi (≥2) loci GSI. The two loci system has been found so far in only four families (De Nettancourt, 1977).

Polygenic GSI.

Within the heteromorphic SSI classes can be identified visually, which facilitates the analysis of even the multi locus system. One locus GSI systems are relatively easy to identify, because the number of allele combination permutations is quite limited. Nevertheless, also some of the more multi locus GSI systems have been analysed. Lundqvist (1956, 1990, 1991) reports complex SI systems with three or four loci in the genera *Ranunculus*, *Beta* and *Lilium*.

In the grasses a less complex two-loci sytem with dry stigmata has been found. In rye (*Secale cereale*), the two unlinked loci *S* and *Z* (Lundqvist, 1956) have been reported to control the system. When for both loci the alleles in the style are matched by those in the pollen, an incompatibility reaction occurs. Until recently, for rye neither female *S* and *Z* products nor incompatibility-related products could be identified (Tan and Jackson, 1988). It has been shown, though, that Ca^{2+} and kinase activity play a role in SI of rye (Wehling et al., 1994) and here too, stigma papillar ligands are expected to be involved. From the grass *Phalaris coerulescens* pollen *S*-alleles were cloned, from which the deduced amino acid sequences shared homologies with thioredoxins (Li et al., 1994).

One-locus GSI systems with a dry stigma and without S-*RNases: Poppy.*

Field poppy (*Papaver rhoeas*) has extensively been investigated on the underlying mechanisms of SI. The incompatibility reaction of poppy is determined by a polyallelic one locus system. The incompatibility reaction occurs on a dry stigma (Lawrence, 1975; Lawrence et al., 1978). From this plant species stigmatic glycoproteins were isolated and identified, and they cosegregated with the *S*-alleles (Franklin-Tong et al., 1989). Subsequently, the cDNA of the stigma papillar *S1* glycoprotein was cloned and sequenced (Foote et al., 1994). To date, the sequence is different from any known in the Brassicaceae or Solanaceae. The *S*-glycoproteins do not possess RNase activity, which the *S*-glycoproteins of the Solanaceae do have (Franklin et al., 1995; McClure et al.,1989). The *S*-glycoproteins, that proved to be *S*-specific, are believed to adhere to (yet unknown) receptors, thus eliciting Ca^{2+} (Franklin-Tong et al.,1993, 1995), which results in a cascade of phosphorylation of specific proteins, in which Ca^{2+} dependent protein kinases and inositol triphosphate may be involved (Franklin-Tong et al., 1995, 1996). An incompatible combination of *S*-alleles results in an increased Ca^{2+} dependent phosphorylation of at least two 26 kD pollen proteins (Rudd et al., 1996) and it also causes the slow-moving calcium wave, regulating the pollen tube growth, to show "rapid and dramatic alterations in $[Ca^{2+}]_i$ within a few seconds of challenge". An unusual high peak is reached, followed by a break-down of the tip-focused $[Ca^{2+}]_i$ gradient (Franklin-Tong et al., 1997). Finally, the pollen tube growth is inhibited. A model for some of the elucidated interactions involved in pollen tube growth and inhibition is presented in figure 3.

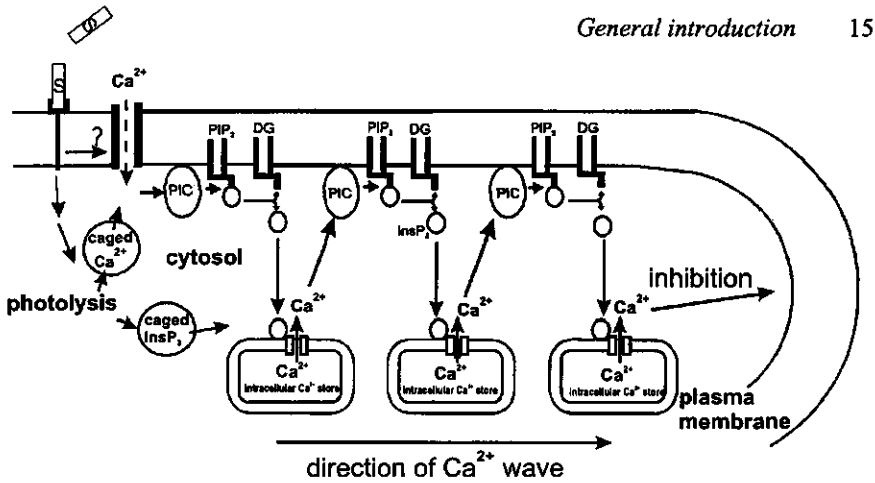

Fig. 3. Model for the propagation of the Ca^{2+} wave in poppy (*Papaver rhoeas*) pollen tubes. The normal growth of pollen tubes of *P. rhoeas* is regulated by a slow moving calcium wave propagated by inositol 1,4,5-triphosphate. Increases of $[Ca^{2+}]_i$ will activate Ca^{2+} sensitive phosphoinositidase C (PIC) , which will then hydrolyze specifically the membrane lipid PIP. The now raised $Ins(1,4,5)P_3$ will stimulate release of Ca^{2+} from $Ins(1,4,5)P_3$ sensitive intracellular stores. Continued slow Ca^{2+} waves can be generated.
It is hypothesized that the binding of a stigmatic *S*-protein (S) to a membrane receptor initiates a signal transduction chain in which Ca^{2+} dependent phosphorylation plays an important role. The normal Ca^{2+} gradient is disturbed. Increases of $Ins(1,4,5)P_3$ and the consequent elevation of $[Ca^{2+}]_i$, inhibition of phosphoinositide (PI) turnover and inhibition of $Ins(1,4,5)P_3$ binding to its receptor lead to an inhibition of pollen tube growth. With small modifications, from Franklin-Tong et al, 1996.

One-locus GSI systems with a wet stigma and with S-RNases: Solanaceae

Single-locus gametophytic self-incompatbility is believed to be the most common incompatibility system within the solanaceous species, although there might exist also solanaceous species that are governed by a two-loci system (e.g. Abdalla and Hermsen, 1971). A remarkable feature of most SI systems is, that SI seems to be very stable and resists to a great extent the spontaneous mutation-induced turn-over into self-compatibility. From the population-genetic point of view new *S*-alleles, and especially self-compatibilizing alleles, would relatively easily accumulate in a population. A large number of natural *S*-alleles that are found, seems to conflict with low frequencies of mutants with a disrupted *S*-locus. Natural selection against those mutants may be one reason, but difficulty in recognizing those mutants may be another. Because, in general, important information can be gained from the analysis of deviant genotypes, spontaneous or induced mutants are regarded as highly valuable research material. Induced mutations facilitate a rational search for those mutants.

Mutation studies (by means of chemical mutagens and irradiation), in order to create point mutations, deletions, duplications and translocations, resulted in nearly all cases in self-compatibility that was either pollen-borne or style-borne. A change in specificity (Van Gastel and De Nettancourt, 1975; Van Gastel and Carluccio, Van Gastel 1976; see also monograph by De Nettancourt, 1977) could not be shown, or can be explained now otherwise by applying the accumulated knowledge about the organisation of the *S*-locus. This holds a lso true for nearly all the cases of believed-to-be pollen-part mutations.

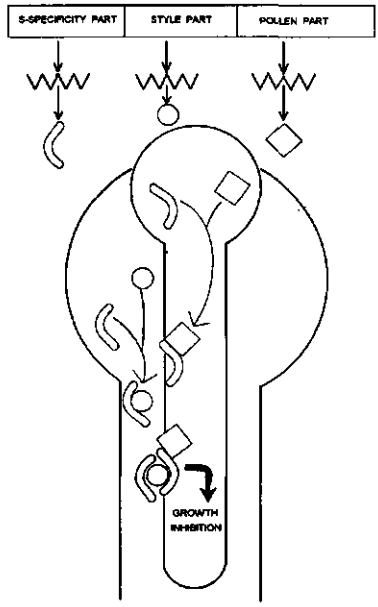

Fig. 4 Envisage of Lewis' hypothesis (1949,1960) of the tripartite structure of the *S*-locus, and the interrelationship of *S*-locus gene products. The model shown here assumes that each part of the *S*-locus encodes a different protein, and self-recognition results from the interaction of an *S* specificity part expressed in both pollen and pistil. Specificity parts form with pollen- or style activity parts the receptor-ligands. Interactions of identical *S*-specificities in pollen and style result in pollen tube growth arrest (with minor modifictions, from Sing and Kao, 1992).

An elegant model of the *S*-locus as proposed by Lewis (1949, 1960), was the so-called tri-partite structure, that could explain satisfactorily most of the results of the aforementioned studies. In this model (Fig. 4), the *S*-locus consisted of an *S*-specificity- (identity), a stylar- and a pollen activity part, all in tight linkage. The specificity part gave rise to a specific protein in both pollen and style, whereas the style and pollen parts were specifically expressed in style and pollen respectively. The combination of activity and identity parts resulted in a specific receptor- ligand pair, causing inhibition of the pollen tube when pollen and style matched in specificity.

There are few reports on a change of the specificity (change of *S*-allele specificity), for instance after anther-culture (Ramulu, 1982) or inbreeding (Maheswaran et al., 1986; Kheyr-Pour and Pernes, 1986). Some of these results may be explained by the expression of accumulated modifier genes (polygenic) that can also bring about reduced self-incompatibility (pseudo-compatibility) up to a level of self-compatibility (e.g. Henny and Ascher, 1976; Robacker and Ascher, 1978). Even the appearance of monogenic pseudo-compatibility genes with strong effects cannot be excluded (see also introductions by Dana and Ascher, 1986; Liedl and Anderson, 1994).

Mutations of SI are, as stated, a valuable source for research. Olsder and Hermsen (1976) detected both a self-incompatible (G609) and two self-compatible dihaploid potato genotypes (G254 and B16) with a high degree of male and female fertility. In successive studies on this material (Hermsen, 1978a, 1978b, 1978c), the underlying genetics was analysed. The self-incompatibility system within the population based on those three clones was very reliable for its expression.

Based on a complete diallel crossing scheme, four *S*-alleles were identified. The self-compatibility was explained by a putative translocation of the pollen-part of the *S1* allele from chromosome 1 to chromosome 12 (Hermsen, 1978 a; Hermsen et al., 1973, 1978b). Heterozygosity

for *S*-alleles in the pollen (e.g., in 2n-pollen and pollen from polyploids; fig. 1b) would then bring about "competitive interaction" (Lewis, 1947), nowadays known as "mutual weakening". This material was originally analysed by classical crossing experiments; biochemical identification of *S*-groups was unsuccessful until the late eighties. Hermsen's material formed the basis for an extensive study on molecular, biochemical and biological aspects of SI in diploid potato (see for instance Kirch, 1993; Van Eldik, 1996; Li et al, 1994).

The *S*-glycoproteins in solanaceous species were shown to co-segregate with the *S*-phenotypes (e.g.Kirch et al., 1989) and different *S*-classes could easily be identified. These *S*-glycoproteins could be traced extracellularly in the same tissue (stigma, transmitting tract of the style and even in a single cell layer) where the SI reactions occurred (Anderson et al., 1989). These tissues showed also an accumulation of corresponding mRNA (Cornish et al., 1987). Within the solanaceous species, a whole range of *S*-alleles has molecularly been cloned. Kirch (1992, 1995) isolated, from the aforementioned diploids, molecular clones of *S1* and *S2* alleles. The translocation hypothesis for *tS1*was investigated by Thompson et al (1991), using RFLP analysis of plant material, coding for this *tS1* with the cloned stylar part of *S1*. With this approach it showed to be impossible to discriminate between plants with and without *tS1* and subsequently a good candidate for the *S*-pollen part or otherwise a useful SI-interacting tool could not be cloned. Based on these data, as well as on sequence data, the tri-partite structure of the *S*-locus, as proposed by Lewis (1961) had to be rejected. The translocation hypothesis for *tS1* could be maintained under the assumption that the translocation would have involved only the pollen part but nothing of the analyzed stylar expressed S1-fragment.

The pollen component plays an essential role in the elucidation of the SI mechanism in the Solanaceae. It was shown that the *S*-glycoproteins have RNase properties (McClure et al., 1989) and are therefore also called *S*-RNases. This led to a range of experiments dealing with sense and anti-sense transformations and transformations with coding regions of *S*-alleles, modified for RNase properties or for presumed identity determining stretches (see for references chapter 5). Based on some of the information gained, two mechanisms were considered for the contribution of the *S*-glycoprotein. One model (Fig. 5, left) is based on the assumption that the pollen part codes for a membrane receptor that is specific in the uptake of the *S*-RNase, the other one is based on a non-specific uptake combined with a specific inhibition of non-self ribonucleases (Fig. 5, right). Both models result in only one specific *S*-glycoprotein being active in the pollen tube. Identification of the membrane receptor (model 1) or the ribonuclease inhibitor (model 2) will play an important role in unraveling the SI mechanism.

To date, many factors have been found that play a role in pollen tube growth and pollen viability, some of them being essential for a successful fertilization, but none proved to be the long-sought pollen SI factor. Recently, however, Ca^{2+}- dependent protein kinases have been isolated from pollen tubes of *Nicotiana alata* that seem to play a role in the SI reaction, presumably shortly after the uptake of the *S*-RNase (Kunz et al, 1997). This discovery is in accordance with the important role of phosphorylation in the SI response of *Brassica*, poppy and rye (see above) and will presumably be very helpful in a further elucidation of the SI pollen pathway.

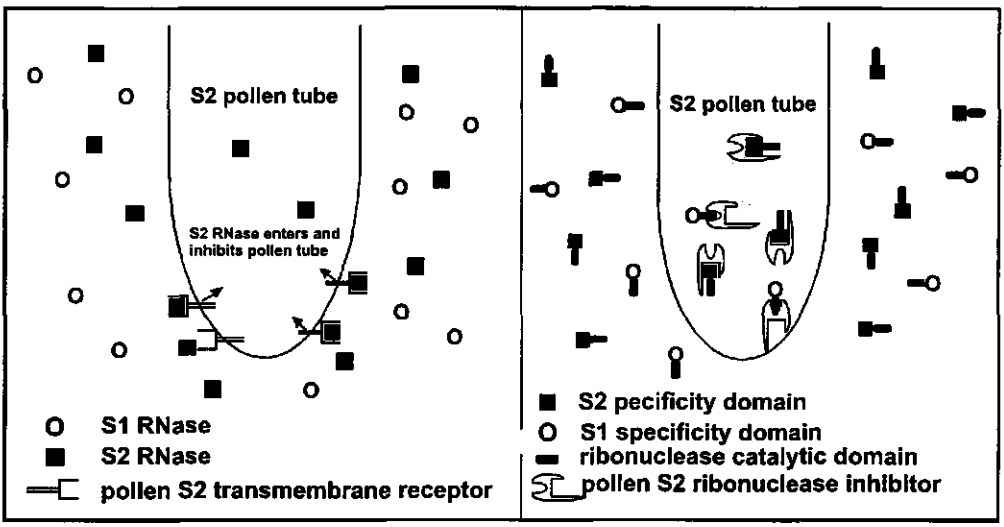

Fig. 5. Two models for self-incompatibility interactions in the one-locus GSI system of the Solanaceae.

Left: model based on the assumption that the pollen *S*-alleles encode membrane receptors. Specific uptake of the *S2* RNase (*S2*-glycoprotein) by the corresponding *S2*-receptor occurrs. *S1* RNase can only be transported over the membrane by an S1 receptor, which is absent here.The *S2* RNase degrades non-specifically the rRNA and /or the mRNA, resulting in inhibition of protein synthesis and finally in pollen tube arrest. Pollen tubes carying other *S*-alleles that S1 or S2 do not transport the S-RNases over the membrane and are hence not arrested.

Right: model based on the assumption that the pollen *S*-alleles encode ribonuclease inhibitors. Here, all *S*-RNases can be transported over the membrane. Pollen S-allele ribonuclease inhibitors have two binding domains: one binding indiscriminately to the ribonuclease activity domain of the *S*-RNases and one binding specifically to the specificity domain of the corresponding *S*-RNase. As a result, in this example, only the self *S2*-RNase is capable of degrading pollen RNA, whereas the (pollen non-self) *S1*-RNase is inactivated. With minor modifications, from Kao and McCubbin (1997).

Unilateral incompatibility in the Solanaceae

It is frequently found that in interspecific hybridisation between a self-compatible and a self-incompatible species, fertilisation is possible in one direction only. This phenomenon is, therefore, called Unilateral Incompatibility (UI) and is found throughout the incompatibility systems. It was, according to De Nettancourt (1977), first defined by Harrison and Darby (1955) but in the early days described by many others (e.g. Anderson and De Winter, 1931; Mather, 1943; Lewis and Crowe, 1958). In general, the SI species can be used successfully as a pollinator, but not as a pistillate parent when pollen from a SC parent is used (SC x SI → F1; SI x SC → –). Because of the strong correlation between one parent being SC and another being SI, there has been a strong opinion among many researchers that the *S*-locus is involved in this process. Lewis and Crowe (1958) formulated the dual function hypothesis for the S-locus, and described an evolutionary

pathway for the development from SI to SC, finally resulting in UI. The two-powers competition hypothesis, described for UI between the SI diploid potato and the SC relative *S.verrucosum* (*ver*), is based on this and on the co-evolution of sympatric SC and SI species (Abdalla, 1970; Abdalla and Hermsen, 1971, 1972; Abdalla, 1974). The presence or the evolution of cytoplasmic male sterility in hybrids derived from SC x SI, plays an important role in this. In this hypothesis, additional genes with various alleles are introduced, that are not necessarily located at the *S*-locus. At the same time, research in other crops indicated that the *S*-locus did not play a role at all in UI. Hogenboom (1973) introduced, based on his work on *Lycopersicon esculentum* (SC) and *L. peruvianum* (SI), the Unilateral Incongruity hypothesis (also abbreviated UI). He made a distinction between SI and UI and argued that incompatibility and incongruity are separate phenomena.

Hermsen et al (1974) detected, in the same material that played such an important role in the research on SI in potato, clones that were "acceptor" for *ver* pollen. Absence of the UI response in species crossing combinations where UI is the rule, is also called "acceptance", and "non-acceptance" stands therefore for UI. Genetic models were tested and similarities were found with a model proposed by Grun and Aubertin (1966). Acceptance segregated independently from both the *S*-alleles and the pollen-borne SC-factor *tS1*, and appeared therefore to support the UI hypothesis of Hogenboom (1973). Chetelat and De Verna (1991) mapped pollen-mediated UI factors on the chromosomes 1, 6 and 10. The factor on chromosome 1 mapped on or near the *S*-locus, thus supporting both the possible involvement of the *S*-locus and *S*-locus independent acting genes on UI.

For a long time, now, a debate is ongoing about the possible involvement of the *S*-locus in UI, and, directly related to this, whether in this connection the term incongruity or incompatibility should be used. The molecular cloning of *S*-alleles and the construction of sense and anti-sense constructs opened the possibility to test whether the *S*-locus has a dual function (causing both SI and UI, or more indirectly, causing SI and contributing to UI) or not.

General aims of the Thesis

The diploid potato material of Hermsen, based on the clones G254, G609 and B16, was maintained over a long period by means of both vegetative and generative propagation. This resulted in material that became weak and diseased. Inbreeding is known to affect the reliability of the SI response and should be restricted as much as possible.The S-homozygous clones that were present, were all based on the SC factor *tS1* and of little value for SI research. The use of pseudo-compatibility (brought about by inbreeding) to create *S*-homozygotes was also not an option for the creation of fertile, well-flowering and SI-reliable clones. Some incompatibility related genes or their products had previously been isolated from this type of material. Molecular constructs, based on these cloned genes, had to be tested in potato on their effect. Well transformable diploid potato material with the proper *S*-allele composition was not available yet, so this was another problem that had to be tackled.

The main aims of the research of which this thesis is a reflection, were:

1 The creation and selection of *S*-homozygous and *S*-heterozygous material and tester clones with a reliable SI reaction, lacking negative factors like inbreeding depression, poor flowering, poor fertility and pseudo-compatibility.
2 Creation and selection of well transformable clones with a proper functioning of the SI response.
3 Elucidation of more biological aspects of gametophytic self-incompatibility.
4 Testing whether or not there is a direct relation between self-incompatibility and the interspecific crossing barrier called "Unilateral incompatibility".

In Chapter 2, the development of well performing diploid potato clones with a reliable SI reaction is described. Some of this material was used to create well-transformable clones (briefly mentioned in the chapters 3 and 6). In Chapter 2, the procedure is also described how the creation of self-incompatible S-homozygotes was achieved, without accompanying effects as break-down of the SI reaction. This material was used as tester clones, as described in some of the following chapters.

In chapter 3, the effect of sense and anti-sense constructs based on the coding region of the *S1* and the *S2* alleles, on the incompatibility reaction is described. An attempt was made to prove the essential role of *S*-glycoproteins (*S*-RNases) in the SI reaction.

In chapter 4, the creation of male and female fertile *S.tuberosum* (*tbr*) x *S.verrucosum* (*ver*) hybrids is described. Potato clones (SI) that are acceptor for *ver* (SC) pollen were selected from the material mentioned in chapter 2. Furthermore, the expression of self-compatibility of *ver* in hybrids and in (backcross) offspring thereof is investigated. The contribution of the pollen part of the *S*-locus of *ver* on self-(in)compatibility and unilateral incompatibility is analysed.

In chapter 5, the materials and results described in the preceding chapters are used in an integrating analysis of the relation between UI and SI. The loss-of function approach, as used in chapter 3, is applied in both *tbr* and *tbr x ver* hybrids. The role of the stylar part of the *tbr S*-locus in UI is examined and various UI and SI phenomena are integrated in a descriptive model.

In chapter 6 the relevance of the developed material is addressed. Some of the results already mentioned in the experimental chapters are discussed in a broader framework. Some significant questions, not addressed in the chapters 2-5, are posed and some speculative approaches and ideas are highlighted.

Chapter 2

Selection of vigorous and fertile *S*-homo- and heterozygous tester clones from self-incompatible diploid potato, *Solanum tuberosum* L.

Abstract

For the selection of diploid (2n=2x=24) potato (*Solanum tuberosum*) genotypes that are useful for the molecular and genetic analysis of the phenomenon of gametophytic self-incompatibility, three different types of basic populations were investigated. These populations were derived from three primary dihaploid clones, G609, G254 and B16, which possessed the *S*-allele combinations *S1S2*, *S1S3* and *S3S4* respectively. In order to select highly vigorous, profusely flowering, fertile and tuberising progenies, three types of populations, derived from the above mentioned diploid genotypes, were screened for performance and classified for the expression of self-incompatibility. Although the selection for well defined *S*-genotypes was sometimes complicated due to the occurrence of pseudo-compatibility and of a self-compatibilising factor, the use of a combination of criteria, viz., Iso Electric Focusing (IEF), pollen tube growth in the styles and the extent of berry and seed set made the selection of sufficient representatives of all six types of *S*-heterozygotes (*S1S2, S1S3, S1S4, S2S3, S2S4* and *S3S4*) possible. After evaluating the strength of the self-incompatibility reaction in these heterozygotes, those with high expression were selfed, and intercrossed within their *S*-allele incompatibility group through the method of counterfeit pollination. In these progenies, well-performing *S*-homozygotes (*S1S1*; *S2S2*; *S3S3*; *S4S4*) for all four *S*-alleles with high expression of self-incompatibility were selected. As a result, all possible S-homo- and heterozygous genotypes with a predictable type of self-incompatibility are available and maintained both vegetatively and as botanical seed. The development of this material has paved the way for more critical analysis of molecular factors involved in self-incompatibility in diploid potato.

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Introduction

The cultivated potato, *Solanum tuberosum* (2n=4x=48), is a self-compatible (SC) crop. Dihaploids (2n=2x=24) from tetraploid cultivars are usually highly sterile, less vigorous and self-incompatible (SI). Self-incompatibility in dihaploids is expected to be similar to the one-locus, multi-allelic, gametophytic system that is found in almost all other tuberous, diploid Solanum species. This expectation was proved to be true from the genetic analysis of self-incompatibility in male fertile genotypes that occurrs rarely among dihaploids (Olsder and Hermsen, 1976; Hermsen, 1978a; Hermsen et al., 1978). These authors analysed three fertile dihaploids, and the presumed tetraploid parent of two of the dihaploids, the cultivar Gineke, and postulated the presence of five *S*-alleles viz., *S1,S2, S3, S4* and *S5*.

Through a complete diallel crossing scheme, the following genotypes were assigned to the three dihaploids that were investigated (Hermsen,1978a): *S1S2*-G609; *S1S3*-G254 and *S3S4*-B16. The two latter clones, G254 and B16, possessed the necessary *S*-alleles for conferring self-incompatibility; nevertheless, they were self-compatible (i.e., set seed after selfing). This anomalous phenomenon was explained as due to the presence of an extra *S1* allele (a duplication) in a presumably translocated segment on a different chromosome and the authors designated this segment as "*tS1*" (Hermsen,1978a ; 1978b). This hypothesis was investigated by Thompson et al (1991), using RFLP analysis of plant material, coding for this *tS1* with the cloned stylar part of *S1*. The tri-partite structure of the *S*-locus, as proposed by Lewis (1961) had to be rejected and the translocation hypothesis for *tS1* could be maintained under the assumption that the translocation would have involved only the pollen part but none of the analyzed genomic *S1*-fragment. In dihaploids, containing such a *tS1* translocation, a fraction of the pollen grains contained the pollen parts of two different *S*-alleles instead of one, and hence inhibited self-compatibility by a competitive interaction (Crane and Lawrence, 1929; see also review, De Nettancourt, 1977) or mutual weakening as, for example, in *Brassica* (Wallace, 1979) or in polyploids (Lewis, 1943). Besides competitive interaction, also the so-called 'pseudo-compatibility' can occasionally bring about berry development with a few seeds in a basically otherwise self-incompatible genotype (Hermsen, 1978b).

Apart from such complications regarding self-incompatibility, the above mentioned genotypes were valuable for the characterisation of proteins that are associated with self-incompatibility in *Solanum tuberosum* (Kirch et al, 1989; Peil, 1995). As a result of this study, it was possible to correlate *S1* to *S4* alleles with specific bands of a number of polypeptides differing in their iso-electric points (Kirch et al, 1989). This observation, obviously, opened up possibilities for a more reliable method

of identification of the *S*-alleles through electrophoresis, and characterisation of the self-incompatibility system in diploid potato more critically. In this context, it was essential to select defined diploid potato material, homozygous or heterozygous for particular S-alleles, that would be suitable for more critical genetic and molecular analysis of the incompatibility system.

Selection of diploid potato genotypes with a defined *S*-allele composition, e.g., *S*-homo- and heterozygotes, with a predictable expression of self-incompatibility is difficult for several reasons. Potato being a highly heterozygous crop, inbreeding depression is a severe problem both for selfing and for intercrossing among individuals within a small group of (diploid) genotypes. This is because the progenies in these cases are generally less vigorous, non-flowering, highly sterile, non-tuberising and frequently segregating for lethal and semi-lethal genes. In order to circumvent these difficulties, a rigid selection of diploid parents based on the performance of their progenies for some of the important characteristics, including the typical expression of self-incompatibility, is essential.

The aims of the present investigation were: 1) to select diploid potato genotypes with highly vigorous, fertile, early and profusely flowering habit, showing good tuberisation characteristics; 2) to isolate homo- and heterozygous tester stocks for different S-alleles with predictable and reliable expression and 3) to produce sufficient plant material of each allelic class (seeds and tubers) for generative and vegetative maintenance.

Materials and methods

Selection of the basic genotypes

Two different types of populations were screened for desirable genotypes. The first of these consisted of 'basic' populations derived from crosses between three dihaploid *Solanum tuberosum* (2n=2x=24) clones, G609 (*S1S2*), G254 (*S1S3*) and B16 (*S3S4*). The origin and the indicated genotypes of these basic clones have been described earlier (Olsder and Hermsen,1978; Hermsen, 1978a). The progeny used for the selection of desirable genotypes had originated (see Table 1, column 2) not only from direct crosses (five original F1's) bet-ween the dihaploid clones but also from intercrossing and selfing of progeny plants (14 populations from selfings and inbreds). Because the *S*-genotypes of each of the parents were homozygous for the marker 'embryo-spot' (Hermsen and Verdenius, 1967), the seeds resulting from counterfeit pollination could be separated from those resulting from the first pollination. In diallel crossing, at least 10 pollinations per combination were made, using flowers from two or more inflorescences in the case of both normal and counterfeit pollination methods.

Besides normal and counterfeit pollinations, "prickle pollinations" (pollination with only IvP-pollen) were made in order to use them as controls for determining the production of spontaneous spotless seeds in such female plants. These particular spotless seeds are believed to be normally the result of diploid or haploid parthenogenesis, the latter leading to monohaploid offspring (Van Breukelen et al, 1977; Uijtewaal et al, 1987).

Statistical analysis

Data on the number of pollinations, obtained berries and number of seeds were analysed with the computer programme Statgraphics Plus v 7.1.

The number of spotless seeds after an incompatible cross plus counterfeit pollination with IvP pollen was corrected within each SI-group by subtraction of the number of spotless seeds produced after pollination with only IvP-pollen .

Table 1. Basic populations (column 1) obtained from SI and SC parents (column 2) with known *S*-genotypes (column 3) used for the selection of well performing SI heterozygotes and SC homozygotes (column 4).
GB = G254 x B16; BG = B16 x G254; *S1S3*, *S2S3* and *S3S4* = self-incompatible tester clones selected from GB, Gx(G x G609) and GB respectively. Numbers behind brackets indicate clone number. ⊠ = selfing, SI = self-incompatible, SC =Self-compatible due to *tS1*. ***Italics bold***: last SC-clone used in a cross.

Population	Parents	Description	*Obtained genotypes*
Selfings			
6107	***{(G254 x S1S3)20 x S1S3)8*** ⊠	***S1S3*** ⊠	S1S3 + S3S3, SC
6108	{***(G254 x S1S3)20*** x S1S3)10 ⊠	S1S3 ⊠	S1S1 + S1S3 + S3S3
6233	6105-6 ⊠	S2S4 ⊠	S2S2 + S2S4 + S4S4*
6234	6105-8 ⊠	S2S3 ⊠	S2S2 + S2S3 + S3S3
Inbreds			
6101	***(G254 x S1S3)20*** x S1S3	***S1S1*** x S1S3	S1S3, SI/SC
6102	S2S3 x {***(G254 x S1S3)20*** x S1S3}1	S2S3 x S1S3	S1S2 + S1S3
6103	{***(G254 x S1S3)20*** x S1S3}1 x S2S3	S1S3 x S2S3	S1S2 + S2S3
6104	S2S3 x ***{(G254 x S1S3)20*** ⊠ ***}4***	S2S3 x ***S1S1***	S1S2 + S1S3, SI/SC
6105	{***(BG112 x GB61)21*** x S3S4}2 x S2S3	S3S4 x S2S3	S2S3 + S2S4
6106	***{(BG112 x GB61)23*** ⊠***}5*** x S2S3	***S4S4*** x S2S3	S2S4 + S3S4, SI/SC
6206	***{(G254 x S1S3)20 x S1S3}9*** x S2S3	***S1S3*** x S2S3	S1S2 + S2S3, SI/SC
6208	S2S3 x ***{(G254 x S1S3)20 x S1S3}2***	S2S3 x ***S1S3***	S1S2 + S1S3, SI/SC, S2S3 + S3S3, SC
6536	(***GB49*** x ***B16***)17 x (***GB53*** x ***G254***)41	***S1S3*** x S1S4	S1S4 + S3S4, SI/SC
6539	***(GB66 x GB65)11*** x S1S4	***S1S4*** x S1S4	S1S1 + S1S4 + S4S4, SI/SC
Original F1's			
6221	***G254*** x ***B16***	***S1S3*** x ***S3S4***	S1S4 + S3S4, SI/SC, S1S3 + S3S3, SC
6222	***G254*** x G609	***S1S3*** x S1S2	S1S2 + S2S3, SI/SC
6223	G609 x ***B16***	S1S2 x ***S3S4***	S1S3 + S1S4 + S2S3 + S2S4, SI/SC
6224	G609 x ***G254***	S1S2 x ***S1S3***	S1S3 + S2S3, SI/SC
6225	***B16*** x ***G254***	***S3S4*** x ***S1S3***	S1S3 + S1S4, SI/SC, S3S3 + S3S4, SC

* *S4S4* not detected.

Selections of spotless seed samples were sown and the seedlings were tested for the accuracy of embryo spot detection, for plant performance and the expression of SI/PC/SC. The statistical analysis of the production of spotless seeds was, however, based on the determination of spotless seeds and not on seedlings without nodal band.

Simultaneous analysis of pollen and style effect was performed on "within incompatibility group" level. On total population level, the analysis of the main effects of pollinator (pollen parent) or recipient (seed parent) was performed separately. Here, pollinator or stylar effect means per clone out of the over-all analysis were consecutively added four times as covariates in an iterative approach after an initial separate analysis. The analysis on the over-all level was also performed by adding within-group means as covariate.

Classification of genotypes for S-alleles

Four criteria were used for the classification of *S*-allele genotypes and their SI reaction: 1) the *S*-alleles were identified by iso-electric focusing of stylar extracts with polyacrylamide gel electrophoresis (PAGE) or precast agarose gels; 2) PAGE results were verified through test crossing and vice versa; 3) the extent of pollen tube growth in the pollinated styles was monitored under a fluorescent microscope; 4) berry and seed set were evaluated after selfing as well as after crossing with tester genotypes.

Biochemical identification-PAGE

Iso-electric focusing of stylar extracts with PAGE was performed as described by Kirch et al (1989) or by means of pre-cast agarose gels (Hypure gel VG 1020, Isolab inc.) following the silver staining procedure based on Tungstosilicilic acid in stead of sodium permanganate, according to company specifications.

Pollen tube growth in styles

Pollen tube growth in styles was studied according to the modified technique of Martin (1959). Briefly, the technique was as follows: receptive styles were pollinated; 48 h later, they were fixed in freshly prepared 3:1 solution of ethanol acetic acid for a day or longer; macerated with 8N sodium hydroxide solution at 65^{0}C for at least 8 min.; rinsed with water; stained with 0.1% aniline blue dissolved in 0.1M potassium pyrophosphate; softened styles were mounted in glycerol and observed under a fluorescent microscope (BG12/4 filter combination in Zeiss microscope).

Estimation of berry and seed set

At least 10 pollinations were made in order to determine whether a genotype was SC or SI. The genotypes that had a high percentage of pollen stainability but failed to set berries on selfing were classified as SI and those that produced berries and seeds in high numbers were considered as SC. Because a SC reaction could result either from the presence of *tS1* or be due to pseudo-compatibility, in ambiguous cases progenies of PC/SC plants were tested in order to verify whether those

parents were SC or PC. Berry and seed set were estimated on the basis of seeds per berry, seeds per pollination and berries per pollination. In the case of counterfeit pollinations and the control pollinations with only IvP-pollen, the spotless seeds were separated from those with spots under a binocular microscope and counted.
Pollen stainability was estimated by mounting fresh pollen grains from three flowers, on different dates in each case, in a drop of 2% acetocarmine solution. On an average, 200 pollen grains were counted per assessment.

Results

Performance of the basic populations

Three types of basic populations consisting of selfs, inbreds and the original F1s, that were investigated in the greenhouse for performance are described in Table 1 with indication of their parents and of the genotypes obtained. In all cases, with the exception of the population 6233, the obtained *S*-allele genotypes of the progenies were fully concurrent with the established genotypes of the parents (Table 1). In the exceptional population 6233, only *S2S2* and *S2S4* genotypes could be detected, whereas the also expected *S4S4* genotype was absent.
There were clear differences in performance (Table 2) among the progeny populations derived from selfs, inbreds and the basic F1s with regard to the average scores for vigour, flowering, fertility, tuberisation and the number of cripples (plants that were tiny, weak and brittle). In general, the progenies of the basic F1s were superior to the other two categories for all the four parameters estimated. For example, the average scores in the five basic F1 populations (6221 to 6225, Table 2) were consistently higher (with 77 useful plants) than in the 14 populations (with 74 useful plants) derived from the selfs and inbreds. Especially the frequency of useful plants after selfing was low. Because of these differences between the three population types, the progenies of the basic F1s were not only more useful for *S*-heterozygotes but also for the selection of the *S*-homozygotes using counterfeit pollination (see later).

Selection of SI and well performing *S*-heterozygous plants out of the basic populations

The evaluation of the basic populations proved that a majority (407/548) of the plants among them were unfit for selection of *S*-heterozygotes since they did not meet the four criteria used for selection (Table 2). In a further round of selection among well performing plants of the populations, a total of 31 useful individuals from different populations were evaluated for SI-expression. All these genotypes were classified on the basis of *S*-allele composition through both IEF and test crossing.

Table 2. Performance of basic populations for the selection of useful heterozygous SI genotypes.
All characters were scored on an ordinal scale for each plant and the average values are presented in the columns. Ranking: 1 = lacking, 2 = present but bad, 3 = poor, 4 = insufficient, 5 = just sufficient, 6 = sufficient, 7 = satisfactory, 8 = good, 9 = very good; between brackets (): # well performing self-compatible clones, - = not segregating SC-plants

Population	# plants	Vigour	Flowering	Fertility	Tuberization	# useful plants	
Selfings						SI	SC
6107	10	2	3	2	1	0	(0)
6108	30	3	3	4	2	0	(-)
6233	11	4	4	4	3	1	(-)
6234	14	5	4	5	4	5	(-)
Inbreds							
6101	30	6	6	7	4	6	(6)
6102	30	5	5	5	5	7	(-)
6103	40	5	5	5	4	9	(-)
6104	30	6	6	8	7	11	(11)
6105	20	6	6	7	6	7	(-)
6106	40	6	6	6	6	7	(10)
6206	30	6	8	7	4	5	(4)
6208	40	7	8	8	3	2	(6)
6536	20	6	6	7	7	6	(6)
6539	20	5	5	7	6	8	(6)
Basic F1's							
6221	40	8	6	8	7	13	(22)
6222	40	8	7	8	7	18	(19)
6223	40	8	7	8	7	18	(15)
6224	23	8	7	8	7	9	(11)
6225	40	8	6	8	7	19	(16)

The expected six classes of four different alleles, i.e., *S1S2*, *S1S3*, *S1S4*, *S2S3*, *S2S4* and *S3S4* were found. Table 3 presents for all six expected SI classes the average scores of the selected plants for each of the four characters vigour, flowering, pollen shedding (scale 1-10) and pollen fertility (% stainable), together with their SI-expression. A notable feature was that 19 out of 31 of the genotypes that showed a typical SI-reaction (Table 3) were derived from the progeny of the five basic F1s (cf Table 1), whereas only 12 of the genotypes originated from the 10 inbred populations and none from the selfings.

In order to evaluate the strength of SI-expression, all six *S*-genotypes, consisting of 30 plants in total, were tested for berry and seed set after selfing (Table 3). A strict self-incompatibility reaction (no berry and seed set) was expected in all plants. However, testing the 30 plants sever-all years, revealed that still nine of them occasionally did set (self)seed, ranging from 10- 80 seeds per berry (compatible crosses give good berry formation and 150 - 250 seeds per berry). This could often be

attributed to pollination of young flowers in which there might still have been an incompletely developed SI barrier (cf. bud-pollination). This phenomenon was considered as a less reliable SI reaction. Excluding these genotypes, the strength of the SI-expression in all others was satisfactory as was evident from the absence of berry and seed set after 30 to more than 100 selfings that were made in different genotypes (Table 3). Berry and seed set in some of the *S*-heterozygotic plants was an indication for the persistence (genetic transmission) of pseudocompatibility. As pointed out already in the population section, a majority of the useful *S*-heterozygotes (viz., serial numbers: 6221, 6222, 6223, 6225) in Table 3 was derived from three of the five populations of basic F1s mentioned in Table 1. This was reflected in the final selection of the clones to be maintained. The populations 6221 and 6225 as well as the populations 6222 and 6224 are the result of reciprocal crosses. The underrepresentation of the populations 6224 and 6225 does not reflect inferiority but was just a matter of random choice.

Selection of *S*-homozygous SI plants from the basic populations

All well performing plants in the self and inbred populations, that could have contained *S*-homozygous genotypes, were investigated. Among those plants, self-incompatible as well as *tS1*-based self-compatible *S*-homozygous genotypes could be present. Because only the *S*-homozygotes with self-incompatibility were essential, the basic populations were screened for such genotypes, and those with self-compatibility were discarded. In populations 6108, 6233, 6234 and 6539 (Table 1), *S*-homozygotes with a typical SI reaction were found. On the other hand, the *S*-homozygotes from populations 6107, 6208, 6221, 6225 and 6539 were all found to be self-compatible and were, therefore, discarded. Among the self-incompatible *S*-homozygotes, the population 6108 consisted of less vigorous individuals, and 6539 had high levels of sterility besides poor tuberization; and these were not suitable for the final selection. Only two populations, 6233 and 6234, gave rise to some desirable genotypes with valuable features (Hermsen, 1978c; Hermsen and Olsder, 1974) despite having a relatively poor general performance. Both of these populations were derived from 6105-06 and 6105-08 which were rare cases of seed set upon selfing (Table 1). This seed set was most probably the result of environment-induced PC. The success rate of this type of selfing was not predictable. The performance and *S*-genotypes of six of the plants selected from the 6233 and 6234 populations are presented in Table 4. Although the selected genotypes were not completely satisfactory in performance, they were typically self-incompatible, and initially useful as testers. A greater disadvantage of these successful populations was that only *S2S2* and *S3S3* *S*-homozygotes were obtained. For the selection of more vigorous *S2S2* and *S3S3* genotypes and of *S1S1* and *S4S4* homozygotes as well, a more effective method of bypassing SI, using many genotypes within an incompatibility group, was required. This was done through counterfeit pollinations.

Table 3 Performance of selected SI-expressing *S*-heterozygous genotypes + key identifiers to table 8.
Vigour and flowering impression: 1 = extremely bad, 2 = bad, 3 = poor, 4 = insufficient, 5 = nearly sufficient, 6 = sufficient, 7 = satisfactory, 8 = good, 9 = very good, n.d. = not determined; pollen fertility expressed as % acetocarmine stainable pollen; no additional mark = standard deviation between periods: 0 -5, * = 5 - 10, ** > 10. SI-expression: totals of seeds/berry/selfing in the first two years; + = caused by pollination of very young flowers; SC = self-compatible

Plant nr.	Key	Genotype	Vigour	Flowering	Pollen fert.	Pollen shed	SI-expression Seeds / berries / selfs		
6102-16	I	S1S2	7	7	84	6	0 /	0 /	32
6104-09	2	S1S2	8	9	93	8	0 /	0 /	71
6104-21	4	S1S2	7	8	92	9	0 /	0 /	43
6222-05	15	S1S2	8	9	96	9	0 /	0 /	39
6222-39	18	S1S2	9	9	87	7	0 /	0 /	49
6101-11	1	S1S3	7	7	90	8	0 /	0 /	73
6104-19	3	S1S3	7	8	80	7	0 /	0 /	78
6104-23	5	S1S3	8	8	97	9	80 /	1+/	148
6223-15	III	S1S3	7	8	78	7	0 /	0 /	30
6225-05	IV	S1S3	8	9	92	8		n.d. (SC)	
6225-15	V	S1S3	8	8	80	8	0 /	0 /	22
6223-40	23	S1S4	9	9	60*	9	0 /	0 /	69
6221-01	8	S1S4	8	8	78	7	0 /	0 /	92
6221-05	9	S1S4	7	9	61*	8	4 /	2+/	64
6221-17	10	S1S4	8	8	64	7	50 /	2 /	50
6221-19	11	S1S4	8	8	64	8	42 /	4 /	62
6221-20	12	S1S4	7	9	75	9	0 /	0 /	107
6105-08	II	S2S3	7	8	50**	9	30 /	1+/	32
6222-06	16	S2S3	7	9	80	7	150/	3+/	51
6222-24	17	S2S3	8	9	91	8	0 /	0 /	48
6222-40	19	S2S3	9	9	74**	9	0 /	0 /	70
6105-06	6	S2S4	7	7	72	8	61 /	5+/	112
6105-15	7	S2S4	7	6	70	8	19 /	2+/	52
6223-01	20	S2S4	8	7	82	6	0 /	0 /	41
6223-29	21	S2S4	9	9	79*	8	0 /	0 /	50
6223-39	22	S2S4	9	9	67*	9	0 /	0 /	51
6536-01	24	S3S4	7	9	71*	9	32 /	1+/	55
6536-02	25	S3S4	8	9	42**	8	0 /	0 /	55
6536-09	26	S3S4	8	8	64	9	0 /	0 /	52
6221-32	13	S3S4	7	9	82	9	0 /	0 /	71
6221-37	14	S3S4	8	9	46**	8	0 /	0 /	54

Production of superior homozygotes from selected S-heterozygotes

All genotypes mentioned in Table 3 have been used in some way, trying to obtain more *S*-homozygotes. Not all the genotype combinations that were made for this purpose have been used for the screening and selection of *S*-homozygotes, particularly because of suspected expression of PC in the offspring or (expected) inbreeding depression. Some parental clones that have been used were excluded from the statistical analysis because of (re)appearance of SC in the selected clones or temporary regrowth problems, leading to bad synchronisation of flowering. Five clones were excluded because of virus infection, resulting in too few observations to be of use for statistical analysis. Those genotypes were indicated by 'Roman numbers" in Table 3. Four of them gave useful *S*-homozygotes though (Tables 4 & 8). The PC genotypes with occasional seed set after young flower pollination were included in this experiment. Unlike the selection of *S*-heterozygotes, it was generally much more difficult to create *S*-allele homozygotes from genotypes that showed the typical SI reaction. The problem in these genotypes was to obtain seeds from selfing, or from intercrossing within an incompatibility group. These difficulties were largely overcome by making a large number of pollinations, followed by counterfeit pollinations.

For making the counterfeit pollinations, different genotypes within each *S*-incompatibility group were selfed and intercrossed, following the general crossing scheme exemplified for *S2S3* genotypes (A, B and C) in table 5. This scheme was applied for all possible six *S*-heterozygous groups. One set of these crosses was carried out without counterfeit pollination (normal incompatible pollination) and the other identical set with counterfeit pollination (normal + counterfeit pollination); this means that 48 hrs after the incompatible cross, a second pollination was carried out but now with pollen from the IvP marker genotypes of *S.phureja*. In this context, approximately 4300 pollinations were made. These pollinations included three groups of within group incompatible crossing: 1) selfing a SI genotype (Self, in Table 5: bold), 2) the same genotype used as seed parent (SP, Table 5: row) with various non-self pollinators (non-bold) and 3) the same genotype used aspollen parent (PP) (Table 5: column) in non-self crosses. These pollinations yielded, approximately, a total of 1100 berries with 125000 seeds of which 9500 were without embryo-spot. In order to assess of the strength of the incompatibility reaction and to determine whether the number of spotless seeds resulting from the counterfeit pollinations was different from the number of spotless seeds obtained from the control pollinations (styles pollinated only with pollen from the marked IvP clones), a statistical analysis was performed (Table 6). The occurrence of spotless seeds from prickle pollination alone (based on LSD-values) was statistically not significantly different from zero, but significantly different from the counterfeit pollination effect (Table 6). This was evident regardless of the consideration of parental effects. The occurrence of the number of spotless seeds after counterfeit pollination or normal single cross was calculated and analysed in

Table 4 Performance of selected SI S-homozygous genotypes derived from selfed SI clones.

Plant	Genotype	Vigour	Flowering	Pollen fert.	Pollen shed	SI Seeds/ berries/ selfs		
6233-11	S2S2	5	5	55**	5	0 /	0 /	70
6234-05	S2S2	5	7	75**	5	0 /	0 /	50
6234-12	S2S2	5	5	50**	5	0 /	0 /	75
6234-01	S3S3	5	5	60**	4	0 /	0 /	50
6234-08	S3S3	6	6	74**	6	0 /	0 /	90
6234-10	S3S3	6	6	41**	5	0 /	0 /	50

The scale of values is ranging from 1 (extremely bad) to 9 (very good). ** = Standard deviation > 10%. SI reaction as seeds/berries/selfed flowers (totals).

Table 5. Crossing scheme of the counterfeit pollination experiment for the production of S-homozygotes

▾Seed parent ▸ Pollen parent	Normal incompatible crossing			Normal + Counterfeit pollination			Control
	A	B	C	A	B	C	IvP
A: S2S3	**X**	X	X	**X**	X	X	X
B: S2S3	X	**X**	X	X	**X**	X	X
C: S2S3	X	X	**X**	X	X	**X**	X

A, B and C belong to the same *S*-allele combination group, e.g. *S2S3*. **Bold X** = selfing. Control with IvP = pollination with IvP-pollen as in counterfeit pollination but without an incompatible first pollination.

Table 6 Analysis of variance (ANOVA) in counterfeit pollination experiment using spotless seed production per pollination or per berry. Main Eff. = Main effect; Seedp. = seed parent; Phu = counterfeit/prickle pollination with IvP's; d.f = degrees of freedom; S.S= Sum of squares; F= (S.S. main effect/df)/(S.S. residual/d.f); γ = significance level.

Spotless seeds/pollination					Spotless seeds/berry				
Main Eff	d.f	S.S.	F	γ	Main Eff	d.f	S.S.	F	γ
Seedp.	24	4293.17	2.82	0.0000	Seedp.	24	13530.17	4.02	0.0000
Phu	1	558.09	7.68	0.0061	Phu	1	2149.44	15.32	0.0001
Residual	207	15039.48			Residual	207	29037.51		

two ways, viz., per pollination and per berry (Table 6). Analyses within *S*-genotype groups and the iterative approaches of the determination of the main effects gave basically the same (nearly identical) results. One conclusion was that the number of spotless seeds/berry was twice as high as the number of spotless seeds / pollination (Fig. 1). From these calculations, it was evident that

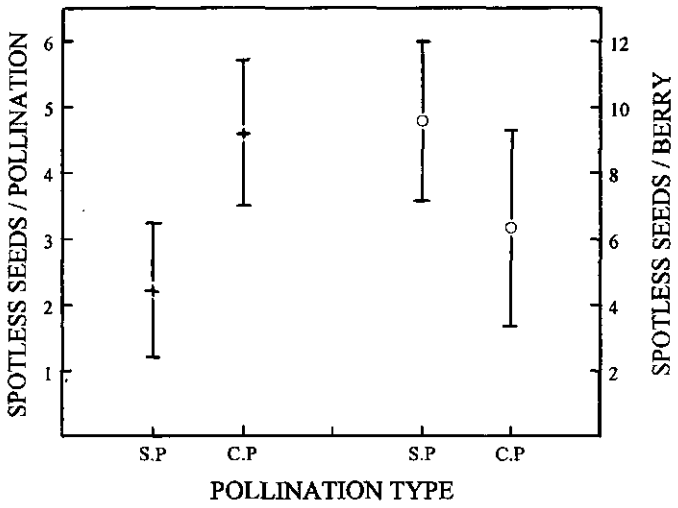

Figure 1. LSD analysis for set of spotless seeds after single normal incompatible pollination (S.P.) or counterfeit pollination assisted incompatible crossing (C.P.).
Y-axes: Left (+): spotless seeds/ pollination; Right (O): spotless seeds/berry. + and O: means. Capped error bars: 0.95% confidence intervals. Based on 473 corrected means.

counterfeit pollinations gave rise to more seeds (spotless) that were potentially *S*-homozygotes than single pollinations in incompatible genotypes. The other difference was that in the case of counterfeit pollinations more berries were obtained and had to be extracted.

Despite a strong selection for typical self-incompatible genotypes, it was evident that certain individuals occasionally set seed after selfing. This was an indication for the occurrence of pseudo-compatibility due to the influence of either the male or the female parent. The genotype 6222-40, for example, was an instance of showing PC effect from the seed parent and 6222-06 from the pollen parent (Table 7). The occurrence of pseudo-compatible genotypes occurred in the progeny in a number of cases, especially when a parental clone (e.g. 6502-38, parent of 1127-14, table 8) showed strong PC (data not shown), clearly indicates the genetic basis of this character. After excluding such PC genotypes, it was still possible to retain a considerable number of genotypes of all *S*-allele homozygotes with high levels of vigour, flowering, pollen fertility, pollen shedding and SI-expression (Table 8). Those homozygotes were either hybrids (intercrosses within incompatibility group) or selfings. Both groups were obtained with and without the aid of counterfeit pollination. As a result of selection of well performing genotypes, most of the selfing-based genotypes (many of them showing inbreeding depression), have been excluded in favour of hybrid types. Because this population was still excessively large, it was narrowed down. The plants indicated by an asterisk (*) have been selected either on the basis of performance, scale of testing for SI expression and their value for other research topics, or have been selected at random. The *S1S1* and *S2S2* genotypes and to a lesser extent the *S3S3* genotypes were the most important for the molecular unravelling of the SI-system (Kirch et al, 1989, Eijlander and Ficker, in prep). The *S4S4* genotypes are slightly underrepresented; this may partly be due to the low priority of obtaining this genotype, but the number was still lower than expected. Numbers are too small, however, to draw final conclusions on *S*-genotype-related fitness or certation.

Table 7. Strength of the SI reaction in selfings and intercrossings within incompatibility groups of selected SI-expressing *S*-heterozygous genotypes with or without using counterfeit pollination. SP = tested as seed parent; PP = tested as pollen parent. Ranking is from "1 = most PC genotype" to "9= most SI genotype", sc = self-compatible.

		Normal incompatible crossing			Normal + Counterfeit pollination		
Plant	Key	Self	SP	PP	Self	SP	PP
6104-09	2	9	9	9	8	8	8
6104-21	4	9	8	9	8	7	8
6222-05	15	8	9	7	5	7	9
6222-39	18	9	8	9	8	9	6
6101-11	1	9	9	6	8	9	7
6104-19	3	9	9	9	8	5	9
6104-23	5	8	7	8	8	8	8
6221-01	8	8	9	8	8	9	9
6221-05	9	7	6	8	7	7	8
6221-17	10	sc	8	sc	sc	7	sc
6221-19	11	7	8	8	8	7	8
6221-20	12	9	6	9	8	8	9
6223-40	23	9	9	5	8	9	7
6222-06	16	4	6	3	5	8	4
6222-24	17	7	6	4	7	8	6
6222-40	19	9	5	8	4	5	8
6105-06	6	8	7	8	7	7	8
6105-15	7	8	8	6	8	9	8
6223-01	20	8	9	7	8	8	9
6223-29	21	7	7	6	6	8	7
6223-39	22	8	7	7	7	7	8
6536-01	24	8	9	8	8	9	8
6536-02	25	9	9	9	8	9	9
6536-09	26	9	9	9	8	8	9
6221-32	13	9	8	9	8	9	9
6221-37	14	9	8	8	8	7	8

Table 8. Selected SI and SC homozygous clones derived from SI and SC clones respectively after (in)compatible pollinations using within incompatibility group pollinations or selfings in combination with or without counterfeit pollination ***Italic-bold*** : SC-genotypes. Method= obtained by counterfeit pollination (cf), selfing (se), non counterfeit pollination aided intra incompatibility class pollination (ii) or by a SC-based compatible cross (co). Fl. = flowering, P.F. = pollen fertility expressed as % acetic carmine stainable pollen, P.Sh. = pollen shed. Scales ranging from 1= extremely bad to 9= very good. SI-expr. = self-incompatibility expression expressed as seeds/berries/selfed flowers. n.d = not determined. ^ = vegetatively maintained

Plant	Parents	Method	Genotype	Vigour	Fl.	P.F.	P.Sh.	SI-expression as totals of Seeds/ Berries/ Flowers		
6496-01^	***IV*** x 3	cf	S1S1	8	9	57**	9	0 /	0 /	60
6496-04^	***IV*** x 3	cf	S1S1	7	8	71**	8	0 /	0 /	64
6499-04^	III x 3	cf	S1S1	7	8	96	7	0 /	0 /	73
1127-14^	6502-38 x 6496-01	ii	S1S1	8	8	88	7	0 /	0 /	58
1130-03	5 x 1	cf	S1S1	7	8	92*	8	0 /	0 /	22
1136-01	12 x 23	cf	S1S1	8	7	65**	7			n.d.
1136-02	12 x 23	cf	S1S1	7	8	74*	6			n.d.
1136-05	12 x 23	cf	S1S1	9	8	77*	7	0 /	0 /	33
1181-02	***G254*** x 6496-1	cf	S1S1	8	8	55**	8	0 /	0 /	25
1138-07	16 x 16	cf, se	S2S2	7	7	62**	6	n.d./	1 /	12
1138-08	16 x 16	cf, se	S2S2	7	7	69++	7	0 /	0 /	20
1139-03	17 x 19	cf	S2S2	9	7	63**	7	0 /	0 /	30
1139-05	17 x 19	cf	S2S2	8	8	70*	7	0 /	0 /	30
1140-01	6 x 22	cf	S2S2	8	8	55**	4	0 /	0 /	20
1140-02^	6 x 22	cf	S2S2	7	7	60**	7	0 /	0 /	54
1140-05^	6 x 22	cf	S2S2	7	7	70**	6	0 /	0 /	48
1146-02	16 x 19	cf	S2S2	8	9	90	5	0 /	0 /	22
6499-01^	III x 3	cf	S3S3	8	9	88	8	0 /	0 /	55
1130-01	5 x 1	cf	S3S3	7	3	50**	3	0 /	0 /	4
1138-04	16 x 16	cf, se	S3S3	7	6	64**	7	52 /	2 /	5
1138-08	16 x 16	cf, se	S3S3	7	7	72*	7	12 /	1 /	32
1142-02^	25 x 26	cf	S3S3	8	7	79	7	0 /	0 /	26
1171-01	24 x 24	cf, se	S3S3	7	7	66*	7	0 /	0 /	23
1095-04	23 x 11	ii	S4S4	7	7	69**	6	0 /	0 /	67
1095-06	23 x 11	ii	S4S4	7	7	75**	7	0 /	0 /	71
1134-01	11 x 11	se	S4S4	6	7	78*	8	n.d/	8 /	18
1147-04	23 x 11	cf	S4S4	7	6	71	6	0 /	0 /	12
6539-10^	see table 1	ii	S1S1-SC	8	9	99	8	2204/	10/	12
1132-07	12 x ***10***	co	S1S1-SC	5	5	51**	7			n.d
1132-20	12 x ***10***	co	S1S1-SC	7	6	75*	5			n.d

Self-compatibility in S-homozygotes

As mentioned before (Table 1), self-compatible clones were found some of which were *S*-homozygous. The *S2S2, S3S3* and *S4S4* self-compatible clones have already been obtained on a routine basis, as is partly shown in Table 1. This was not the case with *S1S1*. The crosses ***IV*** ***(=6225-05)*** x 3 (=6104-19) and ***G254*** x 6496-1, (in italics, bold: SC-clones; Table 8) were made with the secondary aim of testing the validity of the assumption made by Olsder and Hermsen (1976) that *tS1* does not cause mutual weakening when together in a (monohaploid) pollen grain with the complete *S1*-allele. Offspring populations of these crosses did not contain self-compatible *S1S1* plants. Progeny of these *S1S1* plants proved the absence of the SC-factor *tS1*. Plants 6539-10, 1132-07 and 1132-20, however, proved by IEF (Fig. 2) and test crossing to be *S1S1* homozygotes, but they were self-compatible (table 8) and capable of fertilizing other *S1*-expressing plants.The presence of the *tS1*-based SC-clones 1132-07 and 1132-20 (two *S1S1* SC clones out of 20 SC plants, P(k (n=20, p=1/4) $\leq$ 2) = 0.09) proves that even S1-*tS1* pollen is not completely superseded by *S4-tS1* pollen, although a certative disadvantage is very likely.

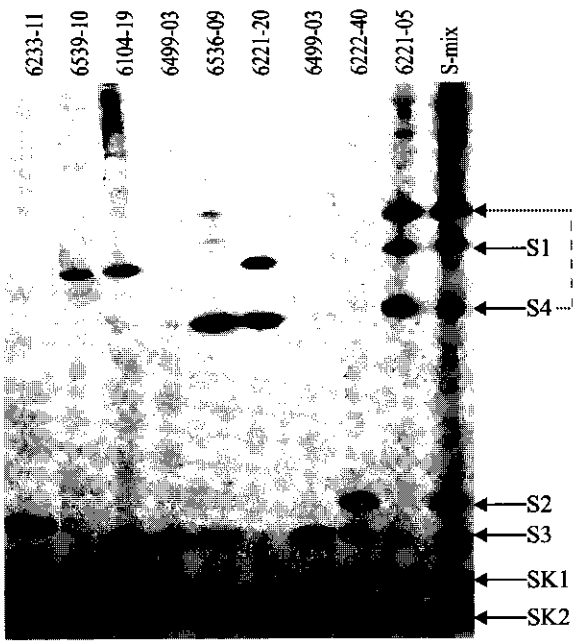

Figure 2 . Iso-Electric-Focusing (IEF) pattern of stylar extracts after silver staining of 3 *S*-homozygotes (6233-11, 6539-19, 6499-03) , 5 *S*-heterozygotes and a control sample. Approximately 15-25μg protein was added to each slot. *SK1* and *SK2* are style specific proteins but not *S*-locus related.. *S1*, *S2*, *S3* and *S4* are stylar expressed *S*-allele specific glycoproteins. *S4* gives facultatively a secondary band.

Maintenance of the selected genotypes by means of in vitro preservation, seed tubers and true seed production.

Most of the selected material is maintained in vitro, and the genotypes mentioned in Tables 3, 4 and 8 are also available in the form of seeds and tubers. Seeds were produced by crossing S-heterozygotes with *S*-homozygotes in order to produce 'single-class' *S*-heterozygotes, i.e., *S1S1* x *S1S2* gives rise to a single-class *S1S2* heterozygote. Such genotypes are available for all six combinations with the four S-alleles. Besides these, a limited amount of single-class homozygotes (in true seed form), derived from counterfeit pollination, is available for *S1S1*, *S2S2* and *S3S3*. One group of *S*-homozygotes, *S4S4*, was recently lost because of viral infection and is now only present in true seed form. The number of *S*-homozygous seeds from *S*-homozygotes with self-incompatibility was relatively small because of the difficulty of producing seed from their well functioning SI, even when counterfeit pollination was used (Table 8).

Discussion

In spite of the presence of a well defined monogenic, multiallelic, gametophytic type of self-incompatibility in diploid potato, the system was not amenable to a critical genetic and molecular analysis in the past for the following main reasons: 1) criteria for the classification of *S*-allele genotypes were less well defined; 2) well performing genotypes, or testers, of *S*-homo- and/or heterozygotes were not available and 3) potato being a highly heterozygous crop, inbreeding depression and the expression of deleterious recessive genes in the progeny were serious impediments for analyses. In the present investigation, an attempt was made to overcome the above mentioned drawbacks by the selection of well performing and well defined SI genotypes.

The classification of the genotypes of *S*-alleles in potato is complicated by the fact that self-compatibility often occurs either due to the so-called pseudo-compatibility or self-compatibilising factors, such as *tS1*, in an otherwise self-incompatible genotype. Classification of such genotypes in afore mentioned cases on the basis of berry and seed set, together with pollen tube growth studies (Hermsen, 1978 a, b), were relatively subjective in some cases. The identification of *S*-alleles through IEF (Kirch et al., 1989) combined with studies on pollen tube growth in styles as well as on test crossing was a step forward for a more reliable classification of the *S*-genotypes. Using a combination of the three criteria, well performing genotypes of both homo- and heterozygous genotypes for four different *S*-alleles have been selected in the present investigation. Accurate determination of *S*-allele genotypes through IEF was especially useful for the selection of self-

incompatible homozygotes as well as heterozygotes and for gaining more insight into the probable inheritance of pseudo-compatibility.

Olsder and Hermsen (1976) found a complete absence of self-compatible *S1S1* homozygotes. Segregation ratios displayed skewness and pollen certation or absence of mutual weakening between the *S1* allele and the hypothesised *tS1* was one of the explanations.
Mutual weakening between two identical *S2* alleles does not occur (crosses were made with a tetraploidised version of clone 1140-2, → *S2S2S2S2*, not giving a compatible reaction pattern in *S2*-containing styles, data not shown). This means that by applying the mutual weakening hypothesis on the ocurrence of *tS1*-based SC *S1S1* genotypes the self-compatibilizing factor *tS1* is *not* expressing the pollen part of the S1 allele but probably an independent gene. This was already suggested by the results obtained by Thompson et al (1991), although they did not rule out the possibility that only the pollen-part was translocated. Re-evaluation of old material of Olsder and Hermsen (1976) by IEF proved the presence of a previously undetected self-compatible *S1S1* plant. *S1 tS1* pollen has probably a certative disadvantage compared with the other three SC pollen types. This means that their *tS1*-hypothesis is not valid anymore, and one of the other six available hypothesis has to be accepted that was previously rejected because of the absence of self-compatible *S1S1-tS1* genotypes. The expression *tS1* is therefore actually an incorrect one. We have strong indications that self-compatibilizing factors like this "*tS1*" do occur much more frequently than is generally believed.

For producing tester genotypes, such as the *S*-homozygotes, showing typical SI-expression, it was essential to self the genotypes that showed strong SI-expression. In certain cases, such as 6105-06 and 6105-08 (Table 1), it was rarely possible to obtain berry set and a limited amount of seeds and progenies. The performance of these progenies with regard to vigour, flowering and pollen fertility (Table 4), however, did frequently not reach the acceptable levels observed in the progenies generated from the basic F1s (Tables 2 and 3). Moreover, the number of really well performing progeny genotypes in the case of 6105-6 and -8 was very low. Obviously, it was necessary to produce more progenies after selfing genotypes that were well performing and showing a strong SI reaction. In other plant species such as *Petunia* and *Nicotiana* , the so-called bud pollinations are practised for producing seeds and progenies from SI genotypes (Pandey, 1963; Shivanna and Rangaswamy, 1969;Clark et al, 1990). This method, however, was not applicable in potato, because the stigma becomes receptive only during anthesis when the exudate becomes available on the stigmatic surface. Self-pollinations, using the pseudo-compatible genotypes for producing seeds and progenies, could be another option for obtaining *S*-homozygotes.

Flaschenriem and Ascher (1979), Dana and Ascher (1985, 1986a,b) and Liedl and Anderson (1994) investigated aspects of PC in *Petunia hybrida*. This species traces back to interspecific hybrids and displays SC or a high level of PC. They often found strong PC at levels difficult to distinguish from SC. Expression of PC could be at either the pollen or the stylar side. Nevertheless, this system may have more in common with the hybrid system of *S.tbr* x *S.ver*, (like *S*-locus linked SC-factors and segregation of Unilateral Incompatibility factors) than with the SI system in diploid potato (Eijlander et al, in prep.). Complications and deviations from the normal SI system in hybrids is discussed by Trognitz and Schmiediche (1993). These authors tried to integrate the incongruity hypothesis of Hoogenboom (1973) with the normal gametophytic self-incompatibility hypothesis. Because the type of pseudo-compatibility investigated here seems to be a heritable character, both from the male and the female side, the SI-expression in the progenies might be weaker and the selections will be more frequently unreliable. It has been observed as likely for several crops that PC may be polygenic and heritable (Mather, 1943; Takahashi, 1973; Henny and Ascher, 1976; Litzow and Ascher, 1983) and offers a good explanation why inbreeding can lead to an increased level of PC.

In view of this, it is essential to avoid PC through careful progeny testing in such genotypes so that completely predictable types of self-incompatible genotypes are selected. The problem with bypassing the SI reaction in the style is that there might be a constant selection for PC expression on the pollen side. Although this is unavoidable, it might be reduced in its effect. For reducing this selection, stylar PC clones can be used when only strong SI at the pollen side is required (and vice versa). The use of PC is out of the question when pollen and style of the desired *S*-homozygotes are used in test crosses, unless a large progeny can be screened for reliable SI-clones. Additionally, the use of young flowers and at least one strongly SI parent is preferred for the production and selection of SI *S*-homozygotes. Here the counterfeit pollination with pollen from appropriate clones like IvP 35, 48 and 101 have proven to be of great help for obtaining otherwise extremely rare genotypes. This was also observed for difficult interploidy and interspecific crosses like *S.tbr* x *S.acl* and *S.sto* x *S.tbr* (Iwanaga et al, 1991; Singsit and Hanneman, 1991; Brown and Adiwalaga, 1991). As was already detected in some of the *S*-heterozygous genotypes that were highly SI after selfing, there still might be a level of PC present that can only be elucidated by test crossing with other clones. This has extensively been done with the selected *S1S1* and *S2S2* tester clones. A pitfall with testing *S*-homozygotes might be that in the style two identical *S*-alleles will be expressed. Because the SI reaction is a quantitative one, the *S*-glycoprotein content might be that high that a pollen expressed PC may remain undetected. Nevertheless, even *S*-homozygotes may be quite PC, as has been detected in genotype 6502-38 (Table 8) and a considerable part of its offspring. Therefore, *S*-homozygotes have to be tested for PC in crosses on heterozygotes as well. On the other hand, various tetraploid genotypes, that were present in our collection, have been tested for their SI

reaction against pollen from diploid relatives, expressing an identical *S*-allele. A *S1S2S3S6* genotype was completely incompatible with *S1*, *S2* and *S3* pollen (data not shown), thus indicating

that there can be a wide range for *S*-glycoprotein content in the style and/or the glycoprotein content needed for a reliable SI reaction. However, differences between expression levels of the *S*-alleles may reduce the efficacy of some of the *S*-alleles when the weakest are down-regulated, as is suggested by results of Kirch et al (1989) and Eijlander et al (in prep, see chapter 3). It also indicates that *S*-homozygous diploids can be quite useful in testing tetraploids.

The term pseudo-compatibility has often been used in other plant species although the definition of this expression is not clear cut. In a broad sense, it has been considered as 'leakage' of a functional incompatibility system. The criteria for considering a genotype to be pseudo-compatible are generally arbitrary. The basis is, however, the level of seed set in a self-incompatible genotype after self-pollination as compared with the mean seed set of the population, expressed as seeds/berry or the number of seed bearing berries/pollination. Such seed set can also result from a system where a self-compatibilising factor is operative (for discussion, see, Rowlands, 1964; Olsder and Hermsen, 1976; Hermsen, 1978a and 1978b). Pseudo-compatibility has also been observed in several crops after some cycles of inbreeding of self-incompatible genotypes (De Nettancourt, 1977). The mechanism of the origin of pseudo-compatibility in these crops is not clear yet. In the present investigation, as was also reported earlier (Olsder and Hermsen, 1976; Hermsen, 1978a and 1978b), there were genotypes that were difficult to be classified either as PC or SC. They were considered to be PC. Genotype 6221-17 (Table 3) is a typical example of the fact that even SC may be unreliable in its expression. It is quite possible that even this pollen expressed factor is influenced by modifier genes as has been observed for a comparable *S.verrucosum*-derived self-compatibilizing factor (Eijlander, unpublished). The level of seed set upon selfing was initially considered as an indication for a putatively useful level of PC in order to easily obtain *S*-homozygotes but this proved to be incorrect because of its inheritable character. In any case, all those genotypes that showed seed set upon selfing were eliminated and, as a consequence of this, only typical heterozygous SI genotypes were presumably selected. Such a careful selection was indeed effective as is evident from the fact that a large majority of the genotypes (20 out of 29, Table 3) was strictly self-incompatible after several rounds of selection during different years.

Because of the importance of plant vigour, fertility, avoidance of lethal genes and the high degree of heterozygosity required in the progeny plants, the performance of the basic populations used in this investigation deserve attention. The three original dihaploid clones, G254, G609 and B16 were known to be vigorous, profusely flowering and fertile (Olsder and Hermsen, 1976). The F1 progenies of these clones, on average, performed much better than the progenies obtained from

selfings or inbreds (Table 2). Furthermore, the number of *S*-heterozygotes that were selected originated predominantly (19 out of 31) from the progenies of the original F1s (Table 3) which is even more striking when the ratio of SI /SC is considered. This clearly indicates that even within a restricted number of genotypes that were used in this investigation, competent SI parents could be selected, giving rise to desirable progenies. From the point of view of the good performance of the progenies of the basic F1s and some of the inbred lines, it should be concluded that the establishment of inbred lines of diploid potato, comparable to those of maize, might be possible.

In view of the recent molecular approaches to elucidate the phenomenon of self- incompatibility in diploid potato (such as gain and loss of function analysis in genetically modified plants), genetically well defined plant material is essential. Part of the material selected in this investigation, which includes both *S*-homo- and heterozygous genotypes, expressing typical SI reaction, was highly valuable for this research. The two most important factors for using such selected clones as testers are the absence of PC and a good pollen fertility when used as a pollinator. This stresses the need for an extensive screening procedure as described here.

Chapter 3

Manipulation of self-incompatibility in diploid *Solanum tuberosum* L using sense and antisense constructs of *S*-RNase genes

Abstract

Diploid potato *(S.tuberosum)* expresses a one locus gametophytic self-incompatibility system. The so-called *S*-glycoproteins are style specific and are held responsible for the stylar part in the self-incompatibility (SI) interaction between pollen and style. The potato genes coding for *S1* and *S2* glycoproteins have been isolated molecularly and used for the construction of various homologous and heterologous sense and anti-sense constructs. Six different diploid potato clones, expressing either *S1* or *S2*, have been transformed with these constructs.

The anti-sense approach was most successful when the *35S* promoter was used, as opposed to anti-sense versions driven by the *S2*-RNase or *SK2* promoters. Transformation of genotypes displaying *S1* or *S2* incompatibility reactions resulted in compatibility with the corresponding *S1* or *S2* pollen, that gave incompatibility reactions in the non-transformed genotypes.

The sense approach confirmed the finding that the *S*-glycoproteins are directly involved in the SI reaction, because the introduction of strongly *S2* expressing constructs resulted in the predicted *S2* pollen inhibition. The constructs based on the *SK2* promoter were much more efficient in this respect, than those driven by the *S2* promoter. Introduction of *S2* driven by the *SK2* promoter resulted not only in gain-of function, but in some cases also in an efficient down-regulation of endogenous alleles like *S3* or *S10*.

Thus, the anti-sense approach gave a specific suppression of the target alleles, whereas the sense approach could not only add a new incompatibility group, but could also simultaneously suppress all other *S*-alleles. This possible effect should be taken into consideration whenever these types of constructs will be used for the production of hybrids in breeding programmes.

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Introduction

The phenomenon of self-incompatibility (SI) occurs in almost all diploid tuberous *Solanum* species, including the diploid forms (dihaploids, 2n=2x=24) of the cultivated (tetraploid, 2n=4x=48) potato, *Solanum tuberosum* L (*tbr*). As in the case of other *Solanum* species, in diploid-*tbr* SI is genetically controlled by the gametophytic system based on a single locus, the *S*-locus, with multiple *S*-alleles. Such *S*-alleles were detected in three dihaploids of *tbr* through diallele crossing and were identified as *S1*, *S2*, *S3* and *S4* by Hermsen (1978 a,b). Although the dihaploids that were heterozygous for *S*-alleles (e.g., *S1S2*) showed typical SI-reactions, there were also similar genotypes that were self-compatible (SC). The SC reaction was caused by a pollen expressed factor, called *tS1*, which was believed to be a translocation of the pollen part of the *S1* allele (Hermsen, 1978a; Thompson et al, 1991). Recently, using the same basic material, all possible *S*-allele heterozygotes (viz., *S1S2*, *S1S3*, *S1S4*, *S2S3*, *S2S4* and *S3S4*) with well defined SI reactions have been selected. In addition, homozygous genotypes for most of the *S*-alleles have been produced and are being maintained as tester stocks (Eijlander et al., 1997).

Besides the traditional methods of detection and classification of *S*-genotypes (Olsder and Hermsen, 1976; Hermsen, 1978a,b), gene products corresponding to the four *S*-alleles have also been molecularly characterised in the above mentioned plant material (Kirch et al., 1989). By analysing protein extracts from the styles of defined *S*-allele genotypes through two dimensional gel electrophoresis, the presence of a group of basic glycoproteins was established. It was further shown that each of the four *S*-alleles was associated with the presence of polypeptides differing in their isoelectric points and with the help of these SI-associated proteins (the *S*-glycoproteins or *S*-RNases) the *S*-genotypes could be clearly distinguished. A comparison of sequence homologies of *S*-associated glycoproteins of *tbr* revealed similarities with those of other solanaceous plants such as *Nicotiana alata* and *Lycopersicon peruvianum* (Kirch et al., 1989; Peil, 1995). Within the Solanaceae, the highly basic glycoproteins have been shown to possess RNase activity and, because of their specific association with the *S*-locus they are called *S*-RNases (Cornish et al., 1987; McClure et al., 1989; Clarke and Newbigin, 1993; Newbigin et al., 1993; Sims, 1993; Kowyama et al., 1994; Royo et al., 1994). In addition to these *S*-RNases, two more abundant proteins, that are designated as *SK1* and *SK2*, were also constantly present in the styles of most of the genotypes and these were non-*S*-linked pistil specific proteins. Of the two non-*S*-linked pistil specific proteins, the most abundant *SK2* polypeptide has been shown to be specifically located (through a immuno-cytochemical method) in the styles and proved to be an endochitinase (Wemmer et al., 1991; 1994), showing homologies with the tomato *ChiP* gene.

Genomic and cDNA clones, corresponding to pistil specific proteins, have been isolated and characterised in potato (Kaufman et al., 1991; Kirch, 1992; Li et al., 1994; Wemmer et al., 1994;

Kirch et al., 1995; Peil, 1995; Ficker et al., 1998a,b). These included two alleles, *S1* and *S2*, of the *S*-locus (Kaufman et al., 1991) and *SK2* of a non-*S*-linked gene (Wemmer et al., 1994). A functional analysis has been carried out for *S1*- and *S2*-RNase promoters as well as the promoter of *SK2* gene by using GUS as reporter (Ficker et al., 1998; and unpublished results). These analyses have indicated that the expression patterns of these genes may be strongly dependent on the type of promoter and the host plant into which they are introduced. Similar functional analyses on *S*-RNases (promoters and especially coding regions) in other solanaceous plants like Tobacco and Petunia have established that *S*-RNases are indeed responsible for SI reaction of the styles (Huang et al., 1994; Lee et al., 1994; Murfett et al., 1994). RNase activity was shown to be essential for a functional inhibition and gradually more information has become available about the identity determinants in the hyper variable regions of the *S*-Rnases (Mc Cubbin et al., 1997; Matton et al., 1997).

In view of the available functional information, together with the cloned genes and defined plant material, it was relevant to test whether the biological activity (i.e., SI-reaction) in diploid potato can be manipulated, as earlier described for *Petunia* and *Nicotiana*, through the introduction of *S*-alleles into appropriate plant genotypes. In this context, antisense versions of homologous and heterologous constructs of *S1* and *S2* alleles as well as a sense version of the *S2* allele were introduced into defined genotypes of potato through genetic transformation. The results of the transgenic expression of *S*-alleles in different types of transformants are described and discussed in this article.

Material and methods

Basic plant material

Two groups of diploid potato (*Solanum tuberosum*, 2n=2x=24) genotypes were used for genetic transformation. The first group consisted of two self-incompatible (SI) genotypes, *S1S4* (code: 195/5, Kirch et al., 1989) and *S3S10* (code: 6618-10-IV, El-Kharbotly et al., 1995). The second group consisted of four self-compatible (SC) genotypes, viz., *S1S3*: 6486-04 (R2); *S1S3*: 6486-19 (R5); *S1S10*: 6486-09 (R3) and *S2S10*: 6487-09 (V). The latter group of clones possessed pollen-based SC (homologous to the so-called *tS1*-like reaction, data not shown) , but expressed stylar specific SI reliably and was related to two interrelated well-transformable genotypes, A16 (El-Kharbotly et al., 1995) and 1024-02 (Jacobsen et al., 1989), see also Appendix 2. In addition, six diploid homo- and heterozygotes for *S*-alleles were used as tester pollinators in order to verify the SI reaction in the transformants. Among these six groups, three were SI-homozygotes, *S1S1* (6496-01, 6496-04, 6499-04), *S2S2* (6233-12, 6234-05, 1140-02) and *S3S3* (6499-01); and the other three were S-heterozygotes: *S1S2* (6222-39), *S2S3* (6222-40) (Eijlander et al., 1997) and *S2S10* (4002-04) (El-Kharbottly et al., 1996).

Plasmid constructions

All the constructs used (without showing vectors) are shown in figure 1.

Plasmids containing those inserts are addressed by the insert names. The construction of inserts and plasmids is described below.

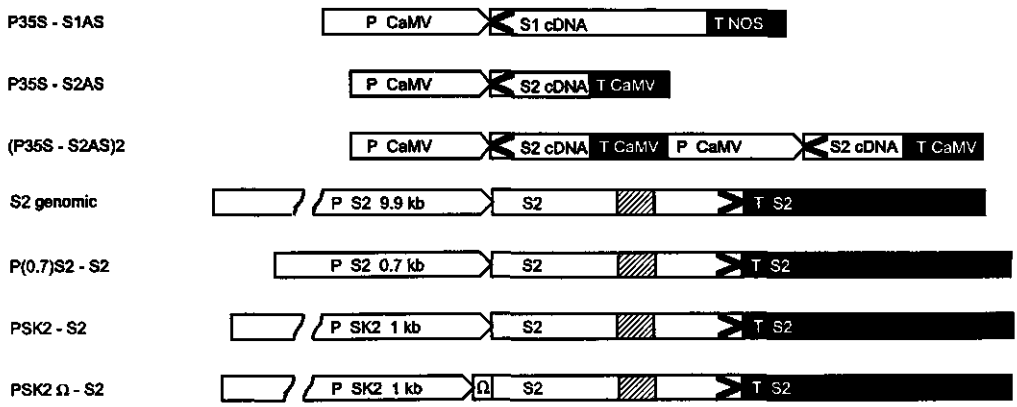

Fig. 1. Schematic representation of the inserts in the binary vectors pGDW32 (P35S-*S1*AS) and pBin 19 (from P35S-*S2*AS to P*SK2*Ω-*S2*). External arrow headed polygons: promoters (P) of CaMV and of the style specific genes *S2* and *SK2*. Promoters larger than 0.7 kb are indicated by broken polygons. Ω = enhancer fragment. Internally arrow headed boxes: sense (>) and antisense (<) orientated *S1* cDNA, *S2* cDNA or the intron (striped box) containing genomic *S2* coding region. Black boxes: polyadenylation sequence (T = terminator) of the CaMV or *S2*-RNase gene.

Anti-sense (AS) *S1&S2*

Type: P35S - *S1*AS. Plasmid pGDW*S1*AS was constructed by cloning into the EcoRI site of pGDW32 (Wing et al., 1989) a partial EcoRI digest fragment (P35S - *S1*AS) of 1,48 kb of pAP*S1*AS. pAP*S1*AS was constructed by insertion of a 0.7 kb SalI/BamHI fragment of *S1* cDNA into a 35S-NOS cassette of vector pAP (Kirch; Pereira, unpublished).Type: P35S - *S2*AS. Plasmid p35S-*S2*AS was constructed by cloning into the *Nco*I and *BamH*I site

of pRT104GUS the 320 bp *Bgl*II/*Nco*I fragment of the *S2*-RNase cDNA (plasmid pHK22, unpublished, genomic clone published by Kaufmann et al., 1991).

Type: P*S2* - *S2*AS. Plasmid P*S2-S2*AS was constructed by cloning into the *Nco*I/ *Hind*III sites of plasmid p*S22*-2 (Ficker et al.,1998 b) a fusion of a 320 bp *Bgl*II/*Nco*I fragment, extending from bp 127 to 447 of the *S2*-RNase cDNA and a 250 bp *BamHI*/*Hind*III fragment of pRT104GUS containing the *CaMV* terminator (Töpfer et al., 1993).

Type: P*SK2* - *S2*AS. Plasmid p*SK2*/1 contains the promoter of the style-specific endochitinase *SK2* (Wemmer et al., 1994) and has previously been described by Ficker et al (1998a, in press). Plasmid p*SK2-S2*AS was constructed by cloning into the *Nco*I and *Hind*III sites of plasmid p*SK2*/1 a 570 bp *Nco*I/*Hind*III fragment of plasmid p*S2-S2*AS, containing a fusion of the *S2*-RNase coding region in antisense orientation and the CaMV terminator.

Sense *S2*

Type: *S2* genomic. Plasmid pBin*S2* was constructed by the insertion of a 12,9 kb *Sal*I fragment of a genomic clone of the *S2* RNase (starting at approximately 9.8 kb upstream from the start codon) out of lG131/1 (Kaufmann, 1991) at the *Sal*I site of pBin19 (Bevan, 1984).

Type: *S2* genomic. Plasmid p(0.7)*S2-S2* was constructed by replacing the GUS-CaMV-terminator-fragment of p*S24* (Ficker et al., 1998b) by a 1.8 genomic *NcoI/ Sal*I fragment of *S2*, the *Nco*I restriction site containing the start codon.

Type: P*SK2- S2*. Plasmid p*SK2-S2* was constructed by cloning into the *Nco*I and *Hind*III sites of p*SK2*/1 a 1.7 kb *NcoI/Hind*III fragment of plasmid pLAT52*S2* containing the *S2*-RNase coding region and *S2*-RNase 3' flanking sequences (Kirch et al., 1995) .

Type: P*SK2 - S2*, Ω-enhanced. Plasmid p*SK2*Ω*S2* was constructed by cutting p*SK2S2* with *Nco*I and removing the nucleotide overhang with *S1* nuclease followed by an *Hind*III digest. The Ω-sequence was constructed by annealing partially overlapping nucleotides, Klenow fill in and cutting with *Nco*I. This Ω sequence is blunt at the 5' end and contains a *Nco*I site at the 3' end. The Ω sequence was fused with a 1.7 kb *NcoI/Hind*III fragment of p*SK2S2* and the resulting fusion was cloned into p*SK2S2* processed as described above. The 68 bp Ω sequence corresponds to the leader sequence of the TMV RNA strain U1 and acts as a translational enhancer (Wilson et al., 1993). The oligonucleotides used for constructing the Ω sequence were:

sense	5'GTATTTTACAACAATTACCAACAACAACAAACAACAAACAACATTACAA3'
antisense	5' CCCCATGGTAATTGTAAATAGTAATTGTAATGTTGTTTGTTGTTTGTTGT 3'

The afore mentioned inserts of plasmids p*SK2*Ω*-S2*, p*SK2-S2* and p*SK2-S2*AS were cloned into pBIN19 as *EcoRI/Hind*III fragments, p(0.7)*S2-S2* as a *Sal*I*/Hind*III, p35S-*S2*AS as a *Hind*III fragment and p*S2-S2*AS as a *KpNI/Hind*III fragment. A tandem insertion of P35S - *S2*AS resulted in (P35S - *S2*AS)2, with a mutated, non-cleavable *Hind*III site. All plasmid constructions were checked by restriction mapping.

DNA methodology

DNA isolation, subcloning, restriction analysis and screening of the genomic library were carried out using standard procedures (Sambrook et al., 1989).

DNA sequencing

DNA sequencing was performed with an automated DNA sequencer (Applied Biosystems model 373A) using the Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturers instructions.

Transformation procedures and vectors

For plant transformation the plasmids were introduced into *Agrobacterium tumefaciens* LBA4404

(Hoekema et al., 1983) by either electroporation (Wen-jun and Forde, 1989) or direct transformation of competent cells according to Höfgen and Willmitzer (1988). Potato transformation was carried out in two ways: 1) as described by Visser (1991) or 2) according to Flipse et al., (1994). Transgenic calli obtained by the method of Visser were selected on MS-medium (Murashige and Skoog, 1962) supplemented with 10 g/l sucrose, 2 mg/l zeatin, 0.01 mg/l NAA, and 0.1 mg/l GA3. Shoots were induced on MS-medium containing 30 g/l sucrose, 0.25 mg/l benzyladenine and 0.1 mg /l GA3. Explants inoculated according to Flipse et al (1994) were transferred two days after inoculation onto selective media (kanamycin 100 mg/l or hygromycin 25 mg/l) with MS medium supplemented with 20g/l sucrose, 1mg zeatin and both 200 mg cefotaxime and vancomycin. Hygromycin resistance was tested under a monthly recurrent selection cycle of two weeks of 25 mg/l hygromycin and two weeks of no selection pressure. Selected shoots were transferred to hormone-free MS-medium. All media were supplemented with 200 mg/l cefotaxime and 50 mg/l kanamycin or 10 mg/l hygromycin.

Protein gel electrophoresis

Up to 50 mg of plant tissue was ground in an Eppendorf tube with 20-100 μl 5 mM potassium phosphate pH 6.0, 2.5 % (w/v) sucrose, 0.1 % (v/v) b-mercaptoethanol, using a ground-glass pestle. Single style extracts were made in a volume of 25 μl extraction buffer.Total anther extracts were made by collecting all anthers of a flower and grinding them in 100μl buffer. After centrifugation of the homogenate at 14000g for 15 min, the supernatant was fractionated on horizontal thin-layer isoelectric focussing (IEF) poly-acryl-amide gels (pH3.5-10) (Schmidt-Stohn, 1979) or agarose gels (Hypure gel VG 1020, Isolab inc). Separated proteins were electro transferred to nitrocellulose filters using a 'semidry' procedure (Kyhse-Anderson, 1984) with a Sartoblot IIS (Sartorius, Göttingen).

Detection of IEF-separated and electroblotted proteins

Immunodetection was carried out using the ECL Western blotting detection system (Amersham Buchler, Braunschweig) basically as described by Kaufmann et al. (1991). Silver staining was performed as described by Kirch et al (1989) for the polyacrylamide gels and according to company specifications for the agarose gels.

Monitoring of incompatibility

Using transformed and non-transformed plants (controls) as pistillate parents, test crosses were made by using pollen from the three classes of *S*-allele homozygotes, *S1S1*, *S2S2* and *S3S3* or with the pollen of the heterozygotes *S1S2* (compatible with all transformants), *S2S3*, *S2S10* or *S3S10* depending upon the genotype and the reaction that was to be monitored.

Styles were harvested 48 hours after pollination and fixed and stained according to the modified technique of Martin (1959) and pollen tube penetration was observed under a fluorescence microscope (Eijlander et al., 1997).

Results

Antisense suppression

Antisense effect of the S1 *allele construct in* S1S10 *transformants*

After transformation of the diploid potato clone, 6486-09 (*S1S10*, pollen-expressed SC), with the *S1* antisense construct pGDW*S1*AS (containing full length *S1* cDNA), plants were selected for hygromycin resistance (hyg+). From the eight transgenic plants, five vigorously growing ones were further tested. They were monitored for: a) the copy number of the T-DNA inserts through Southern hybridisation, b) the presence/absence of *S1* glycoprotein through IEF followed by silver staining and c) the incompatibility reaction through the observation of pollen tube growth of *S1* tester pollen in the styles under a fluorescence microscope. The untransformed potato clone 6486-09 (R3) was used as a control.

A minimum of two copies of the construct was present in all selected transformants except for the clone R3-*S35S1*AS-8 that had a single copy. In agreement with the expectations a reduction in SI reaction, as compared to the control, was observed in tube growth of pollen from *S1S1* homozygotes, whereas in the case of the control plant there was complete inhibition of pollen tube growth of *S1* pollen. The transformants permitted different degrees of pollen tube penetration, with full compatibility being observed in R3-P35*S1*AS-24 (Table 1&2). In order to verify whether the styles of all the transformants confined to the expected norm of the SI reaction, they were pollinated with *S2* pollen and all were found to be fully compatible (Table 2). With regard to the style-specific proteins, IEF revealed that there was no strong reduction of the *S1* glycoprotein detectable in four

Table 1. Expression of antisense *S1* by P35-*S1*AS in five transformants of the *S1S10* - clone 6486-09 (R3). The plants were analysed for minimal copy number of pGDW*S1*as (insert: P35S-*S1*AS), the pollen tube ingrowth of *S1 and S2* pollen from *S*-homozygotes (*S1S1*: 6496-01, 6499-04 ; *S2S2*: 1140-02) in the styles by fluorescence microscopy (C = compatible, PC = Pseudo compatible, I = incompatible) and for the banding pattern after silver staining of single style extracts on IEF gels (-- = absent, - = barely visible, + = clearly visible, ++ = apparently normal level, +++= higher).

Clone number	# copies of pGDW*S1*as	SI reaction against *S1* and *S2*		IEF-pattern		
		S1	*S2*			
Untransformed	0	I	C	*S1* ++,	*S10* ++,	*SK1* ++
R3-P35*S1*as-3	≥2	I - C	C	*S1* ++,	*S10* ++,	*SK1* ++
R3-P35*S1*as-8	≥1	I - PC	C	*S1* ++,	*S10* ++,	*SK1* ++
R3-P35*S1*as-24	≥2	C	C	*S1* +/++,	*S10* +++,	*SK1* +
R3-SP351as-29	≥2	I - C	C	*S1* ++,	*S10* ++,	*SK1* ++
R3-P35*S1*as-32	≥2	I - PC	C	*S1* ++,	*S10* ++,	*SK1* ++

out of five of the transformants. As a comparison, the style expressed *SK1*, together with *S10*, the levels of which were expected not to be altered, are also indicated in table 1. This revealed that transformant R3-P35*S1*AS-24, which was fully compatible with *S1* pollen, showed a detectable relative reduction of *S1*, with an estimated 50% when compared to either its *S10* band or *S1* in the control plant.

Antisense effect of S2 *allele constructs in* S2S10 *transformants*

For the antisense *S2* approach, two constructs were used that were based on a 320 bp *S2* fragment. The constructs P35*S2*AS and (P*S*35S-*S2*AS)2, were used for the genetic transformation of the diploid potato clone 6487-09 (V); the latter construct contained a tandem duplication of the former insert within the vector pBin19. Clone V had the genotype of *S2S10* and showed like R2, R3 and R5, a pollen-factor-based self-compatible reaction.

A total of 30 transformants, 20 based on the P35-*S2*AS construct and 10 on the (P35-*S2*AS)2 construct, was tested for their SI reaction, by monitoring pollen tube growth, using pollen from *S2S2* homozygotes instead of selfing them. The clones had insert copy numbers ranging from 1 to 3.

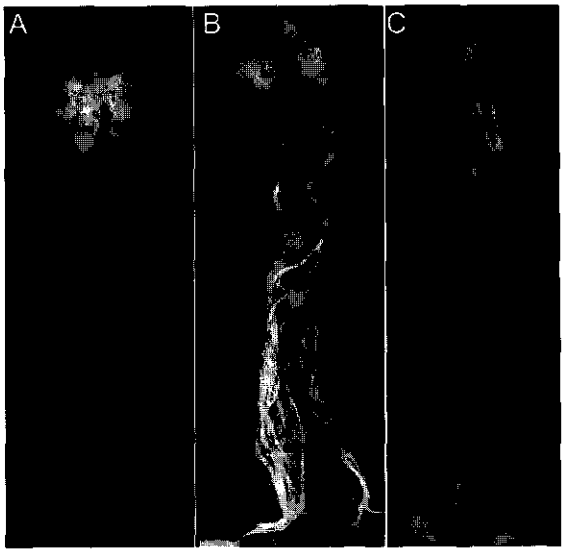

Figure 2. (Left). Fluorescence microscopy pictures of pollen tube penetration in styles.
A: Control pollination with *S2*-pollen on clone V (*S2S10*): incompatible.
B: Pollination with *S2* pollen on clone (P35S-*S2*AS)2-V-6, showing antisense *S2* effect: compatible.
C: Pollination with *S2* pollen on clone P*SK2*Ω-*S2*-VI-2, showing sense *S2* effect: incompatible. Clone VI is *S3S10*. Pollination of clone VI with *S2* pollen reacts as in panel B.

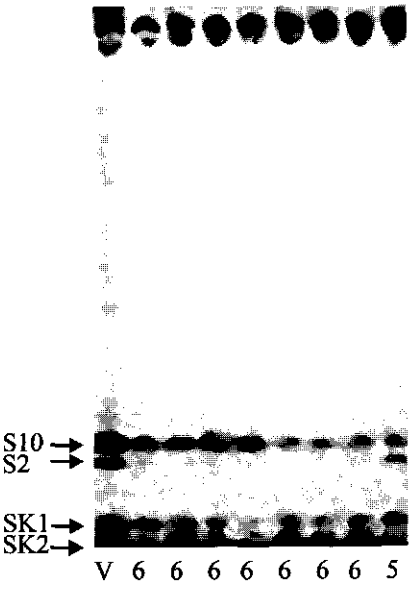

Figure 3. (Right) Silver stained Iso Electric Focusing pattern of style extracts of untransformed (V) and (P35S-*S2*AS)2 transformed clones 5 and 6. Single style extracts show a strong reduction of *S2* only in clone (P35S-*S2*AS)2-V-6 but not in transgenic clone 5 or untransformed clone V. *S2* and *S10* are S-glycoproteins. *SK1* and *SK2* are other style specific proteins.

A majority (23) showed a stable SI reaction. However, in some cases there was a tendency for pseudo-compatibility when the plants were still young, but at later stages typical SI was evident in all of them. The remaining seven transformants showed clearly antisense effects. Among those seven, three were phenotypically unstable by showing full SC and SI reactions in different flowers of one and the same plant when pollinated by *S2S2* plants. In the remaining four transformants, P35-*S2*AS-V-5&8, (P35-*S2*AS)2-V-4&6 (copy numbers 3, 1, 1 and 2 respectively), a strong and stable antisense effect was observed in all flowers. *S2* pollen was compatible and full pollen tube penetration was visible, indistinguishable from compatible control pollinations.

Accompanying the change in SI reaction (Figures 2A and B), IEF showed that the *S2*-RNase band was greatly reduced in intensity (Fig. 3: arrowhead), thus contrasting with the limited effect shown earlier for the antisense *S1* constructs (Table 1). There was clear correspondence between the reduction of *S2* protein and the compatible pollen tube growth of *S2S2* pollen in the styles.

Sense expression studies.

Expression of genomic S2 *constructs.*

pBin*S2* was used to introduce a 12kB genomic *S2* clone into two *S1S3* genotypes (with pollen-mediated SC). R2 (6486-04) gave rise to 38, and R5 (6486-19) yielded 32 flowering transgenics. These 70 plants were tested by IEF for *S2* expression in styles and leaves and in none of them was the *S2*-RNase detected. Some test crosses with *S2S2* pollinator genotypes were made, and , as expected from absence of the *S2*-RNase, all of them reacted compatibly. Two diploid transgenics (pBin*S2*-R2-1 and pBin*S2*-R5-35) with unknown T-DNA copy number were selfed (⊗) and 35 transgenic offspring plants from each were tested for *S2* expression. The two offspring plants pBin*S2*-R5-35⊗-33 & -39 showed a faint *S2*-band when stylar extracts were silver-stained after IEF, in intensity comparable with the *S2*-bands shown in figure 3 for the antisense S2 clones.

The cut-back promoter version of pBin*S2*, P(0.7)*S2*-*S2*, was transformed into *S1S3* clone R2 . One out of 22 flowering transgenic plants showed a weak *S2* band, as described above.

As expected from the low level of expression, none of these weakly *S2* expressing transgenics were incompatible with *S2*-pollen.

Gain of SI function by heterologous sense S2 *constructs.*

The two heterologous constructs P*SK2*-*S2* and P*SK2*Ω-*S2*, containing the *SK2* promoter and the coding region of *S2*, differed only by the absence or presence of the Ω-enhancer fragment, which is a translational enhancer. They were expected to be style specific and to give rise to a high *S2* expression (Ficker et al., 1998, in press). The P*SK2S2* construct was introduced into clone VI (*S3S10*) and P*SK2*Ω *S2* into the clones VI (*S3S10*) and 195/5 (*S1S4*). The presence of T-DNA was confirmed for 14 regenerants of clone 195/5 by PCR analysis and for all 11 tested regenerants of

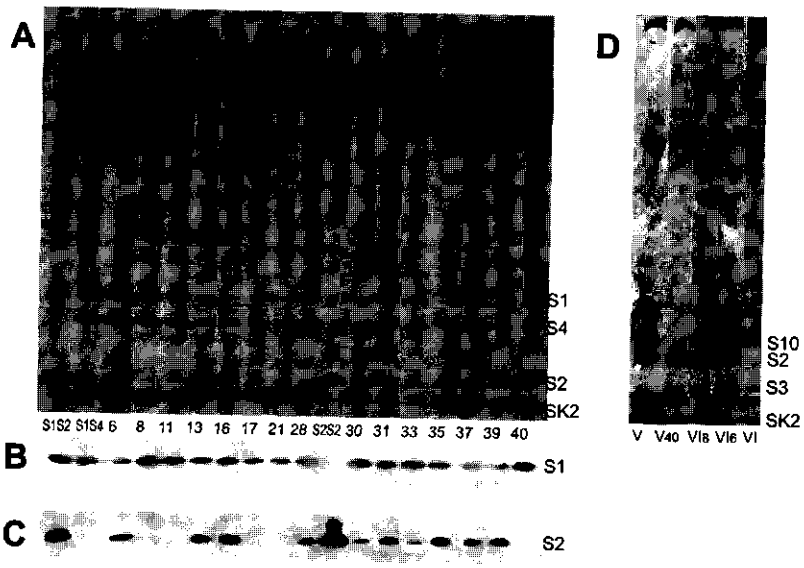

Fig. 4. Expression of the p*SK2* based heterologous constructs, driving *S2*

Panel A (top left). IEF patterns of style extracts of P*SK2Ω-S2* transformed clone 195/5 (*S1S4*) and controls (*S1S2*, *S1S4* and *S2S2*).

Panel B (middle left). Western blot with monoclonal anti bodies against *S1*, as a control on panel C, specifically detecting the presence of *S1* as detected by silver staining in panel A.

Panel C (bottom left). Western blot with monoclonal anti bodies against *S2*, confirming the presence and identity of *S2* in transgenics of panel A.

Panel D (top right). Over-expression of *S2* by P*SK2-S2* and P*SK2Ω-S2* in clone V (*S2S10*) and VI (*S3S10*). V40 shows down-regulation for *S10* and VI8 for both *S3* and *S10*. VI6 shows normal gain of function (*S3S10* -> *S2S3S10*). V and VI are untransformed controls.

the clone VI by Southern blotting. Southern blotting showed a copy number ranging from 1 (e.g., P*SK2-S2*-VI-2 and *SK2Ω S2*-VI-1) to 6 (P*SK2Ω-S2*-VI-2).

Ten out of 14 transformants of clone 195/5 showed IEF detectable *S2*-bands (Fig. 4A), up to endogenous levels of the other *S*-RNases or higher, and so did nearly all (16 out of 18) of the transformants of clone VI (Fig. 4D). Two transgenics (P*SK2Ω-S2* -VI-2&8) showed in several cases *much* higher *S2*-contents than the endogenous level. The identity of the IEF-detected bands on the *S2*-position as *S2*-RNase was confirmed by Western blotting (Fig.4B,C). The strength of the SI reaction coincided with the amount of *S2* glycoprotein present. Plants with high levels of *S2* expression showed strong incompatibility reactions and plants with normal levels allowed for a deeper pollen tube penetration (Fig. 2C) before the arrest was complete. Many plants were unstable

in the expression of the transgene in the course of time. High temperatures reduced the level of *S2* in most of the plants, causing a shift towards compatibility with *S2* pollen. The most stable genotypes (1/3 of the transformants) in VI and 195/5 were incompatible with *S2* under all circumstances. Some genotypes showed a barely detectable amount of the *S2* glycoprotein in entire anther extracts. Pollen from these plants did not show a shift from SI towards SC on non-transformed plants of the same genotype.

S2- *sense inhibition and -over-expression.*

Of the selected 21 P(0.7)*S2-S2* (sense) transformed plants (clone V, *S2S10* SC), none showed a clear-cut inhibition effect on the endogenous *S2* incompatibility reaction, which was in accordance with the apparent normal levels of stylar *S2*-RNase when analysed by IEF.

The constructs P*SK2Ω-S2* and P*SK2-S2* were also introduced into genotype 6487-9 (clone V, *S2S10*). Transformants were tested for SI by pollination with tester clones. None of the 28 tested plants showed a breakdown of the SI reaction against *S2* pollen. IEF showed *S2* levels at least as high as the endogenous concentration and under moderate climatic conditions often an enhanced *S2*-content was found when compared with *S10*. Two clones, P*SK2-S2*-V-8 & -40, synthesised (much) more *S2*-RNase, than that derived from the endogenous *S10* allele. Even excess of complete single style extracts (thus not standardised for total protein content) did show little or no *S10* glycoprotein when silver-stained but only *SK2* and *S2*-bands, as if the clones were *S2*-homozygous. This phenomenon was also incidentally observed for two *S2*-sense transformed *S3S10* plants, P*SK2Ω-S2*-VI-2 & -8 (Fig. 4D). Pollinations with *S2S2*, *S3S3* and *S2S10* plants revealed a loss of both the *S3*- and *S10*- incompatibility reaction, whereas the reaction against *S2* was maintained, thus reconfirming the key role of specific *S*-RNases in the incompatibility reaction. The indicated effects of the constructs used, are summarized in table 2.

Discussion.

S2 genomic clones: sense expression and sense inhibition.

Long *S2*-RNase promoter fragments fail to direct high-level style-specific expression of reporter genes in transgenic potato and tobacco (Kirch, 1992; Kirch et al., 1995; Murfett et al., 1995) and distally located cis acting regulatory elements have been postulated for an optimal level of expression. Ficker et al (1998 a, in press) conducted a functional analysis on middle-long and short versions of the *S2* promoter and hypothesised that there is an interaction between the coding region of the *S2*-allele and its promoter to regulate a proper tissue-specific activity. This hypothesis argues fairly against the expected result. But we (arguably) tested this hypothesis by transformation with a construct having a long promoter and one with a short one. Because all constructs used in this and the previous studies apparently lacked those distally located regulatory elements, high-level

Because the cut-back versions of the *S2* promoter showed nearly the same expression levels in activity studies (Ficker et al, 1998b) when compared with the larger promoter versions (up to 9.8 kb), little systematic effect was to be expected from our transformants based on either the small or the large promoters. Only a fortuitous integration of the homologous constructs in an "activating" site of the potato genome, containing such regulatory elements, might have given a good expression of *S2*. Passing through a sexual cycle, which also involves recombination, can bring about expression of transgenes that were not expressed before (see also review by Stam et al., 1997). For that reason, some offspring was tested on transgene activity as well. The low level of *S2* expression

Table 2. Overview of maximal effects detected in the antisense, sense and over-expression studies.
Blancs: not determined; - = presence not detected / pollen tube growth arrested; + = presence detected / full pollen tube penetration; blank = not tested; ++ = high contents, < = slightly reduced; <<< strongly reduced, barely noticeable.

Construct	Host plant		IEF detected glycoproteins					Pollen tube growth in styles				
	Clone	*S*-genotype	*S1*	*S2*	*S3*	*S4*	*S10*	*S1*	*S2*	*S3*	*S4*	*S10*
none (control)	R2	*S1S3*	+	-	+	-	-	-	+	-	+	
	R3	*S1S10*	+	-	-	-	+	-	+	+		
	R5	*S1S3*	+	-	+	-	-	-	+	-	+	
	V	*S2S10*	-	+	-	-	+	+	-	+	+	-
	VI	*S3S10*	-	-	+	-	+	+	+	-	+	-
	195/5	*S1S4*	+	-	-	+	-	-	+	+	-	
P35 *S1*AS	R3	*S1S10*	<	-	-	-	+	+	+	+		
P35 *S2*AS	V	*S2S10*	-	<<<	-	-	+	+	+	+		-
(P35 *S2*AS)2	V	*S2S10*	-	<<<	-	-	+	+	+	+		-
S2 genomic	R2	*S1S3*	+	+*	+	-	-	-	+**	-	+	
	R5	*S1S3*	+	+*	+	-	-	-	+**	-	+	
P(0.7)*S2 S2*	R2	*S1S3*	+	+	+	-	-	-	+	-		
P*SK2 S2*	V	*S2S10*	-	++	-	-	-	+	-	+	+	+
	VI	*S3S10*	-	++	<	-	<	+	-	+	+	+
P*SK2*Ω *S2*	V	*S2S10*	-	++	-	-	-	+	-	+	+	+
	VI	*S3S10*	-	++	-	-	-	+	-	+	+	+
	195/5	*S1S4*	+	+	-	+	-	-	-		-	

*) = in offspring after selfing; **) = also in offspring after selfing; <<<: in offspring<<

in expressing ofsspring was comparable to that of the single expressor plant containing the 0.7kb promoter version of pBin*S2* and this was in accordance with the findings of Ficker et al., (1998 b) after transformation of a comparable construct into tobacco. It was, due to the quantitative nature of the SI response, not sufficient to promote a functional SI reaction against *S2*-pollen. The detection of sense-inhibition is therefore highly unlikely if this depends on the transcription level of an inserted construct.

Antisense *S1* and *S2* effects.

The antisense effect for *S2* is indisputable, although the phenomenon was not expressed in all plants stably. It was shown that the P35 based antisense *S2*-constructs were transmitted through the gametes. Offspring clones like 1184-01 (= *S2S2* +(P35-*S2*AS)2, derived from crossing (P35-*S2*AS)2-V-6 with the *S2S2* clone 1140-02) showed the expected antisense effect (data not shown). The high percentage (80%) of transformants showing some antisense effect for *S1* (by pGDW*S1*AS, Table 1) is most likely due to the selection procedure, because only a few stably Hyg^+ responding transgenics were obtained, whereas Kan^+ based constructs would have yielded many more transgenics. The reduction of the *S1*-glycoprotein has even in clone R3-P35-*S1*AS-24 not been so dramatic as observed for *S2* in various transgenics derived from clone V. The S10 allele belongs probably to the *S1/ S3/ S1R* family (Kirch et al., 1989) because *S1* and *S3* specific primers allowed for PCR- amplification and *S1* probes strongly hybridised with *S10* in RFLP analyses (data not shown). Comparison of the *S1* signal with that of *S10* can, therefore, give a false impression of absence of antisense effect on protein level when *S10* is reduced as well. Antisense affecting alleles has also been reported by Lee et al (1994) for the *S2* and *S3* alleles in *Petunia inflata*. I.E.F based comparison with the *SK2* signal may be difficult, because this signal is in general very strong, in this way a 50% reduction of *S1* may remain undetected, whereas *SK1* is inappropriate for this due to its unpredictable level of expression. There remained only one transgenic with some demonstrable reduction of the *S*-RNases, but this reduction was not as strong as detected for some antisense *S2* transgenics. Nevertheless, clone R3-P35-*S1*AS-24 was constant and reliable in this acquired *S1* compatibility. It is possible, that the *S1*-RNase content in styles of clone R5 was initially lower than the *S2*-RNase in styles of clone V, and it might also be that in these clones the activity of *S1* and *S2* RNase differs, thus explaining why an antisense induced reduction of *S1*-RNase content with 50% is much more effective than a comparable reduction of *S2*-RNase.

There was no seed set on the stable antisense *S1* clone, because the chromosome number of this plant was spontaneously doubled during transformation. This was also true for some of the *S2* antisense plants. The diploid transgenic clone P*S2-S2*AS-V-6, showing such a strong *S2*AS effect (Fig. 2B, 3), had a disturbed female fertility and set only limited seed in all pollination types. The male fertility, however, was nearly unaffected. Due to this and to the presence of the pollen-expressed self-compatibilizing factor, the aforementioned *S2S2* - *S2*AS clone 1184-01could be obtained, and this allowed testing of the heritability of the AS-effect. The weaker or less stable expression of (P35 -*S2*AS)2 in this clone might be due to a reduction in transgene copy number from two to one by meiotic-recombination. In addition to this, or instead of this, the homozygosity of the *S2*-allele could account for a weaker AS-effect than

in the parental clone. This double contribution in an endogenous *S*-homozygous plant is, according to Mc Cubbin et al (1997), also the most likely explanation why their introduced mutated *S*-protein was less effective in breaking down the stylar part in SI (probably by competition/multimer formation with the homologous endogenous glycoprotein) than when present in an *S*-heterozygous plant.

Sense expression of heterologous constructs.

The sense-expression of *S2* driven by the (Ω)*SK2*-promoter is in accordance with the strong and tissue-specific expression of the reporter constructs (Ficker et al., 1998 a, in press) and is also supported by the results of Murfett et al (1994), using constructs driven by the promoter of the *SK2* tomato homologue *ChiP*. *S2*-RNase content in styles of *S*-allele transgenics can surpass that of the endogenous *S*-alleles The studies by Ficker et al (1998 a,b) pointed out that there might be a specific interaction between promoter, coding region and postulated distally located regulatory elements for a tissue specific expression of the *S*-alleles. The *SK2*-promoter was in this respect different from the *S2*-promoter. The down-regulation of endogenous *S*-alleles might, therefore, be a logical result of over-expression of *S2* when driven by the *SK2* promoter. The heterologous construct may be less sensitive to some down-regulating mechanisms or might even lack some of those regulatory elements. The *SK2* promoter being this effective is in accordance with the success reported for the homeologous promoter *ChiP*. Matzke et al (1989) reported epistatic suppression by reversible methylation, which might be the case here too, because the apparent suppression of *S10* in the transformed V and VI clones disappeared and reappeared with the fluctuation of the greenhouse temperatures. The suppression of *S10*, which was not detected in all *S2*-expressing transformants, stresses the need to combine IEF with the utilisation of *S*-homozygous tester lines, for test crosses with *S2S10* pollinators would have given conflicting results: high S2 expression and nevertheless compatibility in test crosses.

Ficker et al (1998 a, in press) reported that *GUS*-expression of the heterologous P*SK2-GUS* constructs could also be found in anthers, and this was in accordance with the observations (Murfett et al., 1994) on the heterologous constructs based on the *ChiP* promoter (*Chi2;1* encodes, as stated earlier, a tomato homologue of *SK2*). This can explain why some of the transformants showed a low but detectable level of *S2* in total anther extracts during the periods of high stylar expression. It was not determined whether the *S2*-RNase was present in pollen only or that it had a sporophytic origin. The pollen fertility appeared not to be affected by the presence of *S2*-RNase, which is supported by observations of Kirch et al (1995).

It is now confirmed for potato by sense-, antisense- and over-expression of the *S2*-allele that the style-specific *S*-RNases are the key factor in the stylar contribution to the gametophytic self-incompatibility reaction. It has also become clear that a reliable gain and loss of specific SI reactions by a molecular approach is not so evident, but manipulation of the stylar expression of SI in potato is shown to be possible now.

Chapter 4

Expression and inheritance of self-compatibility and self-incompatibility after crossing diploid *S.tuberosum* (SI) with *S.verrucosum* (SC)

Abstract

Diploid potato, *Solanum tuberosum (tbr)*, and *S.verrucosum* (*ver*) can hybridise when the latter species is used as the pistillate parent but not when used as the staminate parent. This phenomenon, called unilateral incompatibility (UI), is frequently observed when a self-compatible (SC) species is intercrossed with a self-incompatible (SI) one. *S.verrucosum* is such a self-compatible species. *Ver* x *tbr* hybrids display cytoplasmic male sterility (CMS) and are, therefore, not suitable for genetic analysis of other crossing barriers. Previously, specific diploid *tbr* clones, called acceptors, were detected that showed bilateral compatibility with *ver* instead of UI. These selected clones were used to realize the reciprocal crosses in order to circumvent CMS and to create, by repeated backcrossing, *ver* with *tbr* cytoplasm. The resulting F1's were both male and female fertile. This "acceptance" for *ver* -pollen is based on the presence of a dominant gene *A* (acceptance), in combination with the absence of an inhibitor gene *I*. The F1's showed only expression of the *S*-allele that was derived from the *tbr* parent. It was shown that this *ver* does not produce style-specific *S*-glycoproteins. *S*-glycoproteins are responsible for the stylar contribution to the self-incompatibility reaction in potato. The F1-populations investigated here, were SC, but skewed segregation ratios for this trait, and disappearance and re-appearance of SC showed up in the following offspring generations. These deviations from the expected behaviour could be explained by postulating a more complex interaction of the acceptance (of *ver* pollen) determining genes *A* and *I*, the involvement of SI governing *S*-alleles from *tbr*, a stylar non-active S_{ver} allele (*S*v) and a weakly *S*-locus-linked pollen-expressed SC factor (SC_{ver}) from *ver*, resulting in "SI by UI based inhibition of SC", is explained hereafter. The presence of the stylar non-active S_{ver} -allele allowed for the penetration of *ver*-pollen in styles of hybrids when the recipient was of the genotype *A* ii* and for any *tbr* pollen that did not express style-active *tbr- S*-alleles. The latter behaviour is normal in any gametophytic SI reaction. Pollen containing simultaneously an active *tbr S*-allele and the SC_{ver} pollen factor was not effective in causing SC when the recipient was of the genotype *aaI**. It caused, however, the expected SC reaction on any other genotype, irrespective of the *tbr S*-alleles active in both parents. Thus, *aaI** non-acceptor genotypes, containing SC_{ver}, are SI by UI based inhibition of SC.

Tetraploid hybrid genotypes, obtained from doubling an SI non-acceptor diploid hybrid, produced

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pollen that showed mutual weakening. This was caused by in interaction of the pollen-active *tbr S*-allele and the, apparently pollen-active, S_{ver} allele from *ver*. Styles of non-acceptor recipients showed for this type of pollen a complete break-down of the SI and UI reaction in the upper part of the style, whereas in the lower part inhibition reactions reappeared, thus indicating at least two different factors in UI, which may coincide with "*a*" and "*I*". Thus, it was concluded that at least four different loci are involved in the expression of UI: the acceptor locus *A/a*, *I/i* for inhibition, the compatibilizing factor SC_{ver} and the pollen part of the regular *S*-locus.

Introduction.

Many plant species have developed systems against inbreeding. They can be based, for instance, on morphological features by which self-pollination is prevented, or on differences in maturation time of pollen and style. In some species heteromorphic characteristics are linked with incompatibility genes controlling pollen-pistil interaction (e.g. *Primula vulgaris,* Richards, 1986), but homomorphic self-incompatibility is more common. Based on the type of interaction between pollen and pistil, two major self-incompatibility (SI) systems can be distinguished: sporophytic and gametophytic. In the sporophytic system the SI reaction is determined by the expression of the parental sporophytic genotypes in the pollen and in the pistil. In the gametophytic system it is based on the expression of the individual alleles present in the pollen (the gametophyte) itself and in the style.
Interspecific and higher order crossing barriers exist in nature. Quite often, related species can be intercrossed reciprocally. Sometimes intercrossing is possible in one direction only. When this is based on processes between pollen landing and fertilization, it can be called unilateral incompatibility (UI), although the expression "incongruity" is also often used instead of incompatibility, when not referring to SI (Hogenboom, 1973). UI is most frequently found in crosses between SC and SI species. UI following the SI/SC rule can be found throughout the two major incompatibility systems. For an extensive review on (unilateral) incompatibility may be referred to a monograph by de Nettancourt (1977), which is still informative. More recently, Mutschler and Liedl (1994) gave a good overview on interspecific crossing barriers in *Lycopersicon*, and they favoured the opinion that SI and UI are discrete barriers, although they admit that there might exist systems in which SI contributes somehow to UI. A more refined and extensive theoretical approach on unilateral incongruity by Trognitz and Schmiediche (1993) deals with all kinds of interactions, involving a limited set of necessary genes with a limited number of essential alleles, but even with this approach it remains difficult to draw conclusions about the correctness of Hogenbooms incongruity hypothesis (1973).
One of the debates that is ongoing over the past 40 years, is whether or not the *S*-locus is involved in the UI reaction. It has always been tempting to assume such a genetic relation, because UI and SI often have several aspects in common, and a strong correlation may be observed between UI on

the one hand and parental species being SI and SC on the other; this suggests to be more than a coincidence. Of course, many arguments have been countered by exceptions and erratic results (Mutschler and Liedl, 1994). Nevertheless, there has been an accumulation of experimental evidence that SI and UI are frequently associated (Lewis and Crowe, 1958; Chetelat and DeVerna, 1991; Hiscock and Dickinson, 1993). Lately Murfett et al (1996) carried out transformation experiments with *S*-gene based constructs in *Nicotiana* and demonstrated that *S*-glycoproteins(also called *S*-RNases, e.g., McClure et al., 1993) can contribute to at least a part of the UI reaction.

One of the reasons why the debate is still ongoing, might be that different taxons may show UI with different strengths and reaction patterns, based on different numbers and types of genes and alleles. The extrapolation of results from one species to another relative may, therefore, be inappropriate. Potato, *Solanum tuberosum* L. (*tbr*) is a self-compatible crop, but this can be attributed to its ploidy level (2n=4x=48). Mutual weakening between different *S*-alleles in the same pollen grain makes that the species is self-compatible. Potato dihaploids (2n=2x=24) are usually SI, although exceptions to this rule do occur (Olsder and Hermsen, 1977; Hermsen, 1978 a,b,c). These dihaploids display UI when crossed with the self-compatible species *S. verrucosum* (*ver*). This species is closely related to potato and even belongs to the same series (Tuberosa). The appearance of SC in a putative SI potato plant does not necessarily cause a shift in the UI/SI relationship. The SC diploid potato clones G254 and B16 (Olsder and Hermsen, 1977) did not cause a break-down of UI when pollinated by *ver*, and remained compatible as staminate parent with all other diploid potato clones. Hybrid offspring of these two clones segregated, however, into acceptor and non-acceptor clones for *ver* pollen. Acceptance is the exception to UI, thus non-acceptance is equivalent to UI. The simplest and best fitting hypothesis is based on two stylar active genes, the acceptor gene *A* and its inhibitor gene *I*. Both genes are dominant, but *I* is epistatic over *A*. So, only *A..ii tbr* genotypes are acceptor (Hermsen et al, 1974). Analysis of other types of plants, including *S.andigena* based dihaploids and other accessions of *ver*, revealed differential behaviour of pollinator and recipient (Hermsen et al, 1977; Hermsen and Sawicka, 1979), thus refining the model. The possibility of the involvement of more alleles and/or more genes was discussed here, and gene-for-gene relationships, pollen penetration capacity, differential reactions to foreign pollen and unilateral incongruity as proposed by Hogenboom (1973) were included, but the results were not conclusive.

The reason why a species is self-compatible can play an important role in answering the question which factors are really involved in the UI reaction between SC and SI species. From *ver* no active *S*-allele products are known (Kaufmann et al, 1992). They claimed that a *ver* x *tbr* hybrid (male sterile), expressing the *S1* allele from *tbr*, was unexpectedly compatible with *S1* pollen from the latter species, thus giving rise to *S1S1* homozygotes. They postulated, therefore, the existence of a *ver* borne style-expressed suppressor of the *S1* function.

The existence of the acceptor lines as described by Hermsen et al (1977) and Eijlander et al (1997) allowed for more detailed analysis of male and female fertile backcross progenies. Now the

segregation of SC related factors, *S*-alleles and UI could be tested for their interrelationship. Here the results of those experiments are presented and it will be discussed which factors play a role in the SC behaviour of *ver* and how they are related to the UI, SI and SC factors as reported for the *tbr* material used here.

Materials and Methods

All diploid potato clones used here, with the exception of clone 1024-02, are offspring from the three basic *tbr* clones G254, G609 and B16, and were used earlier as basic material for the investigation of the gametophytic SI-system and UI (Hermsen et al, 1974; Olsder and Hermsen, 1976; Hermsen, 1978, a,b,c; Eijlander et al, 1997). In this material genotypes were found that were acceptor (*AAii* or *Aaii*) for *ver* pollen, and non-acceptor clones were based on the absence of the acceptor gene *A* (thus *aa***) or on the expression of the dominant inhibitor gene *I* (thus ***I**).
The offspring clone 6233-11 = *S2S2* and non-acceptor (NA) of the type *aaII*, 6234-08 = *S3S3* and acceptor of the type *AAii*, clone 6536-01 = *S3S4* and acceptor of the type *AAii* and full sib clone 6536-02 = *S3S4* and acceptor of the type *Aaii*. These clones and all other diploid *tbr* tester clones (1140-02, 1140-05, 6104-21, 6221-17, 6221-32, 6221-30, 6221-39, 6223-39, 6223-40) used here, have been assesses earlier for their SI reaction by Eijlander et al (1997). Clone 1024-02 (Kuipers et al, 1994) has the genotype *S2S10*, is non-acceptor and, as mentioned before, not related to G254, G609 or B16. The clone was used as pistillate parent in some test crosses.
All *ver* pollen parents were full sibs from *ver* accession PI 195172, or offspring of those sibs. This accession was chosen because among the ver accessions tested on various acceptor *tbr* clones, PI 195172 had the highest pollen penetration capacity (Hermsen et al, 1974).
The backcross population VTV= (*ver* PI 195172 x G254) x *ver* PI 195172, segregates for *S1*, *S3* and *Sv*, all clones being 100% cytoplasmic male sterile. The F1 VT (*ver* PI 195172 -27 x *tbr S2S3*) is also CMS and contains *S2Sv* and *S3Sv* genotypes.
TV^5 = *tbr* (acceptor)-*ver* hybrid, backrossed 4 times with various *ver* accessions (mainly and at least the last time with *ver* PI 195172) ≈ 97% *ver*. It contains *S*-alleles from *tbr*. All plants were male fertile and self-compatible. $TV^6 = TV^5$ x *ver* PI 195172. TV^5 and TV^6 were used in backcrosses with non-acceptor *tbr* genotypes.
The population 6484 = TV^6 x *tbr* 6233-11 (*SvSv* x *S2S2*, *aaII*) consisted of fourteen plants.These F1 hybrids are nearly pure *ver-tbr* hybrids, male and female fertile and non-acceptor (*S2Sv*, *a*I**); the full sib clone 6251-19 = (TV^5 x *tbr S2S3 aaIi*) x (TV^5 x *tbr S2S3 aaIi*) = *S3Sv*, NA and SI; population 1173 (11 plants) = *tbr* 6234-08 x 6251-19 (*S3S3*, acceptor x *S3**,NA).
The population 6541 (25 plants) = *tbr* 6234-08 x *ver* PI 195172 -27 (= *S3S3*, acceptor x *SvSv*, *ver*) = F1 hybrid. Clones from population 6541 (all expected to be *S3Sv*, acceptor) were randomly

picked and backcrossed with *tbr* 6536-01 or 6536-02 (both *S3S4*, acceptor): 1051 (35 plants) = 6541-03 x 6536-01; 1052 (30 plants)= 6541-03 x 6536-02; 1053 (65 plants)= 6541-06 x 6536-01; 1054 (30 plants)= 6541-06 x 6536-02 and 1055 (30 plants)= 6541-11 x 6536-01.
Populations 1061 to 1066 (up to ten plants each) were obtained by selfing of respectively the F1 clones 6541-01, -02, -03, -06, -11, -25.
Pollinations were manually performed. Flowers, used for controlled pollinations, were emasculated prior to anthesis. Compatibility was tested by observing berry and seed set after pollinating at least 5 flowers. In case of doubt pollen tube ingrowth was monitored by means of fluorescence microscopy as described by Eijlander et al (1997). Penetration capacity was classified based on the amount of pollen tubes capable of penetrating upper and lower stylar sections. *S*-alleles and some other style specific proteins (like *SK1* and *SK2*; Kirch et al (1989)), were determined by making test crosses with tester lines (Eijlander et al, 1997) and/or by means of Iso-Electric Focusing (IEF) of stylar extracts on poly-acryl amide or agarose gels. PAGE was performed as described by Kirch et al (1989) or by means of pre-cast agarose gels (Hypure gel VG 1020, Isolab inc.) following the silver staining procedure based on Tungstosilicilic acid in stead of sodium permanganate, according to company specifications.

Results.

Expression studies on *tbr S*-alleles in *ver* cytoplasm.

Kaufmann et al (1992) detected in a *ver* x *tbr* hybrid (*S1Sv*) that some offspring plants after backcrossing with *S1S3* genotypes did not show the expected *S3* allele, but only the *S1* allele; the postulated *ver* borne allele *Sv* does not produce a detectable basic glycoprotein. Two likely explanations for this type of progeny are parthenogenesis (Abdalla, 1970) or suppression of the *tbr S*-allele (*S1*) in the hybrid. Presence of such phenomena could impede research on SC/SI, non-acceptance (NA, here equivalent to UI) and the possible relation between UI and SI in our hybrid system. Therefore, the (*ver* x *tbr*)x *ver* BC1 (TVT, in *S*-alleles: *SvSv* x *S1S3*)x *SvSv*) and the *ver* x *tbr* F1 hybrid *SvSv* x *S2S3* were tested for suppression of *tbr S*-alleles in the styles. A limited set of genotypes was tested for *S*-alleles by means of IEF and of test crosses with *S1S1*, *S2S2* and *S3S3* (Table 1). As expected, all plants were male sterile. All the *S*-alleles of *tbr* that were present in the F1 or BC1, appeared to be functional in respect of *S*-glycoprotein production and incompatibility reaction against the same *S*-allele. Thus, in this material, no indications for genic or plasmic SI suppression were observed, and the types of *ver* that were intended for further investigation on SC and UI, could therefore be used.

Table 1. Incompatibility reaction of VT F1 and VTV BC1 plants in response to *S1*, *S2* and *S3* pollen.
VT = *ver* PI 195172 -27 x *tbr S2S3* ; VTV = (*ver* PI 195172 x *tbr S1S3*) x *ver* PI 195172. *S*-alleles were detected by IEF followed by silver staining. *Sv* = postulated *S*-allele from *ver*, no band visible; *S1/S2/S3*: *S*-alleles derived from the *tbr* clones. Pollinations were made with *S1S1*, *S2S2* and *S3S3* homozygotes (Eijlander et al, 1997). Berry set is indicated by + or -, based on 5 to 10 pollinations ; blank = + expected, but not tested. Average seed set in case of berry formation ranged from 31 to 107.

Clone	*S*-alleles	Berry set or polle tube ingrowth after pollination with:		
		S1-pollen	*S2*-pollen	*S3*-pollen
VT-4	*S3*		+	-
VT-6	*S2*		-	+
VT-7	*S2*		-	+
VTV4-1	*Sv*	+		
VTV4-5	*S1*	-		+
VTV5-8	*S3*	+		-
VTV6-2	*Sv*	+		+
VTV7-1	*S3*	+		-
VTV7-4	*Sv*	+		+

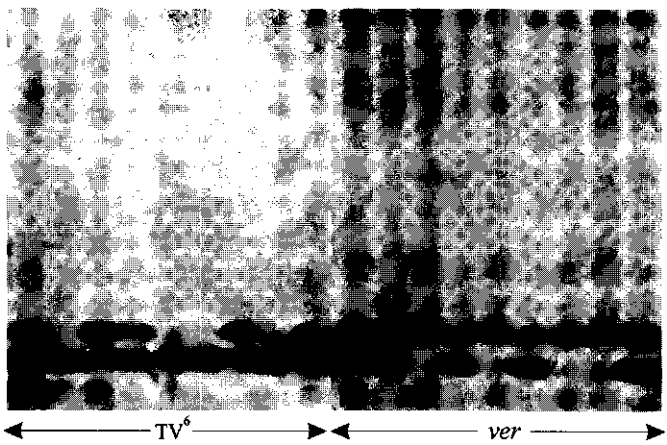


Fig 1. IEF pattern of stylar extracts after silver staining of a population of nearly-*ver* TV[6] (left panel) and *ver*. Two bands of basic proteins are visible: *K1* (upper) and *K2* (lower). Segregation of the bands of TV[6] and *ver* can be explained by assuming that TV[5]=*K2K2* was crossed with *ver* =*K1K2* or vice versa and that the *ver* population tested here, oroginated from the cross *ver1* x *ver2* = *K1K1* x *K1K2*.

Segregation and allelism of style specific proteins in *"S.verrucosum"* and its hybrid offspring.

For *ver* 15 full sib clones and for TV[6] eight clones were tested for SC and the presence of stylar proteins. All 23 genotypes were, as expected, SC. Both populations segregated for two proteins (Fig. 1), abundantly present in the most basic (pH>7) part of the IEF gel (silver stained). Those proteins were not visible in extracts of leaf and stem tissue. Because of their localisation and their presumed non-*S*-allele nature, they were designated *K1* and *K2*, analogous to the style specific non-*S*-linked *tbr* polypeptides *SK1* (presumably an RNase: Lee et al, 1992; Thompson et al, 1995) and *SK2* (an endochitinase: Wemmer et al, 1994). *K1* focused approximately at the *SK1* place, but could be identified by a more redish colouring after silver staining. *K2* focused even more basic than *SK2*. Based on observations as shown in the figures 1 and 2, the ranking from acid to basic of the stylar proteins appears to be as follows: *S4* (1st band), *S1*(not shown here), *S4* (main band), *S2*, *S3*, *K1*, *SK1*, *SK2*, *K2*. The *ver* (selfing) population segregated in 7 *K1* : 8 (*K1*+*K2*) plants and TV[6] segregated into 4 (*K1*+*K2*) : 4 *K2* plants (Fig. 1). Segregation analysis of offspring plants from crosses based on K-bands (*K1*⊗; *K2*⊗; (*K1*+*K2*)⊗ ;*K1* x *K1*; *K1* x *K2*; (*K1*+*K2*) x *K2* and (*K1*+*K2*) x (*SK1*+*SK2*)) showed both normal and skewed segregations, but no genotypes were found missing simultaneously both *K1* and *K2*. This strongly indicates allelism of *K1* and *K2*. If this assumption is correct, then the *S*-alleles of *ver* (*Sv*) produce apparently little or no basic *S*-glycoproteins.

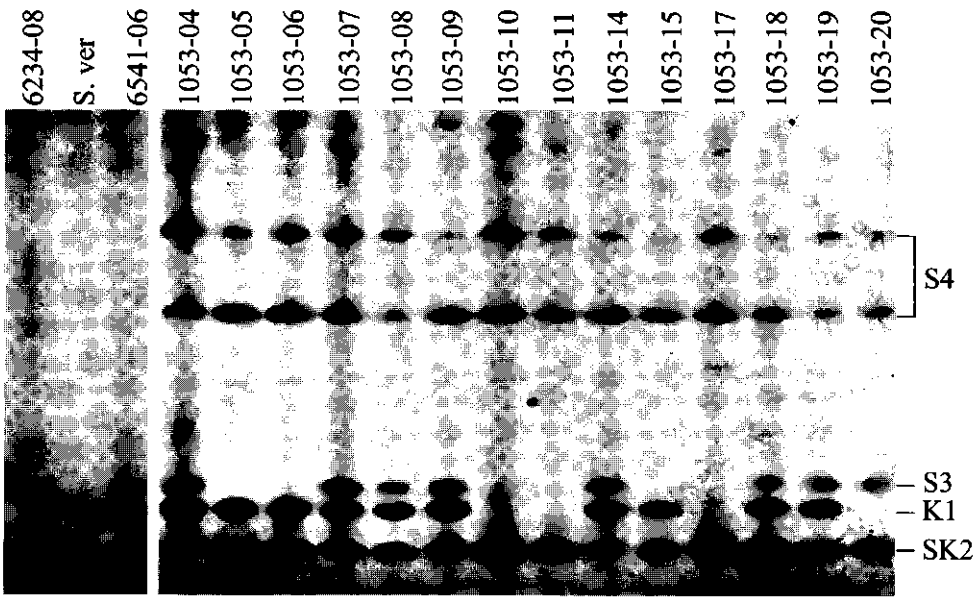


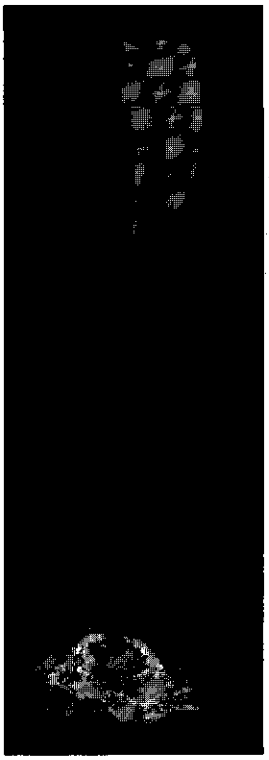
Fig. 2 IEF pattern of stylar extracts after silver staining of (left panel) *tbr* parent 6234-08 (l), *ver* parent PI 195172-27 (m), the F1 clone 6541-06 (r) and (right panel) some offspring BC1 clones of population 1053, derived by backcrossing clone 6541-06 with an *S3S4 tbr* clone. Visible bands: *S4* (upper two), *S3*, *K1* and *SK2*. *SK1* is faintly visible in some lanes between *K1* and *SK2*.

The F1 population 6541 came from the cross *tbr* 6234-08 x *ver* PI 195172-27 (=*S3S3*, acceptor x *SvSv*). The *ver* clone used here, was previously tested for absence of *tbr- S*-allele suppression (see above). Twelve 6541-clones were tested for SC and IEF-band composition. All plants were, as expected, SC and all were showing the *S3* and *SK2* band from the *tbr* parent and also the *K1* band from the *ver* pollen parent (Fig. 2). Seven randomly selected plants were tested for acceptance of *ver* pollen and proved to be acceptors. All seven were as pollen parent compatible on the parental acceptor clone 6234-08 (*S3S3*). Functional activity of the *S3* allele in 6541 plants was directly confirmed by fluorescence microscopy performed on styles of test crosses, showing a proper incompatibility reaction against *S3* pollen (Fig.3). Thus, SI against *S3* pollen was still active, acceptance was maintained and SC characteristics of *ver* were expressed in the 6541 population.

The F1 6541 clones that were tested for acceptance (all *S3Sv* and acceptor) were backcrossed with the *S3S4* acceptor lines 6536-01 and 6536-02 as pollen parents. Five BC1 populations (1051 to 1055), with a total of 143 flowering plants, were investigated in more detail. All these clones showed the *S4*-allele of the *S3S4* pollinator clones (Fig. 2), thus excluding selfing or fertilisation by an *S3* pollen grain and confirming that the *S3* allele was fully functional and effective in the style and not suppressed by some unknown *ver* factor. If there had been a stylar SI-suppressor, the penetration ratio (certation) between *S4* and *S3* would have been at least 98: 2 (at $\gamma = 0{,}05$), so this possibility can be ruled out.

It was postulated that *ver* contained an *S*-locus but that its stylar expression could not be detected through IEF because it lacked an *S*-specific band. The ratio "presence of *S3"* to "absence of the *S3* (= presence of "*Sv*")" was pooled over five populations, and segregated into 70 *S3* : 73 *Sv* (fits 1:1; $\chi^2 = 0{,}06$). The *K1* band segregated in a ratio of 65 present and 78 absent ($\chi^2 = 0{,}59$; $P_{1:1}=0.8$), which is also reflected in the ratio of *K1+S3* : *K1*+Sv : *S3* : *S*v (32:33:38:40), which fits a 1:1:1:1 segregation ($\chi^2 = 1{,}04$; $P_{1:1:1:1}= 0.8$), thus proving the independent assortment of the *S*-alleles and *K1* (fig.2). That *K2* (presumably allelic with *K1*) was not allelic with the *tbr S*-alleles, was confirmed by banding patterns of clone 6251-22, a sibling from clone 6251-19, that had the genotype *S2S3 K2* (fig. 4). So, *S3S4K1* and *S2S3K2* plants were found, indicating, as expected, the non-allelism of either *K1* or *K2* with the *tbr S*-alleles.

The six populations 1061 to 1066 (6541 clones selfed) allowed for 30 plants a proper identification of *K1* and *SK2* bands. Because these genes proved to be non-allelic with *S*-alleles, segregation analysis of the *S*-alleles was not considered here. Seven plants showed only the *K1* band, 13 both the *K1* and *SK2* bands and 10 showed only the *SK2* band. When *K1* and *SK2* would be allelic, then the F1 population (6541) was most likely derived from the cross *SK2SK2* x *K1K1*, resulting in 6541 with the *SK2K1* genotype. In the case that *SK2* and *K1* are not allelic but independent genes, the F1 would then have been hemizygote: *SK2—K1—*, selfing would give different segregation ratios. Testing for these ratios gave a χ^2 of 1,13 for allelism (P=0.57) and a χ^2 of 6,50 for the hypothesis of unlinked genes(P=0.09). Hence *K1* and *K2* are most likely allelic with *SK2*.



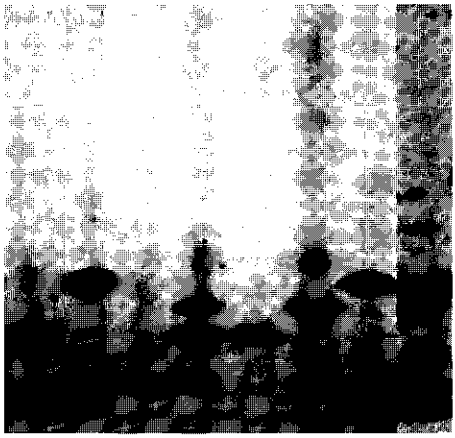


Fig. 3 (left) Incompatibility for S3-pollen as monitored in styles from the *S3Sv* clones from the F1 population 6541 after fluorescence microscopy on anilin-blue stained styles. Top: stigma; bottom: ovary with ovules.

Fig. 4 (top) IEF pattern of stylar extracts after silver staining of *tbr-ver* offspring clones. From top to bottom: *S2, S3, K1, SK1, SK2* and *K2*. Most left lane: 6251-19, expresses *S3Sv SK1K2*. Most right lane: 6251-22, expresses *S2, S3, SK1, SK2* and *K2*.

Self-compatibility based on (differential) acceptance of *Sv* and a pollen-expressed factor, SC_{ver}

The populations 1052 and 1054 (= 6541-03 & 6541-06 x 6536-02 = *S3Sv*, acceptor x *S3S4*, acceptor) segregated into acceptor lines (*AAii* and *Aaii*) and non-acceptor lines (*aaii*) when tested with the hereafter mentioned tester clones, in a pooled segregation ratio of 32 acceptors : 14 non-acceptors (= 3:1; $\chi^2 = 0{,}72$). The populations 1051, 1053 and 1055 (= 6541-03, 6541-06 & 6541-11 x 6536-01< *S3S4*, acceptor>) showed only acceptor genotypes (*AAii* or *Aaii*) when pollinated with the tester clones *ver* PI 195172(clone22 x clone27)-31&-32, but showed in about a quarter of the cases an UI (thus non-acceptor) reaction when tested by another *ver* genotype, (clone 35 x clone 37)-1 (data not shown). No clones were found that were acceptor for the latter *ver* clone and non-acceptor for the former two *ver* clones. It was clear from these observations that in this material a differential non-acceptance reaction against certain types of *ver* pollen was found, as previously reported for this system (Hermsen et al., 1977; Hermsen and Sawicka, 1979).

Table 2. Segregation of *S*-alleles and SC in the acceptor populations 1051 and 1053. The populations are BC1's of the type TVT: 6541-06 x 6536-01 & 6536-02; 6541-06 = *tbr* 6234-08 x *ver*. All are acceptor for *ver* pollen of tester lines 31 and 32 (F1 of PI 195172). Numbers are pooled. *S*-alleles detected by IEF: *S3* and *S4* were derived from the *tbr* clones 6234-08 and 6536-01; *Sv* = postulated stylar non-active *S*-allele from *ver*","detected" by absence of the *S3 tbr* allele. SI = self-incompatible, SC = self-compatible; SC_{ver} - = single SC factor from *ver*, - - = no SC_{ver} factor. The class *S4Sv*, SC contains *S4Sv* plants with and without the SC_{ver} factor.

S-alleles	*S3S4*		*S4Sv*	
compatibility	SI	SC	SI	SC
number found	33	9	2*	33
postulated genotypes	*S3S4*, - -	*S3S4*, SC_{ver}-	*S4Sv*, - -	*S4Sv*, - - /SC_{ver}-

* showing differential reaction against *ver*.

Self-compatibility was predominantly found in the *S4Sv* genotypes (table 2). The SC of the *S4Sv* genotypes can simply be explained by acceptance for the *Sv* self pollen type. The two SI *S4Sv* clones showed a differential reaction against pollen of the *ver* tester clones, but the rest of the *S4Sv* clones with a differential reaction were still SC. Here a quarter of the *S4Sv* plants appeared to be of the differential type. This SC/non-acceptor discrepancy was also detected in the 1052 and 1054 populations. Because nine *S3S4* plants were SC, not attributable to *Sv* (*Sv* is allelic with the *tbr S*-alleles, thus not present in diploid *S3S4* genotypes), an additional pollen expressed SC-factor must be postulated, coded SC_{ver}. The skewed segregations in the *S3S4* and *S4Sv* subgroups indicate that SC_{ver} is likely to be linked with the S-locus. In the mother clone 6541-06 it is in coupling phase with *Sv* and thus in repulsion phase with *S3*. For the *S3S4* part of the population there was a recombinant fraction of 9/42= 0.214 and for the *S4Sv* subpopulation it was (2/35)/ (1/4) = 0.229, so on average there appeared to be 22% recombination between *Sv* and the SC_{ver} factor, with a confidence interval of 13% - 33% ($\gamma = 0.05$). *S4Sv* plants can, therefore, be SC due to acceptance for Sv pollen, and to the presence of the *S*-linked SC_{ver} factor; *S3S4* plants can only be SC beause of SC_{ver} .

From selfing the BC1-clone 1053-27(= *S3S4*, SC_{ver}—, acceptor), a clone with the genotype "*S4S4*, Sc_{ver}*, acceptor" was identified and coded 1144-02. Selfing of 1144-02 resulted, as expected for pollen-borne SC, in SC plants only, all being acceptor. Clone 1144-02 was as male compatible with clones 6221-17 (*S1S4*, NA) and 6223-40 (*S3S4*, NA), but not with 6221-32 (*S3S4*, NA) 6221-37 (*S3S4*, NA) and 6223-39 (*S2S4*, NA). This indicates that a differential reaction not only against *Sv* exists, but against the SC_{ver} factor as well.

Self-incompatibility by UI- based SC inhibition: expression of the pollen-SC_{ver} factor.

The populations TV[5] and TV[6] (both *SvSv* and expected to contain SC_{ver}) are *ver* with *tbr* cytoplasm, yielding male and female fertile hybrid populations when crossed with non-acceptor *tbr* as male parent. Two non-acceptor based hybrid populations were investigated more closely, because the

Table 3. Reaction patterns of population 1173 (*S3S3*, SI, acceptor x *S3Sv*, SI, non-acceptor) in crosses, and deduced genotype composition. The staminate parent of 1173 was known to be *S3Sv aaI**.
Eleven plants were selfed (SC= self-compatible, SI = self-incompatible), all were SC. Ten were tested for acceptance based on berry set and/or pollen tube penetration (A= acceptor, NA= non-acceptor). The genotypes mentioned below (except clones 6, 7 and 11) were additionally tested for pollen tube penetration in styles of clone 1024-02 (*S3S10*, NA). Pollen tube arrest is indicated in fractions compatible (C) and incompatible (I) pollen. *S3* and *Sv*: *S*-alleles; SC_{ver}= SC-factor from *ver*, - = no SC-factor; *A* = acceptor allele; *I* = inhibitor allele. Blank = not determined.

Plant nr.	SI/SC	Acceptor/non-acceptor	Pollen tube arrest in *S3S10*, NA	Deduced genotype
1173-01	SC	NA	1/2 C 1/2 I	*S3S3* , SC_{ver}-, *AaIi*
1173-03	SC	NA	1/4 C 3/4 I	*S3Sv* , SC_{ver}-, *AaIi*
1173-04	SC	A	I	*S3Sv* , - -, *Aaii*
1173-05	SC	A	1/2 C 1/2 I	*S3S3* , SC_{ver}-, *Aaii*
1173-06&11	SC	NA		*S3S**, SC_{ver}-, *AaIi*
1173-08	SC	A	1/2 C 1/2 I	*S3S3* , SC_{ver}-, *Aaii*
1173-09	SC	NA	1/4 C 3/4 I	*S3Sv* , SC_{ver}-, *AaIi*
1173-10	SC	A	1/4 C 3/4 I	*S3Sv* , SC_{ver}-, *Aaii*

expression of SC in the hybrids deviated from expectation. The first investigated population, coded 1173, was derived from a cross in which an acceptor *tbr* and a non-accptor F1 clone (TV^5 x *tbr, S2S3, NA*) were involved. The second population, coded 6484, was an F1 from the cross TV^6 x non-acceptor *S2S2 tbr* clone 6233-12.

The population 1173 (= 6234-08 x 6251-19 = *S3S3* (SI), acceptor x *S3Sv* (SI), non-acceptor) showed the *S3* band by IEF, for the pistillate parent was *S3S3*. Genotypes like *S3Sv* and *S3S3* can be distinguished by test crossing but not by IEF. Therefore, the 1173 -plants were selfed, tested for acceptance of *ver* pollen and test crossed on the *S3S10* non-acceptor 1024-2 (table 3). All eleven obtained clones were self-compatible (Fig. 5a) and all of them, except 1173-02, were tested for acceptance. Five were non-acceptor, the other five were acceptor. The pollen tube penetration fractions (in)compatible pollen and site of pollen tube arrest) of three acceptors and three non-acceptors was investigated in test crosses on clone 1024-02 (*S3S10*, non-acceptor). *S3*-pollen can be inhibited by SI, and *Sv* pollen by UI. The genotypic constitution of the 1173 clones was deduced from combining pollen tube penetration data with acceptor- and SI behaviour. Re-appearance of SC in non-acceptor 1173-plants indicated that parental clone 6251-19 contained the pollen factor SC_{ver} and confirmed the expectation that its non-acceptor background was *aaIi*. Clone 1173-04, which was self-compatible and acceptor, was fully incompatible on clone 1024-2. This proved, as expected, the heterozygosity of SC_{ver} in clone 6251-19 as well as its heterozygosity for the *S*-locus.

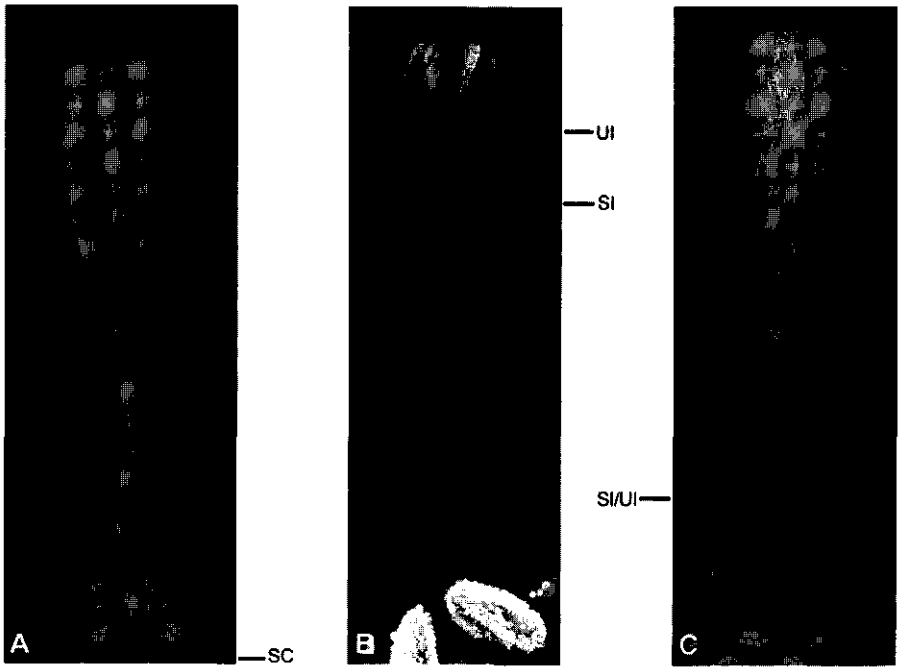


Fig. 5 Pollen tube growth monitored by fluorescence microscopy in anilin-blue stained styles of non-acceptor *tbr-ver* hybrids. Fig. 5a (left): Self-compatibility based on SC_{ver} pollen as monitored in styles from the non-acceptor 1173-08 (*S3Sv* SC_{ver}). Fig 5b (middle) and Fig 5c (right): SI and UI in styles from the non-acceptor F1 clone 6484-06 as function of the ploidy level of the pollen. Middle: incompatible reaction for *S2* pollen after test crossing with an *S2S2* pollinator. Upper arrow: approximate inhibition site when pollinated with *ver* type pollen. Lower arrow: approximate inhibition site when pollinated with *tbr S2* pollen. Selfing results in a mix of those reactions. Right: Pollen tube growth after selfing of the tetraploidised clone 6484-06. *S2Sv* pollen is deeply penetrating the style. Other pollen tube types are earlier arrested.

The second population investigated for a putative suppression of SC was the F1 population 6484: TV^6 x *tbr* 6233-11 (= *SvSv* x *S2S2, aaII*). All fourteen 6484-clones (*S2Sv*, a*I*) were SI, and as expected, incompatible for *S2*- and non-acceptor (NA) for *ver* pollen (Fig. 6b). At least 150 flowers of each plant were self pollinated, 2600 pollinations in total. Twelve seeds were obtained by end-of-season-compatibility, giving rise to six weak and poorly flowering plants with a reduced fertility. All six seedlings showed the *S2*-glycoprotein band and were, based on a limited number of pollinations, self-incompatible and non-acceptor. No *SvSv* plants were found, which has under the assumption that S2 and Sv pollen tubes are equally arrested, a likelihood of at least 17%.
Pollinations with clone 6484-06 (*S2Sv*, non-acceptor) on the clones 6233-11 (*S2S2*, NA) and 6223-39 (*S2S4*, NA) were incompatible, but compatible on 6104-21 (*S1S2*, NA). This observation of a

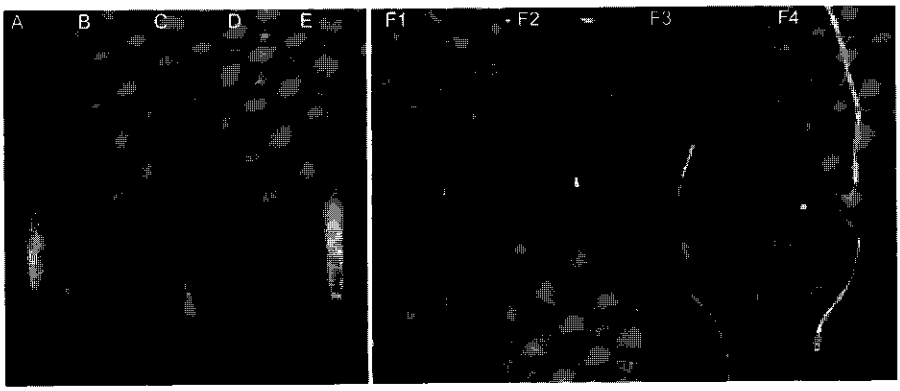


Fig. 6. Detail of fluorescence microscopy images of anilin blue stained pollinated styles. Reaction of pollen tubes in various crosses. Left panel (a-d): rupture op pollen tube tips in normal UI reactions and (e) most common type of pollen tube tips of SI inhibited pollen tubes. Right panel: Deteails of mutual weakening based pseudo-compatible pollen tubes with the presumed genotype *S2Sv* in *S2S2SvSv* styles, as monitored in styles like shown in Fig 5c. Panel f1: directly under the stigma; f2: halfway the style; f3: at 3/5 of the style; f4: pollen tube arrest in the lower part of the style.

differential reaction is highly similar to the observed crossing results with the clones 6541-06 (*S3Sv*, *Aaii*, SC_{ver} -) and 1144-02 (*S4S4*, *A*ii*, SC_{ver} *) (see above). When clone 6104-21 was pollinated with 6484-06, an estimated 10- 20% of the pollen tubes monitored by UV-fluorescence microscopy was of the compatible type.

Mutual weakening by combining *S2* and *Sv* in pollen from a tetraploid NA *tbr-ver* hybrid

Non-acceptor clone 6484-06 (S2Sv a*I*) was somatically doubled by tissue culture (data not shown). Twenty tetraploidised clones were obtained and tested for acceptance and incompatibility against *S2* pollen in test crosses. All tetraploid plants behaved as the original diploid clone 6484-06, they were incompatible for *S2* pollen (from clones 6233-11, 1140-02 and 1140-05) and non-acceptor for *ver* (Fig. 5 b, c). One clone was male sterile, but the remaining nineteen of the tetraploidized clones were sufficiently male fertile and allowed for selfing. These tetraploid clones showed a low level of SC, which was strictly absent in both the original 6484-06 clone and the non-doubled tissue culture derived control plants. From these tetraploids, berries with few seeds were obtained in a much higher frequency than after selfing of the original diploids. Fluorescence microscopy on pollinated styles showed that about 5-10 % of the pollen tubes were of a remarkably different type. In the upper 1/3 of the style they were of a compatible type, with long, thin tubes, with regularly interspaced small callose plugs (Fig. 6: f1, f2), as usually found in normal compatible crosses. At about halfway the style the tubes became broader and irregular, sometimes even branched, with more but irregular callose deposition, and finally the pollen tubes were arrested, with much inflated pollen tubes (Fig.6 f3), differing from the normal incompatibility or unilateral

incompatibility type in this material. The incompatibility reaction was completed at about the end of the style, usually showing swollen pollen tube tips instead of ruptured ones (Fig. 6, f4 versus a,b,c,d).

Doubling of the genome allowed for a type of gene combination that is not attainable in pollen from a diploid. Heterozygosity is, therefore, the most likely explanation, and mutual weakening between *Sv* and *S2* can be held responsible for this. The weakening effect appears to be barely strong enough to bypass the UI reaction, though. So, the pollen part of the *S*-locus of *ver* appears to be still functional, not only in triggering an UI reaction, but also in causing the SI related phenomenon of mutual weakening when together with a *tbr S*-allele.

Discussion

Stylar S-allele suppression. The backcross experiments with the VTV and VT clones indicated that these *ver* accessions neither express a cytoplasmic, style specific incompatibility suppressing factor nor genic suppressing factors, as postulated by Kaufmann et al (1992) in their material. It is possible that their material contained a factor such as reported for *Petunia* (Flaschenriem and Ascher, 1979; Dana and Ascher, 1986, b), which might cause, for instance, (pseudo-)compatibility, although the expression of this factor in *Petunia* appeared to act only in *cis*. Parthenogenesis , as repored to be present in some *ver* lines (Abdalla, 1970), is another explanation for Kaufman's compatibility. Furthermore, the normal incompatibility reaction is not always reliable. Besides, modifiers are commonly found and early pollination can bypass an otherwise fully functional SI system (Eijlander et al, 1997). None of the afore mentioned factors appeared to operate in our material and, therefore, the *S1*, *S2* and the *S3* alleles were fully functional in our system. In the reciprocal type of material, TVT (e.g. the 1053 population), the penetration of *S4* c.q. the arrest of *S3* was fully in accordance with these findings.

Stylar proteins and allelism. Although *K1* and *K2* are accepted to be alleles from the same gene, there is still a small chance (of about 0.2 %) that they are not, because the analysis of the segregation behaviour had to be based on relatively small populations. The presence of *K1* or *K2* together with *S2* and *S3* (6251-22) or *S3* and *S4* (e.g. 1053-27) in a diploid plant proved that they are not located on the same locus as the *S*-alleles. SC can be found in plants that lack both *K1* and *K2* and plants with two different *S*-alleles and *K1* or *K2* can be SI. Thus, mutual weakening caused by *K1* or *K2* is not an explanation for SC. Because of this and because of the relatedness of *tbr* and *ver*, translocation or duplication of *S*-alleles during the evolution of *ver* is therefore not a likely hypothesis to explain SC. Segregation ratios of *K1* and *SK2* in F2 progenies showed that allelism is very likely. It is likely that *K1* and *K2* are located at (approximately) the same locus as *SK2* in *tbr*

and that they are probably allelic. Allelism of *K1* and *K2* with *SK2* implies that they should belong to the family of endochitinases (Wemmer et al, 1994). It is unclear whether they play a role in UI, because the *K1*-, *K2*-, *SK2*- and also the *SK1*-band could not be linked to UI.

Acceptance and differential reactions . The acceptance of *ver* pollen by *tbr* has been reconsidered several times and has been put in a broader perspective by comparing it with UI systems between other species as well (e.g. Abdalla, 1970; Abdalla and Hermsen, 1972; Abdalla, 1974; Hermsen, 1977; Hermsen et al, 1977; Hermsen, 1979; Hermsen and Sawicka, 1979). Differential acceptor series and penetration capacity levels passed in review and this seemed, on the face of it, to conflict with a previous model of Hermsen et al (1974), where only the *Aa/Ii* system (dominant inhibitor epistatic over dominant acceptor gene) was discussed as an alternative for the postulated A_1A_2 system (only double recessives, $a_1a_1a_2a_2$, are acceptors); penetration capacity did not play a role in this analysis. Extending the *AaIi* model by introducing more alleles and various dominance realtionships can bring all results in accordance with each other. Hermsen (1974) used pollen mixtures of *S.ver* PI 195172, and the other articles dealt with various accessions and separate genotypes of *tbr* and *ver*. In our study differences in penetration capacity between *ver* PI 195172 based siblings were detected too. We did not use pollen mixtures, but individual *ver* siblings were used for testing the segregation of acceptance and non-acceptance in hybrid populations. When all individual tester genotypes failed, even those with a high penetration capacity, it was concluded that a tested plant was non-acceptor.

Based on this approach, fully expressed non-acceptance for all *ver* tester clones was detected in the 1052 and 1054 populations. The 1051 and 1053 populations were entirely acceptor for at least one of the *ver* tester clones. The inbred clone 6234-08 was *AA* (data not shown) and, consequently, *ver* must have been *A'a* (*A'* gives an differential acceptor reaction when compared with *A*; *a* must be present because of the segregation of non-acceptors in populations 1052 and 1054) and 6541-06 must have been *AA'*. Backcrosses with 6536-01 (*S3S4 AAii*) resulted in differentials, so 6536-01 must have contained two different co-dominant *A*-alleles, eg. *AA'* or *AA''*.

The self-compatibilizing factors Sv *and* SC_{ver} . There are various ways by which plants can become self-compatible. For instance, a gene can become silenced, but mutation of the coding region is another possibility which has been described for many crops. For *Lycopersicon peruvianum*, a diploid SI tomato species, it has been reported that a single point mutation caused loss of the *S*-RNase activity, resulting in full SC (e.g. Kowyama et al, 1994), but a basic protein remained detectable. A frame shift, as reported for *L.esculentum* (Thompson et al, 1995), truncated the putative *S*-glycoprotein, resulting in another IEF point and loss of its *RN*ase activity, thus causing SC of tomato.

The presence of the not-stylar-active *Sv* allele in a diploid hybrid *tbr-ver* plant means that there can

be only one active *S*-allele. So, all *S*-heterozygous *tbr* clones will be compatible on such a hybrid. A hybrid *tbr-ver* plant will also be SC, as long as the plant is acceptor (*A*ii*) for *ver* pollen. So, the effect of the *Sv* allele in the style depends on the presence or absence of the acceptance determining genes *A* and *I*.

The pollen active SC-factor SC_{ver} that has been found, appeared to be weakly linked with the *S*-locus (chromosome 1). This is in agreement with the location of a pollen active SC factor in *Petunia hybrida* (called pollen inactivator), that was reported to be at a distance of about 20 to 28 map-units from the *S*-locus (whereas also a stylar factor was reported at approximately the same distance: Dana et al, 1986 a,b; Flaschenriem et al, 1979). Therefore, this factor is clearly different from the SC factor reported for the diploid *tbr* clones G254 and B16, which was localised on chromosome 12 and not linked with the *S*-locus on chromosome 1 (Hermsen et al, 1973, 1978). A remarkable characteristic of the SC_{ver}-factor is that its effectiveness of causing a mutual weakening effect depends on the genotype of the recipient. All results obtained so far can be explained by the assumption that SC_{ver} is not effective in overcoming the UI barriers caused by the genotypes *aaII* and *aaIi*, but that other non-acceptor genotypes like *aaii* and *A*I** allow for a SC_{ver} induced compatibility in a combination that would be incompatible when only *S*-alleles were regarded. Thus, genotypes like 6484-6 (*S2Sv, aa*,Ii) and 6251-19 (*S3Sv, aa, Ii*) can contain a self-compatibilizing factor (SC_{ver}) but are SI by an UI-based inhibition of SC. SC can show up in offspring populations, but only when non-aaI* genotypes segregate.

Recognition of the pollen part of the ver-S-locus. The tacitly accepted assumption that pollen of the *ver* type is arrested by non-acceptors, implies that the pollen part of the *S*-locus of *ver* is still capable of causing an UI reaction when penetrating a non-acceptor style (see also below). This is confirmed by segregation ratios in a population based on a non-acceptor (*S2S4*) x 6251-19 (*S3Sv*) cross, where only the *S3* allele penetrated (data not shown). It is also in agreement with the pollen tube penetration types as reported for 1024-02 x 1173, where also the SC_{ver} factor played a role. When the SC_{ver}-factor makes *S3* pollen on 6234-08 (*S3S3,* AA, ii) as compatible as the *S*v-pollen type, the pollen that led to population 1173 will have had the composition of 1 *S3* SC_{ver} *aI* : 1 *S3* SC_{ver} *ai* : 1 *Sv ai* : 1 *Sv aI* : 1 *Sv* SC_{ver} *ai* : 1 *Sv* SC_{ver} *aI*. Not all possible genotypes for this cross have been found, for *S3Sv AaIi* was not detected (see table 3) and this is the only possible SI genotype resulting from the cross *S3S3 AAii* - - x *S3Sv aaIi* SC_{ver} -. This is presumably due to the limited population size, because the segregation found was in agreement with the expected one.

Dual function of the pollen part of the S*-locus: polyploidy-effects.* A most striking result on the *Sv* allele was obtained after doubling of genotype 6484-06 *(S2Sv;* NA). Although it was known that the stylar part of the *ver S*-allele was not active, a dual function of the pollen part (contributing to both SI and UI) could still be present. Somatic doubling of an *S*-heterozygous clone will normally lead to SC, because of heterozygosity of 50 % of the pollen for the *S*-alleles involved. Mutual

weakening, an SI related phenomenon, can then be active. It was known from the diploid that normal recombination does not result in self-compatibility of *S2* or *Sv* pollen, irrespective of the presence or absence of SC_{ver} and will not play a role in a somatically doubled plant. *SvSv* pollen will be inhibited by UI. Pollen being homozygous *S2S2* will be blocked by SI and competitive interaction does not play any role here (Eijlander et al, 1997). The more compatible polle tubes respond in the upper part of the style like *S2S3* pollen tubes in an *S2S3* or *S2S2S3S3* style. Mutual weakening between *Sv* and *S2* in *S2Sv* pollen offers the best explanation for this.
The observation that *Sv* is capable of raising a UI reaction, the aforen mentioned polyploidy based SC and the dual function hypothesis of Lewis and Crowe (1958) justify the assumption that *Sv* is also capable of causing mutual weakening. Thus, the *S*-locus of *ver* contains a pollen-part that is still active and co-dominant in SI. Furthermore, the fact that the combination of *Sv* + *S2* in the pollen (thus being *S*-heterozygous) is also breaking down the UI reaction in the upper part of the style, is a strong indication, if not proof, that the *S*-locus is involved in the UI reaction. However, in the lower part of the style a second reaction type became visible, that has remained unnoticed in the diploid situation. This has presumably been masked or prevented by the much stronger UI reaction in the upper part of the style.

General considerations on SI, SC and UI factors. Chetelat and DeVerna (1991) made it likely that "expression of unilateral incompatibility in pollen of *Lycopersicon pennellii* is determined by major loci on chromosomes 1, 6 and 10", with the remark that the locus on chromosome 1 mapped near or on the *S*-locus. When the tomato linkage maps of Chetelat and de Verna (1991) are integrated with the potato map of Van Eck et al (1994), the flower colour locus maps on or close to the UI related locus. When these results for linkage can be extrapolated to the solanaceous family of *Nicotiana*, more can be said about this locus. Pandey (1981) described for *Nicotiana glauca* (SC species) the phenomenon that SI was very strongly linked with flower colour. Although the explanation was rather speculative and partially flawed by too many reported *S*-alleles present, it can be regarded as SI by UI based inhibition of SC, as reported here. In this study it was shown that within the UI system, that operates between *tbr* and *ver,* the UI reaction in the style is two-fold and directed against the pollen-active part of the *S*-locus in *ver* pollen and that this pollen part of the locus in *ver* has still SI related properties (capable of causing mutual weakening). The UI-reaction is also directed against the pollen active SC_{ver} factor, but differs in the mode of expression from the reaction against the S-locus. All this supports, at least for the pollen part, the dual function hypothesis for the S-locus (contribution to SI and UI) of Lewis and Crowe (1958) and is also in agreement with the observations of Chetelat and DeVerna (1991). It also illustrates that excluding the *possibility* of the SI system from contributing to UI, as proposed by Hogenboom (1973), is not correct and that prevalence of the expression "unilateral incongruity" over "unilateral incompatibility" is not always justified.

Chapter 5

Contribution of the *S*-locus to Unilateral Incompatibility when crossing *S.verrucosum* (SC) with *S.tuberosum* (SI)

Abstract.

Diploid potato, *Solanum tuberosum* (*tbr*), is characterized by a one-locus (*S*) gametophytic self-incompatibility (SI) system. The diploid wild species *S.verrucosum* (*ver*) is self-compatible (SC), and forms an exception to the rule that diploid tuber-bearing *Solanum* species are SI. The cross *ver* x *tbr* is successful, but gives rise to cytoplasmic male sterile F1 hybrids. The reciprocal cross, *tbr* x *ver*, usually fails. This phenomenon is called unilateral incompatibility or unilateral incongruity (UI). Plants showing the UI reaction are called non-acceptors (NA) for the *ver* pollen. However, exceptionally *tbr* plants were found to accept *ver* pollen; the F1 hybrids thus obtained were fully male fertile. Now *tbr* x *ver* offspring could be tested for the contribution of functional *S*-alleles to UI.

An antisense *S2* construct was introduced into an *S2*-homozygous non-acceptor by crossing with a transgenic *S2* antisense expressor, and by transformation of this construct in a *S2Sv tbr x ver* hybrid, that was incompatible for *S2*- and *Sv*-pollen, thus showing SI and UI. Crossing the transformants with *S2S2* and *SvSv* tester clones showed that the suppression of the SI reaction against *S2* coincided with a break-down of the UI reaction against *ver* pollen.

The analysis of the segregation ratios for SI/SC and A/NA in *tbr* x *ver* hybrid populations revealed that *ver* does contain non-acceptor factors against own pollen, not expressed in *ver*, but only in species-hybrid situations where *S*-glycoproteins are expressed. These findings are in accordance with some earlier reports that the *S*-locus is involved in both SI and UI. Here the whole SI and UI system can be explained by a dual function of the *S*-locus (pollen and style genes contributing to both SI and UI), the acceptor gene *A* and its epistatic inhibitor gene *I*, a pollen-expressed SC_{ver} factor. A model is presented explaining observed results as well as allowing predictions based on the aforementioned intergenic interactions.

This chapter is submitted for publication in a slightly modified version as:Ronald Eijlander, Munikote S. Ramanna, Michael Ficker and Evert Jacobsen. Occurrence of Self-Compatibility, Self-Incompatibility and Unilateral Incompatibility after crossing *S.tuberosum* (SI) with *S.verrucosum* (SC): II Contribution of the *S*-locus to Unilateral Incompatibility.

Introduction.

Self-incompatibility (SI) is a mechanism by which many plant species protect themselves from in-breeding by selfing. There are many mechanisms, some based on floral morphology, others on difference of maturation time of male and female reproductive organs within a flower or plant. There are also mechanisms that are based on the interaction between pollen (tubes) and the pistil. This may happen at all stages between landing of a pollen grain on the stigmatic surface and fertilisation. There are two major incompatibility systems: the sporophytic one , where incompatibility is controlled by the interaction of the genotypes of the pollen parent and the style parent (sporophytes), and the gametophytic one, where the incompatibility is determined by the interaction of the genotype of the pistillate parent and the genotype of the pollen grain (gametophyte). Irrespective of the incompatibility system, it is frequently found that hybridisation of related species is possible in one direction only. It is usually found when self-compatible (SC) species are crossed with related SI species (e.g. Anderson and De Winter, 1931; Mather, 1943; Lewis and Crowe, 1958; De Nettancourt, 1977, etc),that the SI species are successful as pollinators, but not as pistillate parents (SC x SI ➜ F1; SI x SC ➜ -). This phenomenon is called unilateral incompatibility or unilateral incongruity (Hogenboom, 1973). Hogenboom tried to distinguish between SI and UI and introduced the concept "incongruity" for inhibitory reactions that are not based on self-incompatibilty, arguing that incompatibility and incongruity are separate phenomena. For a long time a debate is ongoing about a possible involvement of the *S*-locus in UI, and, directly related to this, whether in this connection the term incongruity or incompatibility should be used.

For the Brassicaceae (sporophytic system) it was found likely that the *S*-locus is involved in UI (Hiscock and Dickinson, 1993). The genetic analysis of an interspecific hybrid system in *Lycopersicon,* (Solanaceae), with a one locus gametophytic system, showed the likelihood of (a part of) the *S*-locus being involved in UI (Chetelat and DeVerna, 1991).

For the Solanaceous species, *ver* in particular, a genetic model for the evolution of species from SI to SC was postulated, in which the dual function of the *S*-locus, as proposed by Lewis and Crowe (1958), is crucial. Here the *S*-locus contributes to both SI and UI. The two-powers competition hypothesis is based on this and on the co-evolution of sympatric SC and SI species (Abdalla, 1970; Abdalla and Hermsen, 1972; Abdalla, 1974). The development of CMS (Abdalla, 1970; Abdalla and Hermsen, 1971) in case of interspecific hybridisation with *ver* as pistillate parent is one of the necessary results in this hypothesis, turning most hybrids into a "dead end". The reciprocal cross would be possible only when the SI species has no *UI* genes, that could inhibit the postulated S_c allele of the SC species. Hybrid progeny created with such *UI*-lacking plants could open the possibility to investigate the UI model for the contribution of both stylar and pollen determined factors.

In a diploid potato population originating from two SC parents, not accepting *ver* pollen, Hermsen et al (1974) detected clones that were SI or SC and were acceptor for *ver*. They analysed the genetics

of acceptance by pollinating several *tbr* populations with *ver* pollen mixtures and found segregation of acceptors (A) and non-acceptors (NA). Two models were tested for fitting the ratios. The first model was based on two independent loci, A_1 and A_2, where only the double recessive genotypes ($a_1a_1a_2a_2$) are acceptors. This model was similar to the hypothesis by Grun and Aubertin (1966), but did not fit two out of fourteen observed ratios. Model 2 was based on the independent genes *A* and *I*. *I* is an inhibitor, epistatic over the dominant acceptor gene *A*. Here, only *iiAA* and *iiAa* genotypes could be acceptor. This model fitted the observed segregations very well. The segregation of the *S*-alleles and the SC-factor t*S1*, that was present in the original parents, segregated independenly from the acceptance. A thorough analysis of this and related material by Hermsen et al (1977), in which *ver* pollen was not pooled, revealed a kind of gene-for gene relationship in penetration and barrier capacity of pollen and style, resulting in a differential reaction pattern. Their explanations and expressions correspond with those used for the incongruity hypothesis (Hogenboom, 1973). The results were, however, not conclusive about the exact mechanism, and the authors left open the possibility of other explanations.

It is clear from the study on SC in the hybrid system of *tbr* x *ver* that both the *S*-locus from *ver* or *tbr* and (non-)acceptance play a role in the expression of SC and SI (Eijlander et al., b, submitted). For the pollen part of the *S*-locus, a dual function was made likely, thus introducing at least a part of the *S*-locus in both the SI and the UI hypothesis, which is in accordance with the conclusions of Chetelat and DeVerna (1991) and Foolad (1996). Murfett et al (1996) showed by a molecular approach in tobacco that introduction of an active *S*-allele can contribute to unilateral incompatibility in those solanaceous species, but they did not link this to a genetic model. We used here both a molecular and genetic approach for the hybrid system of *tbr* x *ver* to prove that, like in *Nicotiana*, the stylar *S*-locus product (*S*-glycoproteins, also called *S-RNases*) can contribute to UI. We also integrated the results into an already existing genetic model for UI, explaining why interspecific hybridisation can result in unexpected appearance of SC or SI / UI based crossing barriers between F1 hybrids and the parental species.

Materials and methods.

The material that was used, was based on the expectations that factors like *S*-alleles, (responsible for the SI reaction), the acceptor gene *A* (dominant over non-acceptor allele *a*) that causes acceptance of *ver* pollen, and the acceptor-inhibitor gene *I* (epistatic over *A*, thus in dominant form always causing UI) could be identified by electrophoresis (*S*-alleles), by test-crossing with *tbr*, *ver* or by selfing. The *S2* antisense construct that was introduced here, was earlier proven to be effective against the *S2* incompatibility allele, suppressing the synthesis of the *S2* glycoprotein. With this material (see later for details) material could be created and selected to answer the question whether the *S*-locus contributes to the unilateral incompatibility reaction.

Non-transgenic clones.

Six F2 populations, coded 1061 to 1066 (up to ten plants each) were obtained by selfing of the F1-acceptor clones 6541-01, -02, -03, -06, -11 and -25 respectively. These 6541- plants are all *tbr* x *ver* F1 hybrids (6234-08 x *ver* PI 195172-27) of the genotype *S3Sv AAii* SC_{ver}- or *S3Sv Aaii* SC_{ver}-, and thus acceptor of *ver* pollen. SC_{ver} is a pollen-expressed self-compatibilizing factor, derived from *ver* (Eijlander et al., b, submitted). The F2 populations 1061 to 1066 were expected to segregate *S3S3*, *S3Sv* and *SvSv* genotypes, and, whenever a 6541-parent was acceptor of the type *Aa*, also in *AA* and *Aa* acceptor and in *aa* non-acceptor genotypes. In *tbr*, the *aa* genotypes behave as non-acceptors of *ver* pollen, thus showing UI with *ver*.

Population 6484 (14 plants) originated from the cross of the non-acceptor *tbr* clone 6233-11 (*S2S2*, *aaII*) with the fertile near-*ver* clone TV^6-14 (14th plant of the 5th backcross generation of the F1 acceptor-*tbr* (T) x *ver* (V)). TV^6-14 was of the genotype *SvSv* (no style-activity of the S-allele) and contained the pollen-expressed SC-factor SC_{ver}. *Sv* and SC_{ver} were transmitted to the 6484-population. The plants of this 6484-population have previously been tested for acceptance and for SC. All plants were non-acceptor for *ver* pollen and self-incompatible (SI). SI of the 6484-plants was explained by a combination of gametophytic self-incompatibility with *S2*, UI-based rejection of *Sv* and a special interaction between genes expressed in pollen and style, directed agains S2+SC_{ver} pollen (SI by UI-based inhibition of SC; Eijlander et al., b, submitted).

Transgenic clones.

Clone 6484-06 was a randomly chosen SI genotype out of the afore-mentioned non-transgenic population, with the genotype *S2Sv* and had, presumably, the *aaIi* non-acceptor genotype. As stated above, it contained a pollen expressed "self-compatibilizing" factor (SC_{ver}). This factor appeared to be expressed in combination with a functional *tbr S*-allele (causing a mutual-weakning-like effect), but only when the *tbr* or hybrid recipient was not of the *aaII* or *aaIi* non-acceptor genotype (Eijlander et al., b, submitted). The clone 6484-06 was transformed with the *S2*-antisense construct (P35-*S2*AS)2 (Eijlander et al., a, submitted) in *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983). Transformation was carried out as described by Flipse et al (1994) and Eijlander et al (a, submitted).

Population 1184 was obtained by pollinating *tbr* clone 1140-02 (*S2S2*, SI, NA) (Eijlander et al., 1997; Eijlander et al b, submitted) with transgenic *tbr* clone (P35-*S2*AS)2 -V - 6. This transgenic clone V is of the genotype *S2S10*, NA, expressing the afore-mentioned antisense *S2* construct (Eijlander et al., a, submitted) and it bears a pollen-expressed SC factor (not SC_{ver}) derived from clone 1024-02 (Kuipers et al., 1994)), allowing for *S2*-penetration in *S2* expressing styles. Due to the pollen-SC factor, *S2S2* genotypes can be obtained, that are consequently all expressing this SC factor again. The antisense *S2* construct was transmitted by pollen. So, it was tested whether offspring plants showed an antisense *S2* induced reduction of *S2*-incompatibility. The population was screened by IEF and test crossed with *S2* pollen for presence of *S2S2* homozygotes that expressed the *S2*-antisense construct. Subsequently, the plants were tested for UI by test crossing with *ver*.

Monitoring incompatibility (UI & SI)

Test crosses for monitoring biological expression of antisense effects on SI and UI respectively were performed by pollination with pollen from *S2S2* homozygotes (Eijlander et al., 1997) and pollen mixtures of the *ver* (PI 195172) offspring clones 6555-31, 6555-33 and 1076-06. Styles were harvested 48 hours after pollination and fixed and stained according to the modified technique of Martin (1959). Pollen tube penetration was observed under a fluorescent microscope (Eijlander et al., 1997).

Protein gel electrophoresis

Up to 50 mg of plant tissue was ground in an Eppendorf tube with 20-100 ml 5 mM potassium phosphate pH 6.0, 2.5 % (w/v) sucrose, 0.1 % (v/v) b-mercaptoethanol, using a ground-glass pestle. Single style extracts were made in a volume of 25 ml extraction buffer. After centrifugation of the homogenate at 14000g for 15 min, the supernatant was fractionated on horizontal thin-layer isoelectric focussing (IEF) polyacrylamide gels (pH3.5-10) (Schmidt-Stohn, 1979) or agarose gels (Hypure gel VG 1020, Isolab inc). Silver staining was performed as described by Kirch et al (1989) for the polyacrylamide gels and according to company specifications for the agarose gels.

Results

The effect of antisense *S2* on SI and UI in *S2Sv* and *S2S2* non-acceptor genotypes

The molecular approach of gain and loss of function was very successful in proving that the *S*-glycoproteins play a key role in the SI reaction of the solanaceous species. Here we describe the loss of function approach as used in potato (Eijlander et al., a, submitted), but now applied to two different *S2* containing genotypes of non-acceptors for *ver* pollen: the *tbr-ver* hybrid 6484-6 (*S2Sv*) and a pure *tbr* population: 1184, containing the *S*-genotypes *S2S10* and *S2S2*.

The SI, NA clone 6484-6 was transformed with the antisense *S2* construct (P35-*S2*AS)2. A total of 40 transgenic hybrid plants was analysed for the expression of the *S2* antisense transgene. Testing by pollination with only *S2*-pollen revealed that in one genotype, (P35-*S2*AS)2-6484-6-4, the SI reaction against *S2* was effectively suppressed. This was confirmed by IEF of the stylar extract that showed a reduction of *S2*-glycoprotein content. *S2* pollen was compatible with this transgenic hybrid clone, clearly contrasting with the strong SI reaction in the untransformed clone 6484-6.

The same 40 clones were also tested for acceptance of *ver* pollen by monitoring the pollen tube ingrowth and seed set. Thirty-nine plants showed a very strong UI reaction, comparable with that of the untransformed genotype. Only one clone was altered in this respect. This was the same transgenic clone as the one that had become compatible for *S2* pollen: clone (P35-*S2*AS)2-6484-6-4.

acceptance	non-acceptance	antisense *S2*	antisense *S2*
6536-01 x *ver*	6484-06 x *ver*	(P35-*S2*AS)2-6484-6-4 x *ver*	1184-01 x *ver*

Fig. 1 Reactions of different stylar genotypes on *ver* pollen. Effects of antisense-*S2-RNase* based inhibition of *S2 RNase* production on UI in *S2-RNase* expressing non-acceptors (c, middle right and d, far right) compared with normal acceptance (a, far left) andnon-acceptance (b, middle left) of *ver* pollen. Insets in panel d: callose plugs in the most compatible pollen tubes. See also text.

The UI reaction against *ver,* though still intact (incomplete pollen tube penetration and thus no seed set), was strongly reduced and differed clearly from those in the other clones. Here, many *ver* pollen tubes reached to 2/3 of the style, whereas in the non-transgenic plant *S2* pollen did not surpass 1/4 to1/3 of the style and *ver* pollen was even inhibited at 1/5 to 1/4 of the style (Fig. 1 b, c). In the *ver* pollen tubes there was still a lot of callose deposition and many far-reaching pollen tubes showed inhibition phenomena like thickening of the tubes, irregular shapes, spongy callose deposition along large stretches of the pollen tubes and inflation of the tips.

The cross 1140-02 x (P35 -*S2*AS)2 -V - 6 (= *S2S2* x [*S2S10*, pollen-expressed SC, + 2 copies of *S2* antisense]) resulted, as expected, in *S2S2* and *S2S10* offspring plants. Twenty of them flowered well and were tested for SI and UI reaction by selfing and test crossing with *S2* and *Sv* pollen. Seven clones were SI and were of the *S2S10* genotype. Thirteen were pollen-based SC and seven of them had the desired *S2S2* genotype. Test crossing detected antisense induced *S2* suppression at various

levels in several *S2S2* and *S2S10* genotypes. Clone 1184-01 was *S2S2*, and expressed the strongest antisense induced *S2* suppression among all *S2S2* plants tested. However, this suppression was not as strong as that in the original transgenic parent. Variation in SI for *S2* ranged from pseudo-compatible to compatible with *S2* pollen, which ran parallel to the *S2* glycoprotein content of the styles. Clone 1184-04, also *S2S2*, showed variable reaction patterns from strictly incompatible to compatible with *S2* pollen.

The clones that showed no antisense effect on the SI reaction against *S2* pollen were, as expected, all non-acceptor of *ver* pollen. Reduction of the UI reaction up to acceptance was only detected in *S2S2* plants that showed the *S2*-antisense effect on SI. The UI reaction against *ver* pollen in clone 1184-01 ranged from normal UI to highly pseudo-compatible (Fig. 1d), with limited seed set and clone 1184-04 was slightly less compatible with *ver* pollen. The UI reaction was simultaneously suppressed with the SI reaction for *S2* pollen and ran parallel to the decrease of the *S*-glycoprotein content of the style. When the breakdown of UI was strong, the pollen tubes appeared to be normal, with small, regular interspaced callose plugs (see insets in figure 1d). In the lower part of the style, however, many tubes, but not all, showed reaction patterns as observed after pollination of the antisense-*S2* transgenic 6484-6 clone with *ver* pollen(see above).

The difference in reaction pattern in particular in clone 1184-01 (seed set with *ver*) compared to that in clone (P35-*S2*AS)2-6484-6-4 (inhibition at 1/3 of the style), probably indicates that both clones have different non-acceptor genotypes. Nevertheless, both types of material confirm that reduction of the stylar *S2* glycoprotein content coincides with the simultaneous break-down of the SI and the UI reaction, thus implying that at least the *S2*-glycoprotein contributes to the UI reaction.

Testing for (non)acceptance of *SvSv aaii* genotypes with *ver* pollen

It was shown that the *S*-locus is involved in the UI reaction. The observations suggested that this contribution depended also on the type of non-acceptance, including the possibility that non-acceptance can be expressed even in the absence of an active *S*-glycoprotein. Genotypes with inactive or defective *S*-alleles may shed more light over this question. Those genotypes must have a non-acceptor background (non-*A*ii*) in order to see any effect of such a defect on the UI expression. The *S*-alleles of *ver* show no stylar activity, so the introgression of these alleles in a non-acceptor background may serve as an example of non-functional *tbr S*-alleles.

The hybrid population 6541 (*S3Sv*, SC_{ver}—) is known to contain solely *Aaii* and *AAii* acceptor genotypes, based on test crossing with *ver* pollen (Eijlander et al., b, submitted). After selfing, any *Aaii* acceptor parent is expected to segregate into acceptors and non-acceptors in a 3:1 ratio of *A*ii* and *aaii* genotypes. Self pollen, capable of penetrating the style, is of the genotype *Sv*, *S3* SC_{ver} or *Sv* SC_{ver}. Thus, when no other selection mechanisms play a role, one-third of the genotypes will be of the desired *SvSv* genotype, all the others *S3Sv* or *S3S3*.

Five of the six populations of selfed 6541 clones suffered from inbreeding depression and none of the populations gave over seven testable flowering plants.

Four self populations showed the expected segregation for acceptance (*AAii* or *Aaii*) and non-acceptance (*aaii*) when *S3S3* and *S3Sv* genotypes were tested for acceptance of *ver* pollen, indicating descendence from *Aaii* genotypes. *SvSv* plants were separately selected to be tested for acceptance.The hypothesis Ho is, that any *SvSv aaii* genotype will be non-acceptor, indicating that this type of UI is independent of style-expressed *S*-alleles, and Ha is that *SvSv aaii* genotypes are acceptor because they lack style-active *S*-alleles. Those four segregating populations gave 27 - flowering plants, eight of them being *SvSv* (no *S3* glycoprotein detected). As expected under Ha, all *SvSv* genotypes were acceptor. Under Ho the likelyhood of inadvertently not detecting an *SvSv aaii* genotype is $P(k=0, N=8 \mid p=0.25) = 0.75^8 = 0.10$. These observations show that there is a strong indication for *S*-allele dependence of non-acceptance expression in *aaii* genotypes.

Analysis of *ver* on NA by analysis of likelihoods of expression patterns in population 6484.

As mentioned before, one of the parents of the F1 population 6541 (*tbr* 6234-08 x *ver*) must have been heterozygous (*Aa*) for the acceptor gene *A*, because non-acceptance (NA) segregated in the F2-populations (coded 1061 to 1066), as well as in some of the BC1 populations mentioned by Eijlander et al (b, submitted). The *tbr* parent 6234-08 was *S3S3AAii*, thus implying that *ver* was the most likely source for *a*, because of its origin denoted a_v. This was in accordance with old data on segregation of acceptor lines in *ver* x *tbr* and *(ver* x *tbr)*x *ver* crosses, based on hybridisations of *ver* with the diploid *tbr* clone G254 (*S1S3, AaIi*) (unpublished results). Just like the results from the aforementioned experiment, these unpublished results point to the possible existence of *aaii ver* genotypes, but are not conclusive.

Research on the expression of SC in *tbr-ver* hybrids (Eijlander et al., b, submitted) resulted in a complex hypothesis concerning the suppression of pollen mediated SC in specific non-acceptor genotypes. This hypothesis, explained hereafter, suggested that the near-*ver* line TV^6 could have contained the non-acceptor allele a_v. The cross with the self-incompatible non-acceptor clone 6233-11 (*S2S2, aaII*) would then result in at least some aa_vIi_v genotypes. Theoretically one *a* allele in TV^6 could have been derived from the original *tbr* acceptor clone, but the presence of a second *a* allele (thus $TV^6 = a_va_vii$ or aa_vii) should imply the presence of this recessive allele in the *ver* backcross parents.The inhibitor allele *I* seems to obstruct further analysis of interaction between *S*-locus and *aa*, because of its epistatic behaviour, but the presence of SC_{ver} can bypass this problem. It was hypothesised that the pollen expressed SC_{ver}-factor, as found in the hybrid clones 6484-06 and in 6541-06 (Eijlander et al., b, submitted), is not functioning in *aaI** styles. Clone 6484-06 would then be SI by UI-based SC-inhibition. Based on this, an analysis has been made for the possible genetic constitutions of the parental clones TV^6 -14 and *tbr* 6233-11 of population 6484 (table 1). From this analysis it can be deduced that TV^6-14 being A_va_vii has a maximum likelyhood as low as 2%. With a relative likelyhood of 96% the most likely genotype of TV^6-14 is aa_vii or a_va_vii and that of 6233-11 *aaII.*

The dominant gene *I* could not be tested here on *S*-allele dependance.

Table 1. Likelihood (γ) for erratically missing a SC plant in population 6484 (14 plants) under the assumptions that SC_{ver} is inhibited in an *S2Sv aaIi* style but expressed in an *S2SvAaIi* style and that TV^6-14 can be either SC_{ver} - or SC_{ver} SC_{ver}. This is calculated for all theoretically possible (non) acceptor backgrounds for both parental clones. TV^{6}is acceptor for *ver* pollen and self-compatible, 6233-11 is *S2*-homozygous, self-incompatible and non-acceptor for *ver* pollen.

Genotype of TV^6-14	Genotype of 6233-11	γ if TV^6-14 = SC_{ver} -	γ if TV^6-14 = SC_{ver} SC_{ver}
SvSv, AAii	*S2S2, AAII*	0.00006	0
	S2S2, AaII	0.00006	0
	S2S2, aaII	0.00006	0
SvSv, Aaii	*S2S2, AII*	0.00006	0
	S2S2, AaII	0.0014	0.00000
	S2S2, aaII	0.0178	0.00006
SvSv, aaii	*S2S2, AAII*	0.00006	0
(non-acceptor?)	*S2S2, AaII*	0.0178	0.00006
	S2S2, aaII	1	1

Discussion

Contribution of the S-locus to UI

The approach in testing the contribution of the stylar part of the *S*-locus to non-acceptance of *ver* pollen *tbr* styles was based on three different types of material:

- hybrids that did not expess a *tbr S*-allele but with a putative non-acceptor genetic background (*SvSv aaii*)
- hybrids that were non-acceptors, contained a pollen SC_{ver} factor but did not show SC where it was expected, unless certain interactions were postulated (SI by UI-based SC inhibition, directed by *aaI** styles against SC_{ver} containing pollen). This hypothesis allowed for the analysis of the acceptance of self-pollen in *ver*,
- non-acceptor clones with only one *tbr S*-allele (*S2S2* or *S2Sv*) that were antisense *S2* transformants and showing antisense inhibition of the *S2* alllele, thus disrupting a putative contribution of the stylar product to the UI reaction.

Hybrids that were both male and female fertile and that contained only *Sv* were obtained by selfing F1 hybrids. The five self populations, 1060 to 1065, gave strong indications about the relationship between (non)acceptance and the *S*-locus, because no *SvSv* non-acceptor genotypes were detected

(H_0: *SvSv aaii* = non-acceptor). The alternative hypothesis H_a (*SvSv aaii* = acceptor), that in this material any non-acceptor of the genotype *aaii* needs a style-expressed *S*-allele to exhibit its non-acceptor nature, cannot be proven by direct identification of *aaii* acceptor genotypes, but can only be established by the rejection of the hypothesis that *SvSv aaii* genotypes are non-acceptors at any time. Thus, H_a has a likelihood of 90%.

The postulated suppression of the SC_{ver} factor in *aaII* and *aaIi* styles, as occurs after selfing of clones of the 6484 population (TV^6 x *S2S2 aaII*), enabled a theoretical genetic analysis of TV^6. When the commonly applied confidence level of 5% is applied, it can be concluded that TV^6-14 is *SvSv aaii* SC_{ver}*. This implies that the genotype of *ver* is expected to be *aaii*. TV^6-14 is self-compatible and has successfully been backcrossed with *ver*, resulting in TV^7. Previous data (Hermsen, unpublished) on segregation of *ver* x *tbr* hybrids into acceptors and non-acceptors support our finding that *Aaii* and even *aaii ver* genotypes do exist, that are acceptor for self-pollen.

Another approach to test for the dual function of the *S*-locus was to introduce the sense *S2* glycoprotein constructs by transformation (Eijlander et al., submitted). Any *SvSv aaii* genotype that expresses the *S2* transgene, should then change from acceptor to non-acceptor for *ver* pollen.This transformation has been performed on several *ver* clones with the genotype *Aaii* or *aaii*. The only clone that has been analysed thoroughly for being *aaii*, was TV^6-14. Despite the fact that the transformations were successful and resulted in nearly 100% (transgenic) callus formation, no regenerants were obtained (data not shown), thus disabling this option. Murfett et al (1996) were successful with this approach by causing an UI reaction against *Nicotiana tabacum* and *N. glutinosa* (both SC-species) pollen in *N. tabacum* when the introduced *S2 S*-glycoprotein of *N. alata* (SI-species) was expressed at high levels, but this approach failed for the SC species *N. plumbaginifolia*, indicating different UI backgrounds.

Although in our experiments the approach of sense-transformation-induced UI failed, the approach of knocking down UI by an antisense *S*-allele was successful.The number of antisense *S2* transgenic 6484-6 plants showing antisense effect was much lower than previously reported for clone V (Eijlander et al., a, submitted), the pollen parent of population 1184. The *S2* incompatibility reaction in the non-transformed plants is quite strong. An explanation for this low frequency might be that because of the absence of an additional style-expressed *S*-allele, an up-regulation of the *S2*-allele is obtained. This explanation is not unlikely because down-regulation of *S*-alleles by over-expression of transgenic *S*-alleles has been shown (Eijlander et al., a, submitted). Natural weakening of the SI reaction by modifier genes is mentioned by many authors for many crops (Mather, 1943; Takahashi, 1973; Henny and Ascher, 1976; Litzow and Ascher, 1983) and it is therefore tempting to assume that there are, as an alternative possibility, modifier genes in this clone that enhance the *S2* expression rather than weaken it, thus limiting the antisense effect. That this stronger SI reaction is caused by a non-acceptance background can neither be confirmed nor rejected, because more loci than *A* and *I* are likely to play a role.The extremely strong SI and UI reaction in the original 6484 hybrid

population showed that those plants, including genotype 6484-06, were highly reliable in those reactions. This was confirmed for all the transgenics derived from plant 6484-06, except transgenic number four, that provided the proof for *S*-glycoprotein involvement in UI.

In addition to this, the transgenic clones (in 6484 and 1184 populations) that showed a break-down of UI formed an additional control population by themselves, because the fluctuation in strength of the UI reaction coincided with the fluctuation in strength of SI and in *S2* glycoprotein content.

There is a small discrepancy in the break-down of SI for *S2* pollen and UI for *ver* pollen between the transformed hybrid 6484-06 and the anti-sense *S2* expressing transgenic *tbr* clone 1184-01. Although both clones showed a break-down of UI that coincided with the break-down of SI for the *S2* allele, the latter transgenic showed a stronger break-down of UI, that resulted in seed set after pollination with *ver*. This might be due to a stronger expression of antisense *S2*, but 1184-01 being a non-acceptor of the type *A*I** or *aaii* instead of *aaI**, is probably a better explanation. Based both on the analyses presented here and on the model for SC expression/ inhibition proposed by Eijlander et al (b, submitted), 6484-06 should have the genotype aaIi, thus also blocking the SC-factor from *ver*. This implies an additional UI factor to react upon and a stronger UI reaction in this breaking-down situation. That the 1184 population showed a weaker antisense induced break-down of the *S2* incompatibility reaction, might be due to variation in transmission of the T-DNA, or to the homozygosity of the *S2* allele in the offspring (Heeres et al., 1998).

It was proven (Murfett et al., 1994; Lee et al., 1994; Eijlander et al., submitted) that the stylar SI reaction is determined by *S*-glycoproteins. Chetelat and DeVerna (1991) showed that in *L. pennellii*-pollen UI was determined by at least three loci, one of them mapping on or near the *S*-locus. Foolad (1996) found even more UI related loci, accounting for skewed segregations.That *ver* is still expressing a pollen factor, was proven in our experiments on SC in the interspecific hybrids (Eijlander et al., submitted). Theoretically, a UI determining factor might exist that is closely linked to the *S*-locus. Breaking of this linkage might be as difficult as breaking the linkage between the pollen- and style-factor of the *S*-complex. Because the pollen factor and the style factor are closely linked and together constitute the *S*-locus, the involvement of the *S*-glycoprotein in UI should not be surprising anymore. Murfett et al (1996) showed that in *Nicotiana* the introduction of an *S*-glycoprotein can induce UI characteristics, that were not present before. By antisense suppression they could eliminate SI and UI reaction patterns for respectively *N. alata* (SI) and *N. plumbaginifolia* (SC) from *N. plumbaginifolia* x *N. alata* (SI species, NA) hybrids. These results are comparable with those we found in the antisense *S2* expressing clones 6484-6-4 and 1184-1.

The genes directly responsible for (non-)acceptance *A* and *I* (or a_1 and a_2, Hermsen et al., 1974; or ui_1 and UI_2, Abdalla, 1974) segregate independently of the *S*-alleles. However, acceptance is not completely independent of the *S*-locus. That this has not been detected previously in the *tbr-ver* system, can be attributed to the technical difficulties encountered in the past in detecting *S*-alleles, but also to the rare occurrence of the relevant genotypes in self- and backcross populations.

Descriptive model for interactions of SC, SI and UI determining genes.

All the results obtained on SC, SI and UI and discussed in this and in our afore mentioned article, can be explained by the loci *A* and *I*, the *S*-alleles from the two species (tested here: *S2*,*S3*,*S4* and *Sv*), the pollen-active self-compatibilizing factor SC_{ver} and the earlier discussed interactions between them. The occurrence of differentials (*tbr*-genotypes that are acceptors for one *ver* genotype but non-acceptors for another *ver*) as described by Hermsen and Sawicka (1979) and also observed by the present authors may be explained by the existence of more than two alleles of *A* and/or a dosage effect of the genes involved. The observations made and its predicted (in)compatibility reactions are shown in table 2. This table deals only with the alleles *A* and *a*. Here individual reactions of pollen genotypes in styles of various *tbr* and *ver* based recipients are indicated. Pollen can be of a pure *tbr* type, a hybrid type or a *ver* type. Ploidy effects are also indicated: the left block deals with haploid pollen from diploids and the other block (right) shows some pollen types produced by, for

Table 2. Predicted and/or observed interactions between pollen and style in various acceptor and non-acceptor backgrounds, based on *tbr* and *ver*. *S2* and *S3*: *S*-alleles from *tbr*, *Sv* = *S*-allele from *ver*. *S-* = silenced *S*-allele (by antisense) from *tbr*. Recipients with the genotype *SvSv* react like S-*Sv*. Pollen with *S2S3* and *S2Sv*: diploid pollen as produced by tetraploids or by a restitution mechanism active in diploids. *A* and *a*: acceptor alleles. *A*= acceptor. *I* and *i*: inhibitor alleles. *I* is epistatic over *A*. The genotype *aaii* needs an active *S*-allele to cause UI. SC_{ver} = pollen-expressed self-compatibilizing factor, not effective in a S_{tbr} * *aaI** style. PC= pseudo-compatible= incomplete incompatible reaction, potentially giving some seed set. The question mark ? is placed where the effect of counteracting powers is unknown and depends on dominance relationships. See also text.

Style genotypes		Pollen genotypes								
		produced by diploids						produced by tetraploids		
		S3	*S3* SC_{ver}	*S2*	*S2* SC_{ver}	*Sv*	*Sv* SC_{ver}	*S2S3*	*S2Sv*	*S2Sv* SC_{ver}
tbr	*S2S2, A*ii*	+	+	-	+	+	+	+	+	+
	*S2S2, A*Ii*	+	+	-	+	-	-	+	+	+
	S2S2, aaIi	+	?	-	–	–	–	+	+	?
	S2S2, aaii	+	+	-	+	-	-	+	PC/+	+
tbr x *ver*	*S2Sv, A*ii*	+	+	-	+	+	+	+	+	+
	*S2Sv, A*I**	+	+	-	+	-	-	+	+	+
	*S2Sv, aaI**	+	?	-	-	--	--	+	PC	PC?
	S2Sv, aaii	+	+	-	+	-	-	+	+	+
tbr x *ver, asS2*	*S-Sv, A*ii*	+	+	+	+	+	+	+	+	+
	*S-Sv, A*I**	+	+	+	+	-?	--?	+	+	+
	*S-Sv, aaI**	+	?	+	?	-?	--?	+	+	+
	S-Sv, aaii	+	+	+	+	+	+	+	+	+
ver	*SvSv, aaii*	+	+	+	+	+	+	+	+	+

instance, tetraploids. The inactivation of a *tbr S*-allele (stylar expression) by means of antisense gives, when 100% effective, functionally an *Sv* allele. *SvSv* genotypes are therefore not mentioned. The SI reaction against *S2* pollen in an *S2S2* style is presumably stronger than in an *S2S3* (Mc Cubbin et al., 1997) or an *S2Sv* style (dosage effect). It is postulated that pollen containing both *Sv* and SC_{ver} causes a normal or enhanced UI reaction and that here the interaction like that between *S2* and SC_{ver} is not effective in an *S2Sx aaI** style (Eijlander et al., submitted). Question marks indicate that the final effect depends on the balance of different effects, like in *S2S2aaIi* x $S3SC_{ver}$, where inhibition of SC_{ver} might be epistatic over the compatibility of *S3*. Co-dominance of S3 will lead to a moderately compatible reaction and epistasy to full compatibility. *Sv* pollen will presumably be inhibited in any *I* -containing style, unless *I* too depends on *S*-allele activity. Alleles for UI other than *A* versus *a* and *I* versus *i* are not considered here.

Most of the *S*-glycoproteins of the solanaceaous species have *RNase* properties and are essential for activity of SI (e.g. McClure et al., 1989) and are therefore also addressed as *S-RN*ases. Loss of *S-RNase* activity has been shown to result in self-compatibility (Royo et al., 1994; Kowyama et al., 1994).

That absence of *RNase* activity would be enough to bypass a UI reaction, is not true. There are more factors involved in this, as is exemplified by the UI reaction between *N. plumbaginifolia* (SC-species) and a SC line (SC due to absence of *S-RNase* activity) of the SI species *N .alata* (Murfett et al., 1996). UI was still intact here. *L. esculentum* and *L. peruvianum,* two species that are less related to each other than *ver* and *tbr* are, show a very strong UI reaction. Here too, the cross is only successful when performed on the SC species *L. esculentum*. There is, however, a SC line from *L. peruvianum*, LA2157, that is SC due to a mutation in the coding region for the *S*-glycoprotein, causing loss of *RNase* activity (Kowyama et al., 1994; Royo et al., 1994). Rick (1986) reported, however, that all the lines investigated, retained their UI reaction against *L. esculentum.* Our results confirmed his observation that plants homozygous for this mutation, are highly UI when pollinated by *L. esculentum*. Although the pollen tubes from this pollinator appeared to penetrate perhaps a tenth of a style length deeper into the styles of the SC homozygous plants than in styles from SISI or SISC plants (R.Eijlander, unpublished data), the differences were minute: all plants were definitely UI for *L .esculentum*.The simplest explanation for such a strong UI reaction, even when the *S*-glycoproteins possess no *RNase* activity, might be due to a gene being different from the genes dealt with here. Such a gene might be strongly expressed when species are more distantly related and not as close as *tbr* and *ver*. Other explanations migth be a pleiotropic effect of *I,* expressed in wider crosses, or stronger alleles of this gene in *Lycopersicon.* Another attractive explanation might be that an *S*-glycoprotein may not not need *RNase* properties to trigger a UI reaction (see also later). It would therefore be essential to test whether acceptor plants that are of the *aaii* genotype remain acceptor when transformed with a construct coding for an *S*-glycoprotein that lacks *RNase* activity, like the modified *Petunia inflata S3* glycoprotein that was described by McCubbin et al (1997).

Examples of *S*-glycoproteins not being necessary for a functional UI reaction can be found within *Solanum*.The species *S. brevidens* (*brd*) is a SC species and may lack *S-RNase* activities in the style, for IEF of the stylar proteins of a *brd* clone used here showed a single basic protein at the position of *K1*. Backcross progeny of a *tbr*+*brd* fusion showed this band together with *S*-glycoprotein bands, suggesting similar behaviour of *brd* and *ver* and thus *K1* homology (unpublished results) and thus absence of *S*-glycoproteins. This species shows, apart from crossing barriers due to problems during seed development, also UI reactions when crossed with SI *Solanum* species (Pandey, 1962). Additionally, *brd* and its relative *S. etuberosum* (*etb*) show unilateral incompatibility with *ver*. Despite the barriers at the seed formation level, they could be crossed with *ver* as pistillate parent (Hermsen,1983), supporting the belief that the latter species is a more recent SC one.

Discrepancies

The phenomenon that pollen tubes of the *ver*-type can be arrested by the *S*-glycoproteins of *tbr* cannot be explained when a specific inhibition or transport of *S*-glycoproteins over the membrane occurs, as proposed for the SI reaction (McClure et al., 1989,1990; review by Kao and McCubbin (1997)), because *ver* pollen is not expected to possess those *S*-allele specificities, and should subsequently be indifferent to *tbr S-RNases*. Additionally, the SI reaction is dependent on the *RNase* activity of the *S*-glycoprotein (Royo et al., 1994), which supports the hypothesis of transport over the membrane. Irrespective of which model (selective uptake over the membrane versus random uptake plus selective activation/inactivation of the *S*-glycoproteins: McClure et al., 1989; Clark et al., 1990; Kirch, 1993) is applied, the selective procedure causes a problem. So, when one of these models is basically correct, it must be modified or extended by introducing an additional function for *S*-glycoproteins in UI systems, but outside the pollen tube. As already proposed, the expression of modified *S*-glycoproteins (transgenes) or antisense suppression of the production of *RN*ase-activity lacking *S*-glycoproteins as in the SC lines of *L. peruvianum* can give more insight in other mechanisms of causing pollen tube inhibition.

If those *RN*ase-activity lacking *S*-glycoproteins are capable indeed of causing an UI reaction, the possibility is opened that *S*-glycoproteins cause a signal transduction as presumably takes place in the SI system of *Papaver rhoeas* (Franklin-Tong and Franklin, 1993) or in the sporophytic system of the *Brassicaceae* (e.g. Stein et al., 1991; Nasrallah et al., 1994), with one important difference: here *S*-allele non-matching products are triggering a reaction instead of the *S*-allele matching products. This implies that within a species the compatible crosses, like *S1S2* x *S2S3*, the compatible non-matching of the style and the *S3*- pollen should not trigger a UI reaction. Here the products of the acceptor gene *A/a* and the inhibitor gene *I/i* must either block this signal transduction in case of species-own pollen or, when the membrane-bound *S*-glycoprotein needs an additional factor, to enable the signal transduction in case of non-self-species pollen.

From this it may be clear, that the contribution of the *S*-locus to UI complicates some hypotheses and that both SI and UI might be more complicated than was expected.

Chapter 6

General discussion

Diploid potato expresses a one-locus gametophytic incompatibility (GSI) system. The diploid potato population derived from the clones G254, G609 and B16 (Olsder and Hermsen, 1976; Hermsen, 1978 a,b,c) proved to be a valuable source for research, and for GSI research in particular. The first two dihaploids were reported to be derived from the cultivar Gineke, the dihaploid B16 was derived from a complex interspecific hybrid created by Black. Diallel crosses allowed for a classification of *S*-alleles , randomly assigned *S1* to *S4*. Plant material derived thereof was used for biochemical and molecular classification of factors involved in SI (e.g., Kirch et al. 1989; Thompson et al, 1991; Kirch, 1993;Wemmer, 1994; Peil, 1995). This basic material was used for the creation of well performing *S*-homo- and heterozygotes, and the plant material was tested on SI. Test crosses, iso-electric focusing of stylar extracts, Southern blotting and investigation of SC sources made clear however, that Gineke could not be the direct source of the primary dihaploids G254 and G609, but more likely an indirect one. This uncertainty about the ancestors of this material has, though, no consequences for the validity of the SI results obtained on this material. This is not only corroborated by the consistency of these results, but also by the reports concerning SI in other solanaceous species.

Obtaining reliable S- heterozygous and homozygous self-incompatible tester clones.

It was argued in chapter 2 that pollen-borne PC can be used to create *S*-homozygotes that are, nevertheless, reliable in their stylar SI reaction, and vice versa. The *S*-homozygous tester clones that were required for the SI research described and used here (chapters 2-5), needed to be reliable in their pollen-borne SI response. The counterfeit pollination method proved to be a valuable tool in obtaining material with a strong SI response in pollen and style. Utilisation of strong pseudo-compatibility (PC) already present in *S*-heterozygotes, showed to be unattractive for the production of SI-reliable clones, because of the apparent heritability of PC, causing PC even in the *S*-homozygotes. The *S*-homozygotes that were used as tester pollinators, showed neither pollen-expressed nor style-expressed pseudo-compatibility, and were thus well selected.

Most of the selected clones were tested for their transformation efficiency. Although there was some variation in both regeneration ability (giving regenerants from a stem explant) and transformation ability (= giving transformed cells, see also Kharbotly, 1995; Kharbotly et al, 1995), little progress by breeding for transformation efficiency (= transgenics obtained per transformed explant) was anticipated. Therefore it was decided that this trait could be introduced by crossing with material that was good in this respect (see Appendix 1) and valuable material could be selected. The selected material was reliable in its stylar expression of SI, but all F1 plants were SC due to a pollen expressed SC factor, like the one found in G254 and B16. Transgenics obtained from these clones could be tested for their stylar SI expression. By backcrossing clones without this SC factor have

been obtained. Only a limited number of well performing clones with a reliable pollen- and style expressed SI reaction have been selected, like clone 6618-02 (VI). The continuation of the breeding and selection procedures, with the aim of combining all the good factors of the SI population with the transformation efficiency of the second population was the reason why a wide range of transformable clones was used, instead of sticking to one clone.

Expression of the sense and anti-sense S-RNase based constructs.

It is clear from both the anti-sense experiments with *S1* and *S2* that a complete suppression of the corresponding *S*-glycoprotein production is not required to obtain an efficient break-down of the incompatibility reaction. A slight reduction of the *S-RNase* results in some pseudo-compatibility for pollen carrying that *S*-allele, and a strong reduction results in complete compatibility. This finding is not surprising, knowing that early pollination of flowers from clones that are slow in the building-up of the stylar glycoprotein content frequently lead to seed set. This was also found and described in chapter 2. The gain-of-function approach (introduction of sense-*S2* constructs) in potato showed that the strong *S2* expressing clones displayed a very strong SI reaction, in extreme cases barely allowing the pollen to penetrate the stigma more than half a millimetre. The weak expressors hardly inhibited the pollen tube penetration, thus confirming the results obtained by the loss-of function approach, but then from the other side of the SI reaction spectrum. Analog results were obtained by gain and loss of function experiments with *S*-allele based constructs in *Nicotiana alata* (Murfett et al., 1994) and *Petunia inflata* (Lee et al., 1994). The sense-inhibition approach in potato was not successful in this respect that the endogenous and homologous allele was not silenced or otherwise rendered ineffective. It was in some cases, however, successful in causing a break-down of the incompatibility reaction of the not-targeted non-homologous *S*-allele. In this case the apparent over-expression of the transgene caused some kind of down-regulation of the non-homologous allele. It is also possible that the endogenous homologous allele was down regulated too, but this is impossible to demonstrate because both the transgene construct p*SK2* Ω*S2* and the endogenous *S2* gene code for the same product. This down regulation was also observed in some transgenics with the genotype *S3S10* +p*SK2* Ω*S2*, knocking down both *S3* and *S10*. It might, therefore, be possible to find in nature genotypes with a combination of *S*-alleles that show dominance of one *S*-allele over another by some kind of down-regulation, but there are to date no reports of this yet.
Thus, both the sense and the anti-sense experiments confirm that the *S*-glycoproteins are responsible for the stylar side of the SI reaction, as was also earlier found in *Petunia* and *Nicotiana* (Lee et al, 1994; Murfett et al, 1994).

Contribution of the S*-locus to unilateral incompatibility/incongruity.*

As already stated in the chapters 4 and 5, it is debatable whether the *S*-locus is involved in UI. Until the report of Murfett et al (1996) showing proof for *S*-involvement, only strong indications,

favouring or opposing this involvement, were reported. The apparent absence of *S*-locus contribution to UI, reported in some cases, led to the belief that unilateral incongruity was the proper expression, whereas a contribution supported the expression unilateral incompatibility.

The work of Hermsen and co-workers on UI between diploid potato species made already clear that the expression of UI was independent from the *tbr S*-alleles, because all types of *S*-heterozygotes and even some *S*-homozygotes could be found that showed either UI or the opposite reaction, acceptance. Also, the absence or presence of *tS1*-caused SC could not be linked to acceptance or non-acceptance for *ver*-, *eth*- or *brd*-pollen. In these experiments *tbr* genotypes in which *S-RNase* activity was lacking, were not used, so the influence of absence of active *tbr S*-alleles on UI could not be tested. *Ver*, however, lacks *S-RNase* activity, but nevertheless crossing barriers not based on differences in EBN, were encountered (Abdalla,1970). This supports the incongruity hypothesis, and also the SC mutant found in *Lycopersicon peruvuianum*, that lacked *S-RNase* activity, retained its UI reaction against *L. esculentum*. In the latter case it can be argued that *S*-glycoprotein was present, but it had lost its RNase property due to a point mutation only, which still allowed for a non-RNase dependent UI mechanism. In the case of *ver* this is unlikely, however, because no *S*-glycoproteins have been reported to be present in this species.

The research of Murfett et al (1996), who worked with gain and loss of *S-RNase* expression in *Nicotiana,* showed evidence for the contribution of the *S*-glycoproteins to UI. Because UI related reactions were not present in all the interspecific crossings where they were anticipated, which showed that also in *Nicotiana* more genes or alleles of UI determining genes are involved. This was in agreement with our detection of different levels of UI breakdown after the loss of *S-RNase* activity and with the existence of the previously postulated genes *I* and *A*. We could confirm here that the *S-RNases* can contribute to UI. Is was also clear that the UI reaction is complex, it knows several sites of activity in the style and has different mechanisms. It became also clear that the *S*-locus of *ver* is recognised in the UI reaction when non-acceptor *tbr* genotypes are pollinated with *ver* pollen or with pollen from *tbr-ver* hybrids, which is in accordance with localisation of pollen determined UI linked factors on- or in close linkage with the *S*-locus (Chetelat and De Verna, 1991). The pollen-part of the *ver S*-locus is still active and this might be a prerequisite for raising a UI reaction. Thus it was shown that the *S*-locus, both style *and* pollen part, really can contribute to the UI reaction.

Concurrence of hypotheses on UI

As stated in chapter 5, the model for non-acceptance of Grun and Aubertin (1966) did not differ significantly from one of the models proposed by Hermsen et al (1974). However, the best explaining hypothesis of Hermsen and co-workers, introduced a dominant gene for acceptance in stead of a recessive one. Abdalla and Hermsen (1972) developed the two-powers-hypothesis, an evolutionary model as also discussed by several other authors (e.g. Lewis and Crowe, 1958), but

in the two-powers-hypothesis the defence reaction of the SC species against most forms of introgression of genes from other species plays an important role. The basic assumption in these hypotheses was, that an SI species evolves stepwise into an SC species following the rule SI → Sc → Sc' → SC. According to Abdalla (1970), "the *UI* genes have developed through the challenge of hybridisation between SI and SC populations" and for *ver* "the sensitive plasmons of SC species have developed in the counteracting competition subsequent to crossing of SC and SI populations, particularly after the development of *UI* genes". Thus, finally an SC species will have a genotype like *ScSc uiui* and the SI species for instance *S1S2 UIui.* Little was known then how SI can be converted into SC. *Sc* can therefore also stand for an active *S*-allele linked with a pollen-expressed SC-factor, as has been described in chapter 4. Style-expressed SC factors as described by Flaschenriem and Ascher (1979) and Dana and Ascher (1986b) can thus also be counteracted by the development of *UI* genes. All these hypotheses, including the incongruity hypothesis of Hogenboom (1973) can be brought in accordance with each other, when the *S*-locus is subdivided into at least a pollen part and a style part, against which separate *UI* genes can be developed, which can have various alleles and dominance relationships. Thus, non-acceptance genes can also have dominant acceptance alleles or show intermediate reactions when heterozygous. The appearance of SI affecting genes, like minor genes, causing pseudo-compatibility, the pollen and style SC factors reported for *Petunia* by Dana and Ascher (1986 a,b), *tS1* in *tbr* clone G254, SC_{ver} in *ver* can thus trigger the development of corresponding *UI* genes. When these genes are not located within the *S*-locus complex, the *UI* genes are then *S*-locus dependent evolved, but potentially *S*-locus independent in expression. These *UI* genes are then truly incongruity genes. Thus a mixed system with unilateral incompatibility and unilateral incongruity genes can evolve. The inhibitor gene *I*, as described in chapters 4 and 5, might be such an incongruity gene. It is not known against which factor it is directed, so it might still belong to the unilateral incompatibility genes group.

Pollen expressed SC-factors.

It is already argued that there exists a variety in pollen-expressed SC (chapter 2). There is to date no proof for SC due to mutation of the pollen factor of the *S*-locus, whereas there is ample evidence for SC causing mutations in the style expressed *S*-gene. Like in B16 and G254, which were SC due to the pollen-expressed factor called *tS1*, the well-transformable clone 1024-02 proved to homozygous for pollen-expressed SC. It is tempting to assume allelism with *tS1*, especially after it was shown that *tS1* also causes SC with *S1* pollen. The offspring of 1024-02 did not show any strong preferential penetration of *S1*, *S2*, *S3*, or *S10* pollen. All four *S*-homozygotes were found after selfing of SC F1 offspring clones in more or less equal amounts. Hosaka and Hanneman (1998) detected skewed segregations of SC and SI in offspring from *S. acaule* and *S. phureja*. Their best fitting hypothesis was that the pollen-expressed SC factor *Sli* (S-locus inhibitor) acted sporophytic, and deviates in this respect from the expression pattern of *tS1*, which was a quite remarkable finding. It is, on the other hand, like *tS1*, localised on chromosome 12 (Hanneman, pers. comm),

which still keeps open the possibility of allelism of *Sli* and *tS1*. The appearance of two pollen-expressed SC alleles in 1024-2, unrelated with G254 and B16, shows that SC factors can be more common than believed.

In *Petunia hybrida* Dana and Ascher (1986a) detected a pollen-expressed SC factor at approximately the same distance from *S* as SC_{ver} in the *tbr-ver* hybrids. If these genes are allelic, this *Petunia* SC factor might show acceptance dependent penetration too, but nothing has been reported on this yet. The complex expression pattern of SC_{ver} and its dependence on the proper genetic background of the seed parent is explained in the chapters 4 and 5. The stylar suppression of pollen expressed SC_{ver}, as described in the chapters 4 and 5, explains why intercrossing of two self-incompatible potato species can bring about self-compatibility. For example, species 1 can be described by a basic genotype, *S1S2 AA ii scsc,* which represents a population of self-incompatible genotypes, acceptor for species 2. Species 2 can then be *S3S4 aa II SCsc* (and in some cases scsc), where *aaII* inhibits the penetration of SC, but allows for the penetration of sc and the *S*-alleles, as well as for the pollen of species 1. An F1 of these two species can then be *S1S3/S1S4/S2S3/S2S4, Aa, Ii, scsc/Scsc*. Only the *Aa Ii Scsc* plants will be SC. This phenomenon of sudden appearance of SC after intercrossing SI genotypes is known among potato breeders working with interspecific hybrids (e.g., in complex *phu-stn* hybrids, Hermsen, pers comm.).

Interaction between pollen and style.

The *S-RNase* is transported over the membrane of the pollen tube and is believed to cause an incompatibility reaction by degrading RNA, which finally results in pollen tube arrest. As explained in chapter 1, either the transport over the membrane is allele specific, or the uptake is nonspecific followed by an inhibition of the non-self *S-RNases*. Analysis of DNA sequences and protein structures of *S-RNases* gave already more insight in conserved regions (C1-C5), (hyper)variable regions (V1-V5) and presumed identity (V1-V5, except V3) and activity (C2, C3) determining parts of the *S-RNases* (e.g,. Ioerger et al., 1991; Tsai et al., 1992; Newbigin et al., 1993; Simms, 1993; McCubbin et al., 1997). Mutation studies have been performed on the regions that were expected to be responsible for the activity (e.g. Huang et al., 1994) or the identity. The activity was easily be disrupted by replacing a histidine residue in the activity parts (e.g. McCubbin et al., 1997) and interchanging hyper variable regions could disrupt the identity (Matton et al., 1997; Zurek et al., 1997), but specific identity determining regions within the hyper variable regions could to date not be identified (except, for at least, 4 aminoacid coding triplets). The mechanism of recognition between pollen and style remained still unclear.

The *pSK2*-driven S2 sense constructs, reported in chapter 3, showed in some cases detectable expression in complete anther extracts. Nevertheless, the pollen appeared to stay completely functional. It was not clear whether the pollen produced this *S-RNase*, or the surrounding anther tissue. Expression by the pollen itself could theoretically have resulted in immediate pollen tube growth arrest or pollen death. Kirch et al (1995) expressed already a potato *S-RNase* in pollen of *Nicotiana tabacum*. The pollen remained fully functional and capable of giving seed set. This

showed that there is no cytotoxic effect of the *S-RNase* on the pollen tube, although the authors themselves came with possible explanations why this conclusion might be incorrect. An incorrect genetic background (wrong species) might have been such a reason, or the lack of the necessary protein. Even absence of a proper phosphorylation, directly after *S-RNase* entering the pollen tube (as in a normal situation) might be a reason for this. A Ca^{2+} dependent phosphorylation is a possibility and putative mediating proteins have been detected in *Nicotiana alata* by Kunz et al (1997). That phosphorylation and Ca^{2+} play an important role in the pollen tube growth and/or the incompatibility reaction appears to be likely. It was shown by Li et al (1994) that Ca^{2+} plays a key role in the growth and development of the pollen tube tip. In chapter 1, it is pointed out that Ca^{2+} and phosphorylation play key roles in the SI systems of poppy and *brassica*, so this comes all together not as a surprise.

That a cytotoxic effect in a normal incompatible combination can be rejected, is shown by the style grafting experiments of Lush and Clarke (1997). Incompatibility reactions were, at least partly, reversible and supports the idea that the pollen tube actively synthesises RNA, which is degraded by the self-type *S-RNase* when transported over the membrane.

Discrepancy between pollen recognition in SI and UI.

As stated earlier in chapter 5, there is a problem when explaining the contribution of the *S*-glycoproteins (*S-RNases*) to both SI and UI. In the incompatibility reaction, there is a selective mechanism. Self-type pollen transports selectively the *S-RNase* over the membrane or selectively inactivates non-self *S-RNases*. In the UI reaction, however, non-self *S-RNases* cause an inhibition reaction. This justifies to consider an alternative, or additional mechanism for the interaction between pollen and style. When the *S-RNases* are not transported over the membrane, it might cause a signal transduction over the membrane. Somehow there must be a mechanism that discriminates between species-self and non-species self combinations of pollen tube and *S-RNases*. Probably here the products of the acceptance genes and different acceptance alleles play a role. Species-self is always recognised by this product (from for instance, both *A* and *a*) and should then disable or interrupt the signal transduction cascade caused by , for instance, the combination of *S2-RNase* and an *S1* pollen tube of the same species. Pollen from *ver* is then allowed to penetrate any style of *tbr*, as long as it is of the genotype *A*ii*. Absence of the product from *A* allows now for the *S-RNase* induced signal transduction, finally resulting in pollen tube arrest. There are several ways to test properties of this model. Style grafting as performed by Lush and Clarke (1997) is in the case of signal transduction unlikely to be able to cause a reversion of the UI reaction when analogous with the SI response in poppy. When the introduction of an *S*-glycoprotein, lacking the RNase activity (such as described by McCubbin et al, 1997) in plants that express UI when transformed with correct *S-RNases*, it is clear that identity is the determinant and not activity, thus allowing for a hypothesised additional interaction mechanism.

A key factor in elucidation more aspects of SI and UI still remains the identification and cloning of pollen-*S*-locus factors.

Appendix 1: Selection of well performing and well transformable clones, useful for SI research. Some characteristics and pedigree.

There are from various tobacco species accessions available that are highly efficient for transformation. Additionally, they are easily grown in the greenhouse, with more generations per year than potato. Because *S*-alleles can vary within a species even more that some *S*-alleles between species do, it is tempting to use tobacco for transformation experiments with potato-*S*-allele based constructs. This approach was followed indeed (e.g., Kirch, 1992; Kirch et al., 1995; Ficker et al., (1998), but is not applicable for all aspects of SI research. Interspecific crossing might be necessary for testing the biological effect of a construct. Then not only factors like interspecific crossing barries (e.g., unilateral incompatibility) can play a role, but also the different genetic background of the host can cause subtle interactions that were not anticipated (Kirch et al, 1995; Murfett et al, 1995), changing or inactivating the transgene's effect. Thus, for some basic experiments the constructs should be tested within the species of the *S*-allele origin. Therefore, well transformable potato clones with a reliable SI reaction were a prerequisite.

Screening of the selected SI clones for a high efficiency of transformation ability by *Agrobacterium tumefaciens* showed that there was some variation in regeneration capacity so that some transformants could be obtained, but none of the clones showed both a good transformation ability and a good regeneration capacity (unpublished results). Breeding for these combined abilities would most likely, when using the best performing genotypes, have taken several crossing generations. Some well-transformable diploid potato clones were available that were not related to the SI material. In a combined effort (see also Kharbotly, 1995) crosses were made and progenies were screened for both transformation ability as well as other criteria as mentioned in chapter 2 for the basic clones. A good performance for vigour, flowering, male and female fertility and a reliable SI reaction were prerequisites. The clones had to contain at least an *S1* or an *S2* allele in order to be of use for sense and anti-sense transformation experiments.

Clone 1024-2, one of the best transformable clones available, flowered late but abundantly. This clone was self-compatible, but it was expected to segregate for SC and SI clones in the F1 progenies. However, none of the 60 tested offspring clones was SI. The clones were SC due to a pollen-expressed factor like the one found in G254 and B16, indicating that 1024-2 was homozygous for SC. Segregation patterns of the *S*-alleles showed that 1024-2 contained *S3* and another *S*-allele, that probably belongs to the S1-S3 family. The SI reaction against *S3* pollen was reliable: none of the offspring genotypes originated from fertilisation by *S3*-pollen (Fig. 1). The stylar SI reaction of the selected clones was also very reliable (for *S1 and S2* in the 6486 and 6487 population respectively, for *S3* only on *S1S3* or *S2S3*), thus eligeble as good clones for testing of the stylar contribution of SI. The selection of well transformable clones was more labourious. The screening method developed by Kharbotly (1995) proved to be a powerful tool, but the performance in SI research formed a time bottle neck in this. Only few clones were found that met all criteria to a satisfying extent.

Plant A16, an offspring of clone 1024-2, was superior in transformation efficiency and was very vigorous, but had poor characteristics like, late flowering, functional male sterility, very poor in tuber set, easily wilting, wild branching, and it did not express the desired *S*-alleles. Most of these characteristics were transmitted to its progeny. Thus, only few transgenic clones out of hundreds screened, were suitable for our research on SI. Well performing, reliable self-incompatible clones were under represented, but found in a late stage of the project, which explains also why the pollen-mediated SC clones predominate in this thesis. It also explains why a less easily transformable clone like 195/5 was used. All selected clones were, however, reliable in their stylar expression of SI. Only the transformed SI clones that were diploid (thus not spontaneous somatically doubled) could be used directly to test the influence of the constructs on the male expressed SI reaction.

The pedigree of the transformable clones is presented in table 1, as well as the *S*-allele composition of the clones or populations, as far as investigated. Most of the clones in the 6618-population suffered from a low degree of flowering and fertility problems. Population 1122 had an excess of SC clones and a tendency to flower malformation. Many clones, though vigorous, suffered from a low male fertility. Population 1120 segregated for some useful clones, but the vigour was somewhat reduced. Many clones had a tendency for pseudo-compatibility under unfavourable climatological conditions.

Clone V is excellent in its transformation efficiency (an average of 80% in 5 weeks). It flowers middle late and fairly well, pollen fertility is excellent. Its tuberisation is late and only acceptable for vegetative propagation, when raised in early spring or grown under short day conditions.

Clones R2 and R5 are slow in transformation: nine weeks are required for 50% transformation efficiency. The plants are vigorous, flower early and profusely with excellent fertility. Tuberisation in small pots is early and good.

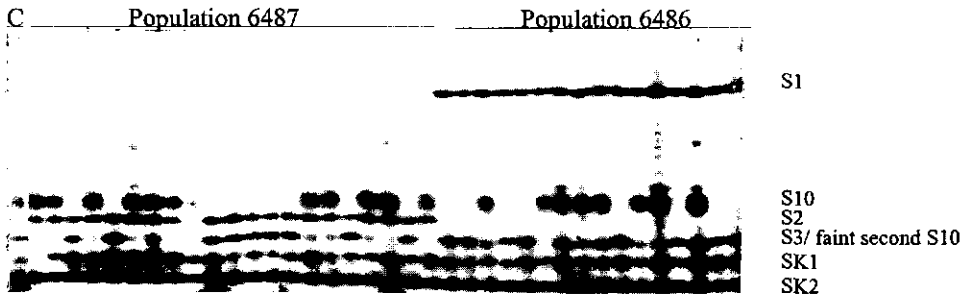


Fig 1. Silver stained Iso Electric Focusing patterns of offspring of clone 1024-2. Most left: clone 1024-02. Left: population 92-6487 (*S3S10* x *S1S2-> S2S3 + S2S10*); Right: 92-6486 (*S3S10* x *S1S3-> S1S3 +S1S10*). The *S10* allele shows sometimes a faint second band, here at the position of *S3*. The penetration of only *S1* in population 6486 and only *S2* in population 6487 confirms that the band on the *S3* position has the *S3* identity.

Table 1. Pedigree of well transformable clones and some characteristics. See for clone numbers and references also chapters 2 and 3

Clone	Mother	Father	S-alleles	SC/SI	Remarks
86-04-176					doubled monoploid, amf amf
87-10175-5	86-04-176	87.0008			
87.0007					= SH82-62-247
87.0008					= SH82-70-297
87.1024-1	86-040-231	87.0007			
87.1024-2	86-040-231	87.0007	*S3S10*	SC	well transformable, Amf amf
87.1029-31	87.1017-5	87.1024-1			
91-6222-40	G254	G609	*S2S3*	SI	see chapter 2
91-6104-19	*S2S3*	*S1S1*, SC	*S1S3*	SI	see chapter 2
91-6167-2	Her-64	87.1029-31	*S9S11*		
A16	91-6167-2	1024-2	*S9S10*	SC	functionally male sterile
93-4002-3	91-6222-24	A16 !!!	*S2S10*	SI	
VI (93-6618-02)	A16	91-6222-40	*S3S10*	SI	
R2 (92-6486-4)	1024-2	91-6104-19	*S1S3*	SC	
R3 (92-6486-..)	1024-2	91-6104-19	*S1S10*	SC	
R5 (92-6486-19)	1024-2	91-6104-19	*S1S3*	SC	
V (92-6487-09)	1024-2	91-6222-40	*S2S10*	SC	
94-1120-...	R5	91-6105-06	*S1/S3+S2/S4*	SC/SI	
94-1122-...	R5	93-4002-03	*S1/S3+S2/S10*	SC/SI	

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Summary

In chapter 1 an overview is given of the major mechanisms operating in Angiosperms that prevent or limit the degree of inbreeding. The two major systems that function on the basis of interaction between pollen and stigma/style, are the sporophytic and the gametophytic self-incompatibility systems (SSI and GSI). The plant is called the sporophyte and pollen and egg cells are called gametophtytes. In the sporophytic system, the pollen grains carry the information about the pollen donor in their coating. Thus, the pollen coating does not reflect the pollen genotype but deposits in it reflects the genotype of the pollen donor and the dominance relationships between the self-incompatibility alleles (*S*-alleles). When the recipient has incompatibility characteristics in common with the pollen coating, the combination will be incompatible and pollen germination and pollen tube growth will be arrested on or in the stigma. In the Brassicaceae, a group displaying SSI, signal transduction seems to be an important mechanism for triggering an SI response.
In the gametophytic self-incompatibility system, the pollen reflects the genotype of the pollen grain itself. When the incompatibility allele(s) of the pollen grain are met by a similar allele in the recipient, the pollen tube growth will be arrested. Thus, selfing provokes a gametophytic self-incompatibility (SI) response. Non-matching of *S*-alleles between plants of the same species results in a compatible combination. Most diploid Solanaceous species display GSI. The styles contain extracellularly the products of the style-expressed *S*-alleles, the *S*-glycoproteins. About the pollen components, contributing to SI, little is known, but *S*-heterozygosity in the pollen causes self-compatibility. The cultivated potato, *Solanum tuberosum* L.(*tbr*), is a tetraploid and behaves, due to *S*-heterozygosity in the pollen, as self-compatible species, whereas diploid potato generally possesses an active operating GSI system.
There exist, however, also diploid species that are self-compatible (SC). Frequently, regardless of SSI and GSI, the SI species can be crossed with related SC species only when the latter are used as female parents. This means, the SC species can be used as the pollen acceptor (acceptance), but the SI species rejects the pollen of the SC species (non-acceptance). This phenomenon, in which interspecific hybridisation can occur in only one direction, is called Unilateral Incompatibility or Unilateral Incongruity (UI).

In chapter 2 it is described how the basic plant material, used for SI research, was developed and selected. Vigour, abundant flowering and a good fertility were prerequisites for this material, but the most important characteristic was a reliable SI reaction in pollen and style. The combination of these characteristics is rarely found in diploid *tbr*. From a diploid *tbr* population, expressing four different *S*-alleles, plants could be selected for all six S-heterozygosity classes, that met all the afore mentioned criteria. *S*-allele composition could be tested by performing test crosses, but in addition to this, stylar extracts were analysed by iso-electric focusing, followed by silver staining. The *S*-glycoproteins, also called *S-RNases* because of their RNase properties, focus in the basic part of the gels. The selected material was used for the creation and selection of SI plants that were homozygous for the *S*-alleles. Normally, the SI system will prevent *S*-homozygotisation, unless the SI system is weakened by pollen- or style expressed minor or major SI-suppressor genes. A weakening of the SI response can cause seed set after selfing. This is called pseudo-compatibility (PC). Occasionally, however, some pollen tubes manage to penetrate the style , even when the SI

system is fully functional and PC can be excluded. The seed set will then, however be too low to establish a sink-source relationship that is strong enough to cause berry formation: the flowers will drop. The *S.phureja* (*phu*) clones IvP35 and IvP48 are normally compatible with diploid *tbr*, but the hybrid seed has the remarkable and useful characteristic, that the embryo's have a nodal band, which is visible through the seed coat as a seed spot at the first node between hypocotyl and the cotyledons. Pollination with those *phu* clones after making crosses that were incompatible, caused berry formation. This additional pollination is called "counterfeit pollination". Spotless seed, harvested from those berries, yielded both *S*-heterozygotes and *S*-homozygotes. Analysis on the seed set and the strength of the SI reaction in this offspring showed that, even when the original parents were selected for their good SI reaction, weakened SI was present, that could be expressed in either the pollen or the style. It was shown that this had a heritable character. From this material, *S*-homozygotes could be selected that were reliable in their SI reaction and that served as tester clones, as described in the chapters 3, 4 and 5.

The selected material, described in chapter 2, was poor in its transformation efficiency. For the functional analysis of, for instance, *S*-allele based constructs, an efficient transformation system is essential. It was decided, therefore, to select for this trait. Transformation efficiency was introduced from other unrelated sources. Well transformable clones with a reliable stylar SI expression could be selected from this material (Appendix 2), that were used for a gain-and loss of function approach. Sense (chapter 3) and antisense *S-RNase* constructs (chapters 3 and 5) were introduced by genetic transformation. Indeed, sense *S2* transgene constructs, driven by the promoter of the style-specific endochitinase *SK2*, were able to cause an incompatibility reaction against *S2* pollen in plants that did not contain the *S2* allele when not transformed. Some of those constructs showed such a high level of expression, that due to some mechanism, the endogenous *S*-alleles were down-regulated and became compatible for the endogenous *S*-alleles, whilst remaining incompatible for the transgene *S*-allele. The antisense *S1-RNase* and *S2-RNase* constructs were able to reduce the expression of the corresponding *S1* and *S2*-alleles, which resulted in a break-down of the incompatibility reaction against the corresponding *S1* and *S2*-pollen. Thus, the gain and loss of function approach showed the key role of the *S-RNases* in the stylar side of the self-incompatibility reaction.

In chapter 4 it is described why *ver* is self-compatible and how this is expressed in hybrid offspring, when crossed with self-incompatible *tbr*. When the former species is used as recipient, the hybrids suffer from cytoplasmic male sterility, thus disabling a further analysis of inheritance and expression of SI, SC and UI. The reciprocal cross fails normally, as already stated, due to UI. However, some of the potato clones, described in chapter 2, accepted *ver*-pollen and yielded male and female fertile hybrid offspring. Those particular potato clones are called "acceptors"for *ver* pollen, as an exception to the rule of UI. Plants that show UI are thus called "non-acceptors". It was shown that the species *ver* can be SC due to at least two different reasons: 1) there is no stylar S-glycoprotein and a stylar SI response is therefore disabled, and 2) there is a pollen-expressed self-compatibilizing factor, SC_{ver}. This SC_{ver}-factor was linked with the *S*-locus of *ver*, at an estimated distance of 18 cM. SC_{ver} is also capable of suppressing the SI reaction against pollen-expressed *tbr* *S*-alleles. This suppression depends, however, on the genotype of the pollen recipient. Acceptors

allow for the penetration of SC_{ver} carrying pollen, but specific non-acceptors can inhibit this type of pollen. It was shown that there exist differential reactions against *ver* pollen, and in particular, also against the SC_{ver} factor. Experiments with somatically doubled hybrids showed that where the stylar part of the *S*-locus of *ver* is inactive, that the pollen part of the *S*-complex is not only capable of triggering a UI reaction, but also in causing the SI-based phenomenon of mutual weakening. Mutual weakening is the phenomenon that when two different *S*-alleles are expressed in a pollen grain, the GSI reaction in the style will not take place anymore, even when those *S*-alleles are expressed in the style as well. Thus, a dual function of the pollen part of the *S*-locus is made likely.

In chapter 5 the gain and loss of function approach, as described in chapter 3, was used to test whether the stylar part of the *S*-locus is involved in the UI response too. The sense approach failed, due to the absence of transgenic *ver* regenerants, but the loss-of-function was successful. Both a transgenic non-acceptor *tbr* x *ver* hybrid and a transgenic non-acceptor *tbr* clone, both expressing only the *S2* allele in the style, showed a collapse of the UI reaction that coincided with the antisense *S2* caused break-down of the SI response against *S2* pollen. The *S*-locus complex shows thus a dual function for both the pollen part and the stylar part, both contributing to the SI and the UI response. It was made likely that *ver* can have a putative non-acceptor background for self pollen, but that its expression requires S-glycoproteins to be expressed. In this chapter it is discussed why the most important hypotheses about UI are not necessarily conflicting with each other. An explaining and predictive model with interactions of a range of genes and alleles is presented. The most important genes and their properties are:

- the acceptance gene *A,* which causes acceptance (*aa* genotypes being non-acceptors), but knows different alleles that cause differential reactions against *ver* pollen,
- the inhibitor gene *I,* which causes non-acceptance and is epistatic over *A*,
- the pollen-expressed SC factor SC_{ver}, which is in weak linkage with the *S*-locus, causes pollen to be compatible in any style, except those with the genotypes *aaII* and *aaIi,* in which it is inactive or even causes a UI reaction,
- the *S*-locus complex with both a pollen component and a style component, in which the pollen *ver* factor triggers a UI response, and the active stylar part is needed for a UI reaction in *aaii* non-acceptor genotypes. The latter explains why the introduction of an active *S*-allele in a SC species (such as reported by Murfett et al., 1996) can bring about a sudden SI or UI response and why *ver* can be a putative non-acceptor for self pollen, without becoming self-incompatible.

As a consequence of this, the expression "Unilateral Incompatibility" cannot completely be replaced by the expression "Unilateral Incongruity". The latter expression is valid in cases where the *S*-locus does not contribute to the UI response at all.

In the final chapter some of the results are discussed in a broader context. The last part stresses that the dual function of the *S*-locus implicates that, within the existing model of *S-RNase* activity in the SI system, a second function of the *S*-glycoproteins must be postulated. This can be triggering a signal transduction, resulting in a SI like response, resulting in the arrest of the pollen tube, but which may be independent of the RNase properties of the *S*-glycoproteins.

Samenvatting.

In hoofdstuk 1 wordt een overzicht gegeven van de belangrijkste mechanismen in bloeiende planten om inteelt te beperken of te voorkomen. Sommige mechanismen zijn direct herkenbaar zoals het voorkomen van uitsluitend mannelijke of vrouwlijke bloemen op een plant. Andere mechanismen zijn gebaseerd op het wel of niet doorlaten van pollenbuizen door de stijl en zijn niet direct zichtbaar. Verschillende van deze mechanismen worden nader toegelicht. De twee hoofdgroepen zijn "sporofytische zelf-incompatibiliteit" en "gametofytische zelf-incompatibiliteit". De plant zelf wordt de sporofyt genoemd, en het stuifmeel de gametofyt. Bij de sporofytische incompatibiliteit wordt, vereenvoudigd gesteld, de informatie van de bestuiverplant meegegeven met de stuifmeelkorrel. Deze informatie zit aan de buitenkant. Als de bestoven plant een "incompatibiliteitskenmerk" gemeenschappelijk heeft met de buitenkant van het stuifmeel, wordt de bevruchting onmogelijk gemaakt. Wat er aan genetische informatie in het stuifmeel zit, is dan niet meer van belang. Dit heet daarom sporofytische incompatibiliteit. Bij gametofytische incompatibiliteit is niet zozeer de buitenkant van het stuifmeel van belang, als wel de genetische inhoud van het stuifmeel. Als de genetische inhoud van de stuifmeelkorrels (pollenkorrels) voor "incompatibiliteit" wordt weerspiegeld in de te bevruchten bloem, wordt de doorgroei van de pollenbuis geremd en gestopt. Zelfbestuiving leidt dus tot een "gametofytische zelfincompatibiliteits-reactie". Het mechanisme dat in de meeste aardappelsoorten actief is, is de zogenaamde "gametofytische zelfincompatibiliteit".

In hoofdstuk 2 wordt verteld hoe het basis-onderzoeksmateriaal is geselecteerd en wat daar uit is gekomen. Het basismateriaal vloeide voort uit kruisingen die ooit gemaakt waren tussen diploïde klonen (24 chromosomen), die uit de normale, tetraploïde aardappel (*Solanum tuberosum* (*tbr*), met 48 chromosomen) verkregen waren De te gebruiken planten moesten uiteraard goed bloeien en mannelijk en vrouwlijk vruchtbaar (fertiel) zijn, maar bovenal heel betrouwbaar in hun zelf-incompatibiliteitsreactie (SI). Op dit soort eigenschappen is er in de eerste ronde geselecteerd. Om duidelijk onderzoek te kunnen doen aan heel specifieke varianten van de incompatibiliteitsgenen, de "*S*-allelen", is geprobeerd planten te maken die slechts één type van zo'n *S*-allel bevatten, de zogenaamde *S*-homozygoten. Dit druist eigenlijk tegen het "incompatibiliteitsmechanisme" in, wil men toch nog een betrouwbare SI reactie behouden. Soms lukt het een pollenkorrel toch om door te groeien en de eicel te bevruchten, waar dit op grond van de SI reactie niet verwacht was. Omdat een lage zaadzetting meestal tot vroegtijdige vruchtval leidt, wordt dit soort zaad zelden verkregen. Daarom is er gebruik gemaakt van een aanvullende bestuiving, waarbij er wel voldoende zaad gevormd wordt. Het zeldzame zaad zit dan verstopt tussen het "reddende" zaad. Het reddende zaad is eenvoudig te herkennen aan kleine vlekjes op het embryo, wat door de zaadhuid zichtbaar is, en het zeldzame zaad heef zo'n vlekje dus niet. Uit dit zeldzame zaad zijn *S*-homozygoten geselecteerd die vitaal waren en betrouwbaar in hun SI-reactie. De identificatie van de *S*-genotypen gebeurde niet alleen middels toetskruisingen, maar ook door stijlextracten via gel-electroforese (IEF) en zilverkleuring te analyseren. De *S*-allelen produceren in de stijl de zogenaamde *S*-glycoproteïnen, vanwege hun RNA-afbrekende eigenschappen ook wel *S-RNasen* genoemd, en deze zijn na IEF goed te herkennen. Deze techniek is door alle experimentele hoofdstukken gebruikt.

Al dit materiaal heeft aan de basis gestaan van de aardappellijnen die gebruikt zijn in het

vervolgonderzoek. Omdat er ook getransformeerd moest worden, wat ook wel "genetische modificatie" wordt genoemd, is er bovendien gezocht naar lijnen die redelijk efficient getransformeerd konden worden. Hiervoor moest er verder gekruist en gescreend worden. Het materiaal wat hieruit is voortgekomen (weergegeven in de appendix en kort besproken in het discussiehoofdstuk), is gebruikt voor de transformatie-experimenten zoals beschreven in de hoofstukken 3 en 5.

In hoofdstuk 3 worden de resultaten van zogenaamde "gain and loss of function" experimenten beschreven. Door het via transformatie toevoegen van een extra gen dat codeert voor een *S-RNase,* is het mogelijk aan de stijlkant een extra incompatibiliteitsgroep tot expressie te brengen. Dit is gebeurt voor het *S2*-allel. Als dit overmatig tot expressie komt, kan dit er toe leiden dat de plant de productie van de andere, niet-transgene, *S*-allel producten terugschroeft. Daardoor kunnen de ander incompatibiliteitsreacties komen te vervallen. Zo is aangetoond dat de *S3* en S10 allelen, die weinig overeenkoms vertonen met *S2,* zo goed als uitgeschakeld konden worden. Het bleek ook mogelijk om het *S1* en het *S2* allel uit te schakelen door het introduceren van "antisense" constructen. Deze antisense-constructen bevatten een deel van het coderende stuk DNA van een gen coderend voor een *S-RNase,* maar dan omgedraaid. Deze nonsens-informatie ontregelt op de één of andere manier de expressie van het correcte gen. Het *S1*-gen produceerde nog redelijk wat *S1-RNase,* maar toch bleken sommige planten compatibel geworden te zijn voor *S1* pollen. Het *S2*-allel kon vrijwel volledig uitgeschakeld worden; er was in sommige planten zo goed als geen *S2-RNase* meer te detecteren. Ook deze planten waren nu hun zelf-incompatibiliteit voor *S2*-pollen kwijt. Hiermee werd aangetoond dat de *S-RNases,* die co-segregeerden met de incompatibiliteitsgroepen, rechtstreeks betrokken zijn bij de incompatibiliteitsreactie.

In hoofdstuk 4 wordt een eerste link gelegd tussen zelf-incompatibiliteit en een interspecifieke kruisingsbarrière, de zogenaamde unilaterale incompatibiliteit. *Solanum verrucosum* (*ver*) is een zelfcompatibele wilde soort die nauw verwant is aan de cultuuraardappel. De diploïde variant van onze cultuuraardappel kan normaal gesproken deze soort wel bevruchten, maar omgekeerd niet. Enkele diploïde *tbr* klonen, die reeds in hoofdstuk 2 besproken waren, vormden een uitzondering op deze regel. Met deze klonen bleek het mogelijk fertiele hybriden te maken die in daaropvolgende kruisingsgeneraties konden worden geanalyseerd. Het bleek dat *ver* om twee redenen zelfcompatibel kon zijn: 1) er was geen *S-RNase* productie, dus er kon aan de stijlkant geen SI reactie veroorzaakt worden en 2) er was een gen dat aan de pollenkant in bepaalde gevallen een incompatible pollenkorrel toch door kon laten groeien. Het mechanisme achter dat laatste bleek een ingewikkeld in elkaar te zitten. Voorts bleek dat de pollenkant van de *S*-locus van *S.ver* nog steeds actief was, dit in tegenstelling tot het stijl-expressiedeel. Bovendien bleek de pollenkant een tweeledige functie te hebben: het droeg bij aan de SI reactie maar ook aan de UI reactie. Verder bleek ook nog dat de UI reactie tussen *tbr* en *ver* op tenminste twee en mogelijk drie verschillende reactiemechanismen berust.

In hoofdstuk vijf werd er verder geanalyseerd aan zowel *ver* zelf als aan transgene *tbr-ver*

hybriden en transgene nakomelingen van antisense *S2* planten uit hoofdstuk 3. Het uitschakelen van het enige actieve *S*-allel in deze planten met behulp van antisense veroorzaakte een ineenstorten van de unilaterale incompatibiliteitsreactie. Verder bleek dat *ver* zelf een zogenaamde "nonacceptor" achtergrond voor eigen pollen kon hebben, hetgeen door het ontbreken van een actief *S*-allel echter geen enkele consequentie bleek te hebben. Al deze resultaten kunnen met al reeds bestaande modellen goed in overeenstemming worden gebracht, mits er enige flexibiliteit wordt betracht aangaande dominatieverhoudingen van genen en allelen. De zelf-incompatibiliteitslocus blijkt wel degelijk bij te kunnen dragen aan de unilaterale incompatibiliteitsreactie.

Als een consequentie van deze bijdrage van de *S*-locus aan zowel de zelf-incompatibiliteit als aan de unilaterale incompatibiliteit moet het model voor de interactie tussen pollenbuis en stijl uitgebreid worden. Alleen een specifieke opname of activatie van het *S*-RNase in de pollenbuis blijkt niet meer te volstaan om e.e.a. met elkaar in overeenstemming te brengen, aangezien de unilaterale incompatibiliteitsreactie niet-allel specifiek is. Bij handhaving van de bestaande modellen moet er een tweede pad gepostuleerd worden, dat mogelijk een signaaltransductie inhoudt.

Curriculum vitae

Ronald Eijlander werd op 4 juni 1958 geboren te Amsterdam. In 1977 behaalde hij het VWO-diploma aan het Berlingh College te Beverwijk. Aansluitend begon hij met de studie Plantenveredeling aan de Landbouwuniversiteit Wageningen (LUW). De doctoraalstudie omvatte twee stages en drie afstudeervakken. De eerste stage was in de aardappelveredling bij de ZPC in Friesland, de andere in de rubberveredeling aan het IRCA te Ivoorkust. Het eerste afstudeervak betrof cytogenetisch onderzoek aan rogge bij de vakgroep Erfelijkheidsleer, het tweede betrof de epidemiologische ontwikkeling van de schimmelziekte "wolf" in spinazie bij de vakgroep Fytopathologie en het derde vak betrof hybride-groeikracht in aardappel na kruising van cultuurmateriaal en een aangepaste wilde verwant, aan de vakgroep Plantenveredeling. Hij studeerde af in januari 1987. Hierna volgen een aantal onderzoeksbanen die vooral betrekking hadden op aardappel, bij het voormalige SVP en ITAL. Deze instituten zijn later opgegaan in het huidige CPRO-DLO. In april 1991 is de overstap gemaakt naar de LUW, om daar aan een AIO-schap te beginnen bij de voormalige vakgroep Plantenveredeling. Het betrof een EEG-gefinancierd project dat ten doel had mechanismen van gametofytische zelfincompatibiliteit te onderzoeken. Aardappel was daar een geschikt modelgewas voor, en aldus werd er door de promovendus wederom aan aardappel gewerkt. De resultaten van het promotie-onderzoek staan beschreven in dit proefschrift.