BIOCHEMICAL ASPECTS OF SELF-INCOMPATIBILITY IN *PETUNIA HYBRIDA*.

by

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PREFACE

Part of the work described in this thesis has been published or presented at scientific meetings as indicated below:

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Self incompatibility in *Phalaris coerulescens.*
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Abstracts Plant Molecular Biology Conference, University of Auckland, New Zealand **1**,2 (1987)

Stigma proteins of the two loci self-incompatible grass *Phalaris coerulescens.*
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DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, no material described herein has been previously published or written by another person except when due reference is made in the text.

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Lor-wai TAN

LIST OF ABBREVIATIONS

bluscrb	plasmid vector bluescribe
bp	base pair
cDNA	complementary deoxyribose nucleic acid
2,4-D	2,4-dichlorophenoxyacetic acid
dATP	deoxyadenine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanidine triphosphate
dTTP	deoxythymidine triphosphate
EDTA	ethylenediamine tetra acetic acid
IAA	indole-3-acetic acid
IEF	isoelectric focusing
IPTG	isoproplythiogalactoside
kinetin	N-furfurylaminopurine
mRNA	messenger ribonucleic acid
NAA	α-naphthalene acetic acid
PMSF	phenyl methyl sulphonyl fluoride
SDS	sodium dodecyl sulphate
Tris	2-amino-2-hydroxyl methyl amino methane

X-gal	5-bromo-4-chloro-3-indonyl-galactopyranoside

UNITS AND SYMBOLS

^{o}C	degree Celsius
Ci	curie
g	unit of gravitational force
gm	gram
h	hour(s)
kda	kilodaltons
M	molar
mCi	millicurie
mg	milligram
mm	millimetres
mM	millimolar
min	minute
µCi	microcurie
µl	microlitre
%	percent
s	second(s)

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SUMMARY

1. The S2- and S3-related proteins in pistils of *Petunia hybrida* W166H were expressed uniformly throughout the length of the mature styles.

2. There were no detectable differences in the level of expression of these proteins in either immature or mature styles of *P. hybrida* W166H.

3. Two-dimensional gel patterns of pistil proteins revealed a prominent cluster mainly in the acidic region in contrast to the basic nature of these proteins reported earlier (Kamboj and Jackson, 1986).

4. By the use of tissue culture techniques, callus was successfully generated from style tissues of *P. hybrida* W166H.

5. The protein profiles of the dedifferentiated callus tissue were very similar to those of stigmas and ovaries of *P. hybrida* W166H.

6. A cDNA library constructed from *P. hybrida* W166H styles showed no homology when screened with a 30 base sequence from a stylar-specific *Nicotiana alata* glycoprotein.

7. Messenger RNA prepared from immature styles were found to be a better source of templates for cDNA synthesis than mature styles.

8. The production of stigma surface secretions in *P. hybrida* W166H coincided with the onset of the self-incompatible reaction. By the method of fluorescence microscopy, only compatible pollen tubes were observed to traverse the lower portion of the styles intact.

9. Differences in the stigma protein patterns observed in the two loci self-incompatible system of *P. coerulescens*. could not be correlated simply to the S- and Z-alleles.

1. INTRODUCTION

Sexual reproduction in flowering plants can be viewed basically as an interaction between pollen and pistil where the pollen grain carrying the haploid genome contains the male gametes; the diploid pistil consists of the stigma, style and ovary which houses the female gametes. With a compatible reaction, the pollen grain after alighting on the stigma imbibes water, germinates and produces a pollen tube. The tube then enters the stigma, growing down through the style to the embryo sac where double fertilization occurs i.e, one sperm fertilizes the ovum while the other fuses with the polar nuclei.

In contrast, self-incompatibility promotes genetic diversity within a population by preventing inbreeding. Although the mechanism responsible for this phenomenon has yet to be fully elucidated, it is known that in most flowering plants a polyallelic S-locus is an important controlling element (de Nettancourt,1977).

Self-incompatibility can either be heteromorphic or homomorphic. In the former, cross-compatible plants display differences in floral morphology as opposed to the homomorphic type where no such differences are observed. In turn, homomorphic self-incompatibility can either be gametophytic or sporophytic. With gametophytic self-incompatibility, a pollen grain which carries an S-allele identical to one of those in the pistil will not be functional on that particular pistil. With sporophytic self-incompatibility, the genotype of the sporophyte determines whether the pollen grain is rejected or not.

1.1 Sporophytic self-incompatibility

In the Cruciferae exemplified by *Brassica* , inhibition of self-pollen occurs on the surface of the stigma. Within minutes of pollination, self-pollen which germinate develop malformed tubes which rarely penetrate the stigma cuticle. However, should the pollen germinate, callose plugs form in the tubes thus preventing further growth (Dickinson and Lewis,1973). A significant difference between self- and cross-pollen in the capture and

adhesion process is that binding of cross-pollen appears to be stronger. Interaction between the cross-pollen coat and the stigmatic papillar cells results in hydration of the pollen grain. This is accompanied by a change in its shape from an ellipse into a sphere (Roberts *etal.* ,1980). Earlier work on the physiochemical aspects of pollen and stigma surfaces have shown the pollen exine is composed of sporopollenin, a polyester of antheraxanthin (Brooks and Shaw,1971) impregnated with hydrophobic lipids (Stanley and Linskens,1974) ; the papillar cuticle a polyester matrix (cutin) is also impregnated with hydrophobic waxes (Roggen,1972; Kolattukudy,1981). On this basis, Ferrari *etal.*, (1985) have proposed that pollen-stigma interaction, (using *B. oleracea* var. *capitata* as their model), can be divided into four distinct stages. The initial or primary pollen grain binding due to weak nonspecific van der Waals forces resulting from interactions between the sporopollenin and cutin containing long chain fatty acids. The secondary binding stage following capture occurs at a slower rate and is specific for cross-compatible stigmas. The third stage consists of the pollen tube tip coming into contact with the cuticle; again only in the case of a cross-compatible mating. During the final stage cross-compatible tubes grow into the papillae beneath the cuticle and eventually penetrate the stigma (Kano and Hinata, 1969).

Self-incompatibility in most of the Cruciferae is governed by a single S-locus although multigene systems have been reported (Lewis,1979). Nasrallah *etal.*, (1985) have demonstrated that these S-glycoproteins are synthesized in the papillar cells of the stigma and are not present in the styles, ovaries or seedlings. The increase in the synthesis and accumulation of these S-glycoproteins can also be correlated with the onset of self-incompatibility. These glycoproteins are characterized by their alkaline isoelectric points and their molecular weights range from 55-65 kda (Nasrallah and Nasrallah,1984). By the method of cDNA analysis, a clone coding for an S-locus-specific glycoprotein has been isolated from *Brassica oleracea.* Subsequent analysis of N-terminal protein sequences encoded by three other S-allelic clones has shown extensive homology between them. Similar protein sequences have also been

detected in three S-locus-specific glycoproteins of *B, campestris* (Nasrallah *etal.*, 1985; 1986).

1.2 Gametophytic self-incompatibility

In this system, self-incompatibility is determined by the haploid genotype of the pollen grain. Extensive studies on Solanaceae and Rosaceae (Heslop-Harrison,1975) have shown that fertilization is prevented when the S-allele carried by the pollen grain is identical to one of the S-alleles present in the diploid stigmatic tissue. In contrast to the sporophytic system, pollen tube growth is inhibited not on the stigma surface but in the transmitting tissue of the style. Pollen tube growth is inhibited in the upper third of the style. Callose plugs form just behind the tube tip which then may burst (Harris *etal.*, 1984). This is characteristic of the Solanaceae where self-incompatibility is controlled by a polyallelic S-locus.

Initial emphasis was placed on biochemical studies of the pollen grains. Serological and immunological studies conducted using *Oenothera organensis* pollen showed the S-proteins present in the grains were readily diffusable from the intact pollen (Lewis, 1952; Lewis *etal.*,1967). Li and Linskens (1983) have demonstrated differences in the composition of pollen tube walls of *Lilium longiflorum* following self- and cross-pollination. *Petunia* pollen can be germinated in liquid medium containing 10% (w/v) sucrose and 0.01% (w/v) boric acid at 25°C. The release of proteins from germinating grains has been shown to be an energy driven process (Kamboj *etal.*, 1984) some of which are necessary to trigger the recognition event in the stigma. In *Secale cereale* , callose has been indentified as the substance deposited in pollen tube walls following a self-incompatible reaction. The sugar component was shown to have a large number of 1,4 β-glucosidic linkages (Vithanage *etal.*, 1980). Howlett and Clarke (1981a;1981b) have isolated and partially characterized two glycoproteins from rye grass (*Lolium perenne*) pollen both of which are effective allergens. The carbohydrate component of these glycoproteins contained a high proportion of the monosaccharides glucose and galactose.

Oligosaccharins present in plant cell walls have been shown to serve as regulatory molecules involved in such varied functions as growth, development defence against disease as well as for reproduction (Albersheim and Darvill, 1985). Futhermore, sugars have been implicated in the pollen-pistil interaction by Callan and Thompson (1986). By pretreating hazelnut (*Corylus avellana* L.) stigmas with concentrated sugar solutions prior to self-pollination, they were able to overcome the self-incompatibility barrier.

The S-allele specific glycoproteins were shown to be be correlated with known genotypes and van der Donk (1974) noted that synthesis of mRNA was enhanced in cross- instead of self-pollinated *Petunia hybrida* stigmas. Based on the results of isoelectric focusing, Bredermeijer and Blaas (1981) have reported that S-specific proteins could be detected in the styles of *Nicotiana alata* . Subsequently, both Clarke *etal.*, (1985) and Kamboj and Jackson (1986) have by SDS-polyacrylamide gel electrophoresis demonstrated the presence of these S-allele specific glycoproteins in *N. alata* and *P. hybrida.* respectively. Anderson *etal.*, (1986) have successfully cloned cDNA coding for the S_2 allele associated stylar glycoprotein. N-terminal sequence analysis of proteins associated with self-incompatibility alleles in *Lycopersicon peruvianum* show considerable homology with the N-terminus of the *N. alata* S_2 glycoprotein. Maheswaran *etal.*, (1986) have by raising an interspecific hybrid between *L. esculentum* and *L. peruvianum*, identified a new allelic specificity generated at the S-locus after inbreeding the self-incompatible *L. peruvianum.*

In the Gramineae, a more complex gametophytic system exists. Here, control of self-incompatibility is dependent on two loci, S and Z. Lundqvist (1954) showed in *S. cereale* that two independent polyallelic loci are responsible which behave in a complementary manner. Here, each pair of alleles, one from each locus determines the specificity. Rejection occurs when the alleles at each locus in both pollen and stigma are identical e.g., with a genotype S_{12} Z_{22}, half the pollen grains would bear the S_1Z_2 allele and the other half the S_2Z_2. A stigma bearing the S_{22} Z_{22} genotype would accept half the pollen (S_1Z_2) as this combination is not

present in the stigma. These two loci gametophytic self-incompatibility system has since been found in *Festuca pratensis* (Lundqvist,1955), *Phalaris coerulescens* (Hayman,1956) and *Hordeum bulbusom* (Lindqvist,1962).

However, the grasses possess physical characteristics that are similar to the sporophytic types. The rye inflorescence consists of a dense, terminal spike 10 to 15 cm long. The spikelets are solitary at each node and are alternately arranged on a zig-zag rachis. Each spikelet consists of only two fertile florets, each floret containing three anthers and a pistil consisting of two feathery stigmas and an ovary. The grass ovary is uniovulate and the micropyle can only accomodate a single pollen tube. This so-called stigma is composed of two stylodia with secondary branches emerging from the upper part. These secondary branches are made up of five layers of cells with the papillate tips acting as the pollen-receiving surfaces. Capture of pollen grains by the stigma leads ultimately to the death of the papillae (Heslop-Harrison,1979). The pollen grains at the time of anthesis are trinucleate, consisting of a vegetative cell and two gametes. The grass pollen is short-lived with environmental factors such as high temperatures and low humidity reducing viability. As well it has a higher respiration rate than that of binucleate pollen species normally associated gametophytic types (Hoekstra and Bruinsma,1975;1979). Heslop-Harrison and Heslop-Harrison (1987) reported on the independent way in which the organelles and gametes appeared to move in the elongating pollen tube of *S.cereale* within specific cytoplasmic lanes. *In vitro* germination of grass pollen has proved to be notoriously difficult. By using a 2.5% (w/v) agar medium containing 25% (w/v) sucrose and 0.02% (w/v) boric acid, Pfahler (1965) succeeded in germinating *S.cereale* pollen. Another feature which distinguishes the grasses from most genera of the gametophytic type which have free-flowing surface secretions is the apparent 'dry' stigma surface common to sporophytic self-incompatibility types. However the papillar cells of the grasses have been shown to bear a thin secretory layer (Heslop-Harrison,1980). Physiological features of the self-incompatibility response in grasses can be summarized as follows: (i) the initial hydration and germination of pollen occurs for

both compatible and incompatible grains and their rates are similar (Shivanna *etal.*, 1978), (ii) in an incompatible state, pollen tube growth ceases when the tip touches the stigma surface (Hayman, 1956) although Shivanna (1982) has observed that in *Alopecurus pratensis*, the incompatible tubes grow through the cuticle of the stigma papilla into the intercellular spaces before growth ceases (iii) the formation of nodules composed of microfibrillar pectins is associated with tube retardation followed by deposition of callose (iv) the general metabolism of the incompatible pollen grain continues after the arrest of tube growth (Heslop-Harrison,1982).

It must be stressed that new breeding systems are still being discovered. Lundqvist *etal.*, (1973) have shown that self-incompatibility in *Ranunculus acris* and *Beta vulgaris* are controlled by three or four separate genes which are usually linked. In these gametophytic systems all four genes must be allelically matched to produce an incompatible reaction. A four gene sporophytic system has also been found in *Eruca sativa* a member of the Criciferae (Verma *etal.*, 1977; Lewis, 1977).

1.3 Plant tissue culture

Plant tissue and cell culture has become a much used technique in plant physiological research. Various workers have successfully induced callus and plantlet formation from a wide variety of plant organs and cells. (Review, Withers and Alderson, 1986). This type of research work has resulted in an improvement in the production of cash crops eg., coffee, propagation of ornamentals, orchids, cereals and citrus plants, all of which are highly vulnerable to virus infections. Just as importantly, tissue culture allows genetic improvement of plant stocks and is a viable method of germplasm storage of commercially valuable plant stocks (Review, Reinert and Bajaj, 1977).

1.3.1 Anther culture

Anther and ovary culture are the most useful tissue culture techniques employed by plant breeders since they involve the regeneration of whole plants from either haploid spores or zygotes. The former was first described in *Datura innoxia* by Guha and

Maheswari (1964). Since then, this technique has been applied successfully to over 200 species of plants including members of the Gramineae and Solanaceae (Baenziger and Schaeffer, 1983; Sunderland and Dunwell, 1977; Vasil, 1980).

The genotype of the donor plant affects the yeild of the callus. Guha-Murkherjee (1973) concluded from experiments on *Oryza sativa* that the response in the anthers to tissue culture depended mainly on the donor plant genotype. The most encouraging results have been obtained with the cereals. Thus Foroughi-Wehr *etal.*, (1982) noted that the best yield of anthers producing microspore derivatives were obtained from *Hordeum vulgare* and *Triticum aestivum.* The external environment is also a contributing factor where both photoperiod and light intensity affect the yield of microspore plantlets. Studies on *Brassica campestris* (Keller *etal.*, 1983) and *Triticum aestivum* (Lazar,1984) have shown that the temperature under which the donor plants are grown influences culture response. Depending on the age of the donor plant, higher yields of plantlets were obtained from anthers collected from the first flowers in a number of species investigated (*Nicotiana* ; Dunwell,1976; *Brassica:* Thurling and Chay, 1984; *Oryza:* Luppoto, 1982; *Triticale:* Orlikowska,1977). The developmental stage of pollen was found to have a profound effect on yield. Dunwell (1976) noted that a difference of 2mm in corolla length of Nicotiana flowers led to a four-fold difference in the number of pollen plantlets which subsequently developed. The stage of pollen development is determined by cytological analysis or more simply by measuring corolla length. Prolonged treatment of rice inflorescence with ethanol or hypochlorite, two methods of sterilization, was shown to reduce anther response (Lai, *etal.*, 1980). Culture medium employed for anthers vary with plant type eg., the salt mixture recommended for the Solanaceae are half strength of the MS medium (Murashige and Skoog,1962) and N6 mixtures have been used for cereals (Chu,1978). Optimum sucrose concentrations also vary with the species to be propagated; in the Solanaceae where the pollen is binucleate, 2-5% (w/v) is sufficient whereas the trinucleate pollen of the Gramineae and Cruciferae require higher levels 6-15% (w/v) as well as the addition of the auxin 2,4-D (Misoo and Matsubayashi,1978). Most Solanaceae do

not however require the auxin for callus production (Dunwell,1986). The orientation of the anther on the surface of the medium has been shown to be crucial for callus formation. Sopory and Maheswari (1976) noted an inhibition in callus production when anthers were submerged in the medium; anthers laid flat with full surface contact yielded the best results. Optimum incubation temperatures of these calli are usually between 25-30°C. Experiments are usually carried out in the dark although Sharma *etal.*, (1983) found that a light intensity of 300 lux produced optimal growth for *Nicotiana paniculata.* A pretreatment of anthers at 35°C for 48 h was found to be necessary to induce high yields of *Brassica oleraceae* (Keller and Armstrong,1983) and *Brassica hirta* (Klimazewska and Keller,1983).

1.3.2 Ovary culture

The female megaspore has also been used as the source of haploid induction. San Noeum (1976) developed a technique for culturing unpollinated ovaties and ovules of *Hordeum vulgare.* Other species successfully cultured in this way include *Beta vulgaris* (Hosemans and Bossoutrot, 1983), *Gebera jamesonii* (Meynet and Sibi, 1984) *Nicotiana rustica* (Zhu *etal.*, 1983), *Nicotiana tabacum* (Zhu and Wu,1981), *Oryza sativa* (Zhou *etal.*,1983), *Triticum aestivum* (Zhu *etal.*,1981) and *Zea mays* (Troung-Andre and Demarly,1984).

As with anther cultures, differences in donor cultivars also affect the response in ovary and ovule culture. In *Gerbera jamesonii* the percentage of ovules producing gynogenic calli varied from 8-17% (Cagnet-Sitbon,1980). Wu and Chen (1982) noted that in two cultivars of *Nicotiana tabacum* the induction frequency was as high as 80% in contrast with a low frequency of 8% for *N.rustica* . The developmental stage of the embryo sachas also been shown to be an indicator of yield. As in *Hordeum vulgare,* the best results were obtained with late stage ovaries i.e., almost mature embryo sacs (Wang and Kuang,1981) while Kuo (1982) noted that callus could be induced with ovaries ranging from uninucleate to the mature embryo sac stage. It appears that ovary culture is possible with the embryo at various stages of development. Nitsch medium was used originally for ovary culture but recently either Miller, MS or N6

medium are used (Yang and Zhou,1982). The addition of auxins and cytokinins are important supplements in this tissue culture. Thus IAA (0.5-1 mg/L) and kinetin (2-4mg/L) were found to be the optimum concentrations for *Nicotiana* (Zhu and Wu,1979) in contrast with *Gerbera*, where another cytokinin BA (2mg/L) was also required for maximum yields. Sucrose requirements depend on the species used both *Nicotiana* and *Gerbera* required low concentrations (3-6% w/v) whereas the Gramineae utilized higher amounts of sucrose of between 8-14% (w/v) (Zhou and Yang,1981). The orientation of the ovary tissue when placed in the medium is also an important factor. Wang and Kuang (1981) observed better growth of *Hordeum* ovaries when the cut surface was in contact with the medium. Photoperiod effects on optimum growth in cultures were also variable ranging from 10-16h at 500 lux for *Gerbera* (Cagnet-Sitbon,1980) to 3 000 lux for *Hordeum* (San Noeum,1976) with incubation temperatures between 25-28^{o}C.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1 Plant Materials

Pistils and ovaries were collected from flowers of Petunia hybrida W166H at various bud lengths grown under glasshouse conditions. They were snap frozen immediately in liquid nitrogen and stored at -80°C until required. Pollen was collected from flowers one day prior to anthesis while still enclosed in anther sacs. They were then allowed to dry at room temperature overnight before use in pollination experiments.

Phalaris coerulescens stigmas kindly supplied by Dr. D. Hayman (Dept. Genetics, University of Adelaide) were first examined for pollen contamination, collected and then stored in liquid nitrogen until required.

2.1.2 Chemicals

Tris (hydroxymethylaminomethane), acrylamide, N,N`-methylene-bis-acrylamide and b-mercaptoethanol were purchased from Sigma Chemical Co., U.S.A. Carrier ampholytes were obtained from LKB Bromma Sweden and molecular weight standards were purchased from Pharmacia Fine Chemicals, Sweden. All other chemicals of the highest purity were obtained from Ajax Chemical Co. Australia.

2.1.3 Solutions and buffers

All aqueous solutions, buffers and reagents were prepared in double glass distilled water.

2.2 Methods

2.2.1 Preparation of protein samples

Proteolytic degradation was prevented by carrying out all operations as rapidly as possible and including the protease inhibitor PMSF (1 mM final concentration) in all extraction buffers.

2.2.1.1 SDS-polyacrylamide gel electrophoresis

Tissue extract were prepared by grinding samples (0.1 gm fresh weight) in mortars precooled in liquid nitrogen, then 0.1 ml extraction buffer (37.5 mM Tris-HCL pH 8.8, 1% (w/v) SDS and 4% (v/v) b-mercaptoethanol) was added. The homogenate was centrifuged for 20 min at 11 500 x g in an Eppendorf centrifuge. An equal volume of loading buffer (0.125 M Tris-Hcl pH 6.8, 4% (w/v) SDS. 20% (v/v) glycerol, 10% (v/v) b-mercaptoethanol and 0.01% (w/v) bromophenol blue) was then added to each sample, mixed and heated to 100°C for 90 s before electrophoresis.

2.2.1.2 Isoelectric focusing (IEF)

Protein samples were dissolved in IEF buffer solution (6.7 M urea,2% (v/v) ampholines pH range 3.5-10.0 and 3% (v/v) b-mercaptoethanol. The mixture was centrifuged for 1 min at 11 500 x g before loading onto the gel.

2.2.2 Polyacrylamide gel electrophoresis

2.2.2.1 SDS gels

SDS-Polyacrylamide slab gel electrophoresis was carried out using a modified Laemmli discontinuos buffer system (Laemmli,1970; Laemmli and Favre,1973). The gels were calibrated with the following marker protein standards: Phophorylase b (94 kda), serum albumin (67 kda), ovalbumin (43 kda), carbonic anhydrase (30 kda), trypsin inhibitor (20.1 kda) and a-lactalbumin (14.4 kda).

2.2.2.2 Two dimensional gels

Two dimensional slab gel electrophoresis was carried out using a modification of the methods of O`Farrell (1975) and Iborra and Buchler (1976) as described by Hallenbeck et al., (1982). First dimension isoelectric focusing was carried out with slab gels comtaining 4.5% (w/v) acrylamide, 8.4 M urea and 2% (v/v) ampholines pH range 3.5-10.0

2.2.3 Protein determination

Using the equation 1.5 x O.D280 nm-0.75 x O.D260 nm, protein

concentration (mg/ml) of all the samples were measured in a spectrophotometer (LKB Ultrospec 4050).
Dithiothreitol and b-mercaptoethanol both absorb strongly at wavelengths below 280 nm. To allow for this, a buffer blank similar to that containing the sample was used.

2.2.4 Detection of proteins

2.2.4.1 Using Coomassie Blue

After electrophoresis, the gels were immersed overnight in a staining solution (0.1% (w/v) Coomassie Brilliant Blue R-250, 45% (v/v) ethanol and 10% (v/v) acetic acid). The gels were then destained with a solution containing 25% (v/v) ethanol and 7.5% (v/v) acetic acid.

2.2.4.2 By silver staining

After treatment in Section 2.2.4.1, the gels were fixed in 10% (v/v) glutaraldehyde for 30 min and washed in several changes of distilled water overnight. They were then treated in a solution of dithiothreitol (5mg/mL) for 30 min. After soaking in a solution of silver nitrate the gels could then be developed for proteins as described by Morrisey (1981).

2.2.5 Molecular biological techniques

2.2.5.1 Extraction of total RNA

In all experiments involving RNA isolation and use, all glassware was washed with 0.5 M KOH and baked for 4h at 200^{o}C to minimize RNAase contamination. All solutions were prepared in autoclaved, double glass-distilled water and sterilized immediately after preparation by autoclaving.
Freshly harvested pistils were weighed and ground with crushed glass in liquid nitrogen in a precooled mortar. For every gm (fresh weight) of tissue 10 ml extraction buffer was used (5M guanidine hydrochloride, 50mM Tris-HCl pH 7.5, 10mM EDTA pH 7.5 and 5% (v/v) b-mercaptoethanol). Solid N-lauroyl sarcosine was then added to the slurry and mixed vigorously to give a final concentration of 4% (w/v). For every ml of homogenate, 0.15 gm of

caesium chloride was added and the mixture centrifuged at 15 000 x g for 20 min to remove cellular debris. The homogenate was then layered onto a caesium chloride cushion (5.7 M caesium chloride in 100mM EDTA pH 7.) in polycarbonate tubes and spun for 16h at 37 300 x g in a Beckman L265B Ultracentifuge, fixed angle rotor type 65. The RNA pellet was then dissolved in a small volume of 4M guanidine hydrochloride pH 7.5 and phenol/chloroform (1:1) was used to remove small amounts of protein and RNA was then precipitated using 0.3 M sodium acetate pH 4.8 and absolute ethanol. The yield and purity of the RNA was determined by scanning its UV spectrum from 320 nm to 230 nm. The yield was calculated using the equation 1 O.D 260 nm = 45 mg/ml RNA.

2.2.5.2 Purification of messenger RNA (mRNA)

Poly (A+) RNA was purified by affinity chromatography on oligo dT-cellulose following the method of Aviv and Leder (1972). One mg of total RNA in distilled water was denatured at 65^{o}C for 2 min and quickly cooled on ice. This sample was then passed through a column of oligo dT-cellulose and binding buffer quickly added to the column(10 mM Tris-HCl pH 7.2, 0.4 M NaCl and 0.3% (w/v) SDS) and the eluate collected in a sterile Eppendorf tube. The eluate was the run through the column again and the procedure repeated twice. The column was then washed with more binding buffer and the poly A- fractions collected. To release the poly (A+) mRNA from the column, elution buffer was then added (10 mM Tris-HCl pH 7.2 and 0.3% (w/v) SDS) and these fractions were pooled and precipitated with ethanol and stored at -20^{o}C.

2.2.5.3 First and second strand cDNA synthesis

Approximately 1 mg of mRNA was dissolved in sterile water. Oligo dT12-18 primer(100ng) was annealed to the mRNA after heating at 95^{o}C for 1 min and quickly cooling on ice. First strand synthesis was carried out using buffer(25 mM Tris-HCl pH 8.3, 4mM NaCl, 3 mM MgCl2, 2.5 mM DTT, 5U human placental RNAase inhibitor, 5U AMV reverse transcriptase (Pharmacia), dGTP, dATP, dTTP (1.25 mM) and 1 ml 32P-dCTP (10mCi/ml) in a total volume of 30 ml. Each incubation was carried out at 50^{o}C for 15 min. Cold dCTP (1 ul of a 10 mM stock) was added to the incubation mixture which

was allowed to proceed for a further 60 min. The reaction was terminated by adding EDTA to a final concentration of 20 mM.
An equal volume of phenol/chloroform was added to remove all the proteins present and the upper aqueous phase containing the cDNA:mRNA hybrid precipitated with sodium acetate/ethanol. After washing with ice cold ethanol the pellet was dried under vacuum and redissolved in sterile water. An aliquot was removed and electrophoresed on a 7% (w/v) polyacrylamide gel in order to assess the size range of the hybrid. To the remaining sample, second strand synthesis buffer was added (0.2M Tris-HCl pH 7.5, 8 mM MgCl2, 10 mM (NH4)SO4, 40 mM KCl, 0.15 mM NADH, 50 mM dNTP`s, 2U E.coli RNAase H (Pharmacia), 5U E.coli DNA ligase (Pharmacia) and 10U Klenow fragment of DNA polymerase I (BRESA)) to a final volume of 50 ml.
The sample was incubated at 15^{o}C for 60 min and then for a further 60 min at 30^{o}C. The reaction was terminated by the addition of EDTA to a final concentration of 20 mM and the mixture extracted with phenol/chloroform, precipitated and dried as decribed previously. The cDNA pellet was dissolved in S1 nuclease buffer (5mM sodium acetate pH 4.5, 0.2 M NaCl, 1 mM ZnSO4 amd 0.05% (v/v) glycerol) and incubated with 10U S1 nuclease (BRL Research Laboratories) for 45 min. The reaction was terminated with the addition of 1M Tris-HCl pH 8.0 and the cDNA precipitated and recovered as previously described.
Blunt ends were obtained by dissolving the cDNA in 5mM Tris-HCl pH 7.4, 7mM MgCl2 1 mM DTT and then adding 1U Klenow fragment of DNA polymerase I and incubating at 30^{o}C for 30 min.
Eco R1 linkers were attached to the cDNA dissolved in buffer (20 mM Tris-HCl pH 7.4, 10 mM MgCl2, 1 mM DTT and 0.1 mM ATP) in an overnight ligation process at 15^{o}C using 5U T4-DNA ligase.
After restriction endonuclease digestion of the cDNA, the unligated linkers were removed by electrophoresing the sample on a 1% (w/v) agarose gel and the cDNA of the required size isolated using DEAE membranes previously inserted along the length of the gel. The cDNA was then ligated to the plasmid vector bluscrb at 14^{o}C for 16h.

2.2.5.4 Transformation of E.coli cells

The E.coli strain JM 101 was prepared for transformation by the SOB/SOC method of Hanahan (1986). Transformants were detected by plating onto LM agar and ampicillin plates previously coated with 0.1 M IPTG and 2% (w/v) X-gal.

2.2.5.5 Screening recombinant colonies

Recombinant colonies were spotted onto a filter membrane (Hybond-N) from Amersham (U.K) which had been placed on a LM+ampicillin plate and allowed to incubate at 37°C for 5h. The colonies were then lysed using 0.5 M NaOH and 1.5 M NaCl followed by 0.5 M Tris-HCl pH 8.0 and 1.5 M NaCl and then baked in a vacuum oven at 80°C for 2h.

Prehybridization and hybridization techniques were carried out as described by Maniatis etal., (1982).

2.2.6 In situ pollination experiments of Petunia hybrida W166H

2.2.6.1 Self- and cross-pollination experiments

Petunia hybrida flowers of various bud lengths were emasculated and bagged one day prior to anthesis. These were then pollinated by gently brushing the receptive stigma with either the cross- or self-pollen obtained from freshly dehised anther sacs. After the appropriate time interval the pistils were then excised and examined for pollen tube growth.

2.2.6.2 Observation of pollen tubes using fluorescence microscopy

Pistils were fixed overnight in Carnoy`s fluid (absolute ethanol: chloroform: glacial acetic acid; 6:3:1), then hydrated sequentially in 70% (v/v), 30% (v/v) ethanol and finally distilled water. The stigmas were softened by soaking in 0.8 N NaOH for 1h at 60°C, stained overnight in 0.1% (w/v) aniline blue in 0.1 N K3PO4 and squashed using 80% (v/v) glycerol before viewing.

Pollen tubes were observed with a Zeiss photomicroscope II with reflected light from an HBO 100 high pressure mercury vapour lamp, Zeiss exciter filter BG3 and Zeiss barrier filters 65/50. and photographed using Kodak High Speed Extachrome (daylight) film ASA 400

2.2.7 Plant tissue culturing techniques

Glassware, media and metal instruments were heat sterilized at 130°C for 3h. Culture media M DAUC (see appendix) was supplemented with 2% (w/v) sucrose, 0.8% (w/v) agar and the pH adjusted to 5.8. Various concentrations of kinetin and NAA were then added to this media in 50 ml plastic tubes before heat sterilization. Excision and transfer of stylar tissue into culture media was carried out in a laminar flow cabinet under sterile conditions. The tubes were placed in a rack and exposed to white light(photon irradiance of 20 mE/m2/s, temperature of 23°C and 16h daylength) and examined daily for signs of callus growth.

3. RESULTS

3.1 *Petunia hybrida*

3.1.1 Expression of S-related proteins from different tissues of *Petunia.*

Extracts from the three sections of mature styles (a, b and c) were examined for the expression of the S-related proteins. 100 µg of each sample was loaded onto a 12.5% (w/v) SDS-polyacrylamide gel and electrophoresed for 3.5h. The S2 and S3 associated proteins were detected in all three samples co-migrating with the 30 kda marker. There appeared to be no gradation in expression of the S2 protein along the length of the mature style with region (a) exhibiting the same quantity of protein as region (c) (Fig.1).

The S-associated proteins were observed even at the immature bud stage (<20 mm in bud length). There was no difference in the level of expression in any of the three samples. In addition, the protein profiles of the stigmas at all three different developmental stages were shown to be almost identical (Fig.2).

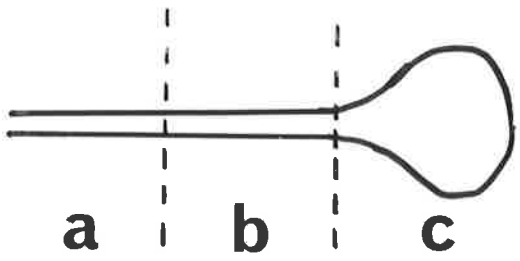
The expression of the S2 and S3-related proteins were detected in mature ovaries, callus and stigmatic tissue at the different developmental stages. The protein profile of mature ovaries was shown to be similar to that of stigmas. Only the S3 related protein was present in immature ovaries(Fig.3). A prominent protein band of approximately 43 kda, common to callus and stigma tissue was not expressed in mature ovaries. Only a low molecular weight band of approximately 20 kda present in both mature and immature stigma and ovaries was not present in the dedifferentiated callus tissue.

No differences in the intensity and distribution of the proteins were noted in the IEF patterns of the stigmas at various developmental stages (Lanes 1, 2 and 3). A band with pI of ~5 present in mature ovaries (Lane 4) and also in the three stages of

FIG. 1 SDS-PAGE (12.5 %) protein patterns of mature style sections stained with Coomassie Blue.

No visible difference in the expression of the S2- and S3-related proteins could be detected between the three sections of the style (depicted schematically) which correspond to Lanes a, b and c.

Low molecular weight markers were loaded in the unmarked lane: 1. phosphorylase b (92 kda)
2. albumin (67)
3. ovalbumin (42)
4. carbonic anhydrase (30)
5. trypsin inhibitor (20)
6. α-lactalbumin (14.4)


a
b
c

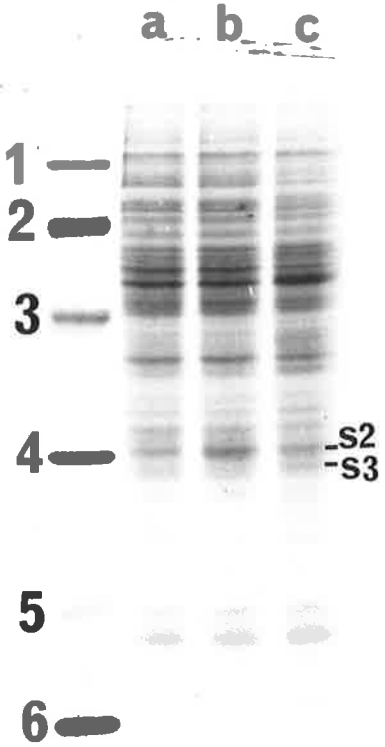

a
b
c
1
2
3
4
5
6
S2
S3

FIG. 2 Silver-stained SDS-PAGE (12.5%) protein patterns of styles selected at different stages of maturity.

Styles were selected according to their bud lengths. Equal concentrations of proteins were loaded and electrophoresed according to the methods in Section 2.2.1. No differences between the levels of expression of the S2- and S3-related proteins at the different developmental stages were observed.

Lane 1 Styles from buds of less than 20mm in length
Lane 2 Styles from buds of between 20-30 mm
Lane 3 Styles from buds of more than 40 mm

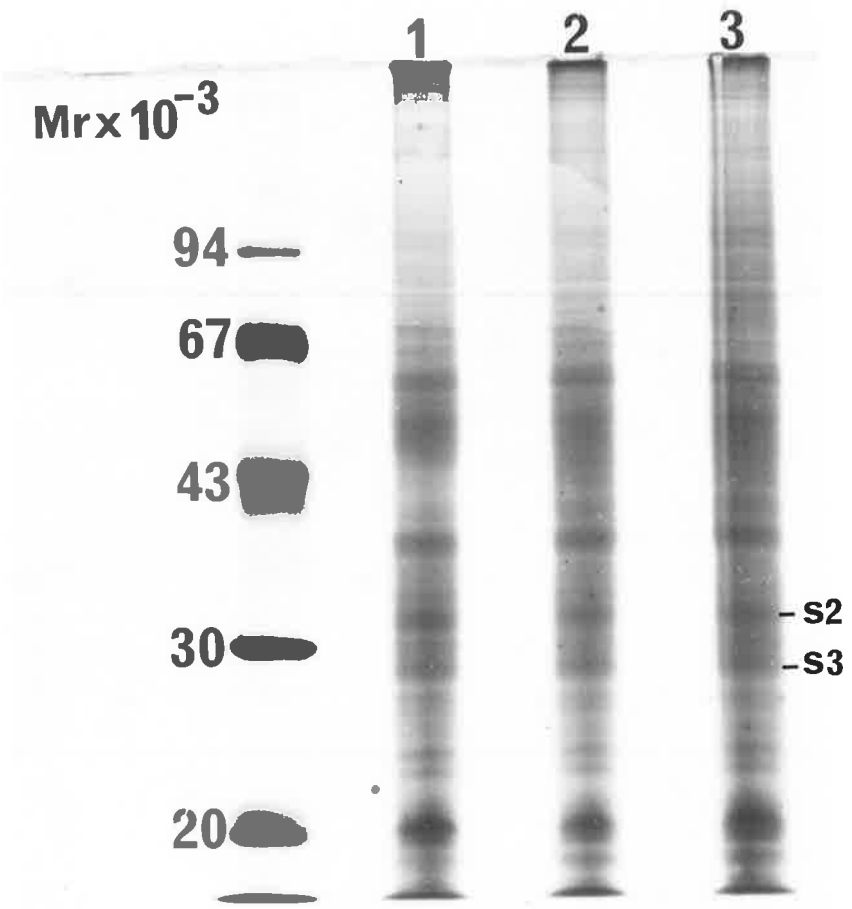

1
2
3
Mr x 10^{-3}
94
67
43
30
20
S2
S3

Fig. 3 Silver-stained SDS-PAGE (12.5%) protein patterns of mature and immature ovaries and callus tissue derived from styles of *P. hybrida* W166H.

Both the S2- and S3-related proteins were detected in mature ovaries (Lane 1) and callus tissue (Lane 2). Only the S3-related protein was observed in immature ovaries (Lane 3).

A callus specifc protein of 47 kda was also detected (◀). A prominent 43 kda protein was found to be present only in mature ovaries and callus (●). In contrast, a 20 kda protein was shown to be common to the immature and mature ovaries (◁).

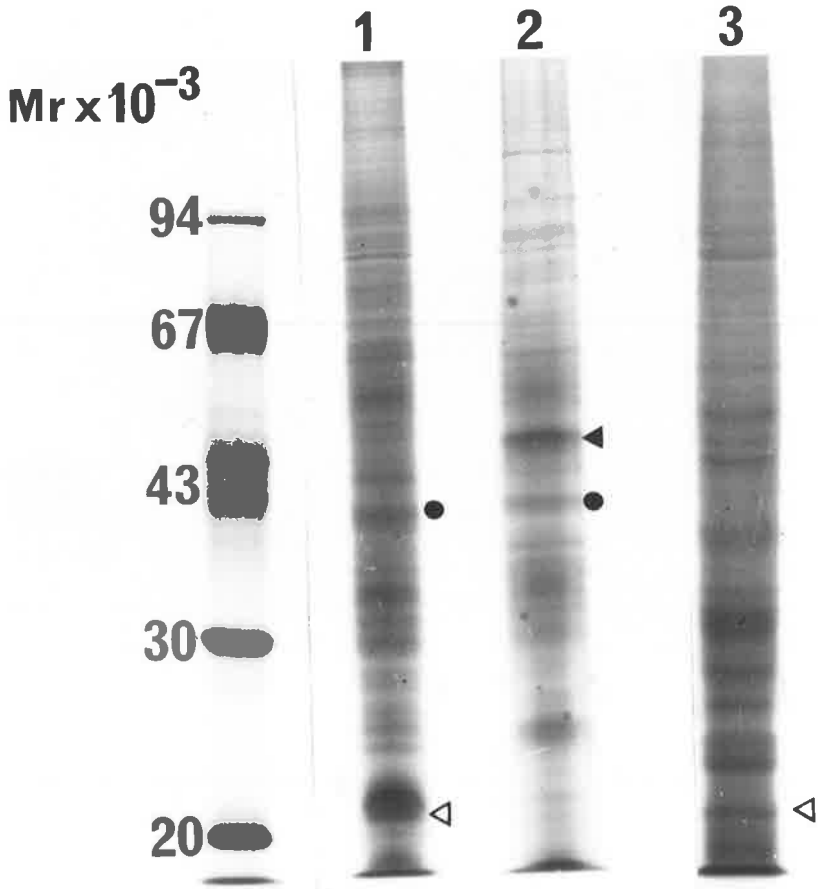
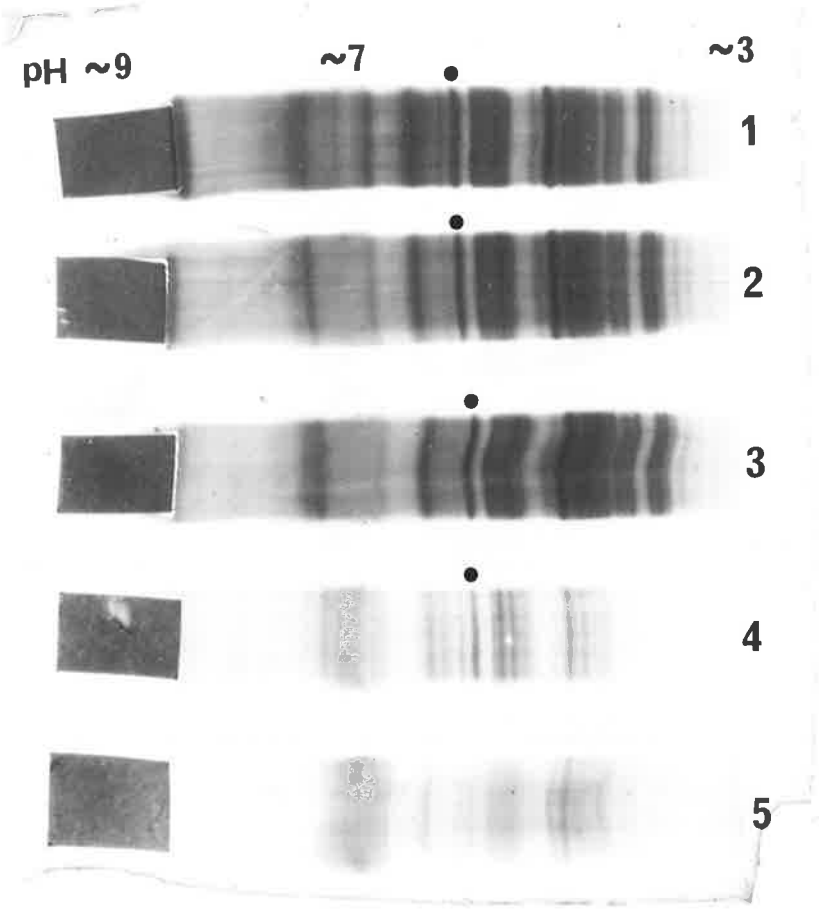

1
2
3
Mr x10^{-3}
94
67
43
30
20

Fig. 4 Silver-stained IEF patterns of proteins from style, ovary and callus tissue.

Lanes **1** (<20mm bud length), **2** (20-30 mm length) and **3** (>40 mm length) of styles at the different stages of development showed ver similar patterns. A band focused at pI ~5.0 was detected in all three stylar and the mature ovarian (Lane 4) extracts (●). Callus tissue (Lane 5) exhibited marked differences in its banding pattern in the pI region of 4.0-6.0 and 7.0-8.0.


pH ~9
~7
~3
1
2
3
4
5

stigma development was evident in the callus (Lane 5). In contrast, there were marked differences in the callus protein patterns notably in the region of pH 4.0-6.0 and 7.0-8.0 (Fig.4).

3.1.2 Comparison between 2-D gel electrophoresis patterns of stigma, ovary and callus.

On separation in a second dimension (12.5% (w/v) polyacrylamide), the proteins were observed to co-migrate with low molecular weight markers spanning a range from 92-20 kda (Fig.5). The three stigma samples exhibited patterns which were essentially similar, with marked protein separation in the acidic range. The callus appeared to lack proteins in region A (acidic) which were present in all the gels of the three stages of stigma development and ovary extracts. In contrast, the callus exhibited some basic proteins (region B) which were not detected in either the stigmas or ovaries.

3.1.3 Conditions affecting callus formation.

One factor which appeared to be criticial for callus formation was the stage of development at which the stigmas were selected. Callus was not produced from tissues either at the undifferentiated stage when the stigma was morphologically indistinguishable from the ovary or after elongation of the style had been initiated. Both kinetin (3.0 mg/L) and NAA (0.2-3.0 mg/L) were essential growth factors (Table 1). There was a lag period of approximately 4 weeks before callus production was observed (Fig. 6). However when growth of the dedifferentiated tissue had been established, callus tissue was maintained on the same MDAUC medium without any apparent detrimental effect.

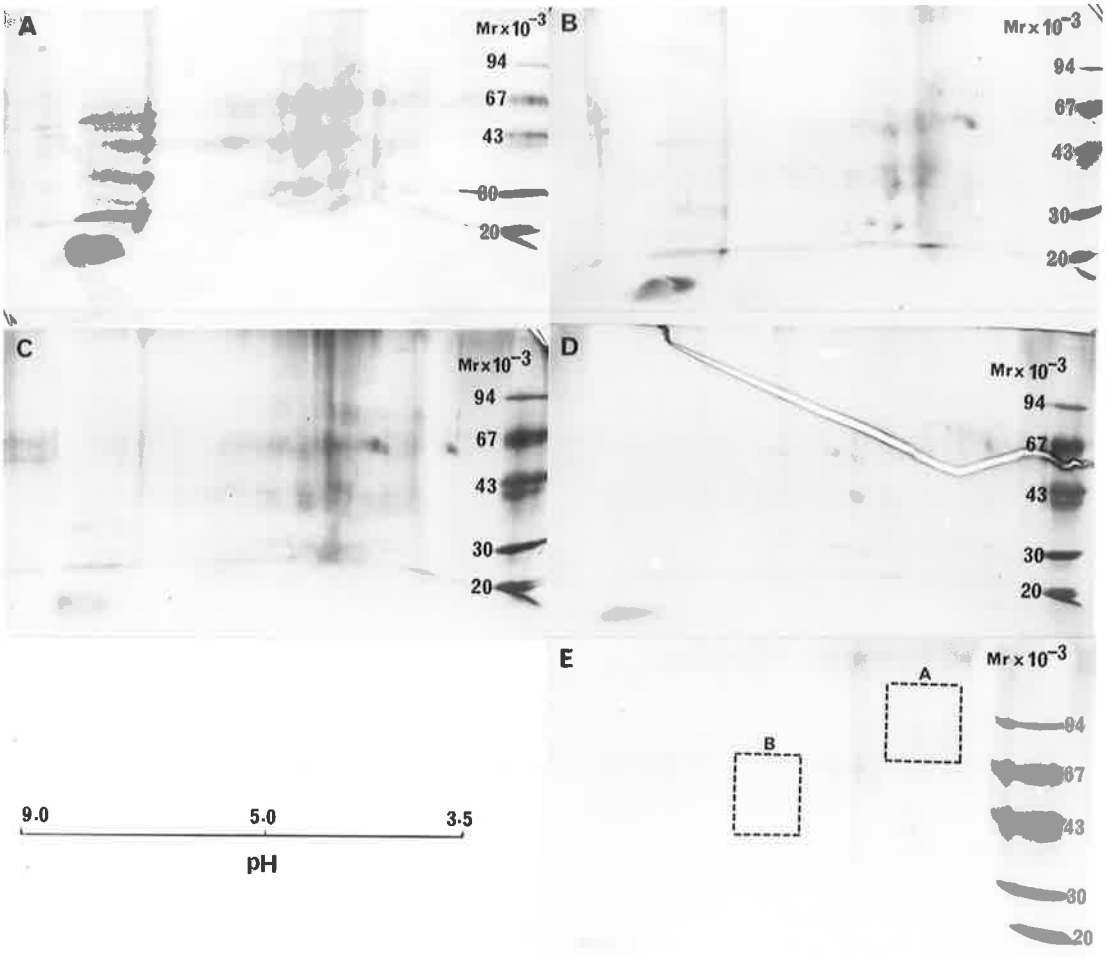
3.1.4 The effects of self- and cross-pollination on *Petunia* flowers

The time required for pollen tubes to grow through half the length of the style was about 24h (Table 4). Prior to and at this stage, no difference in the rate of growth was observed

Fig. 5 Silver-stained two-dimensional gel eletrophoretic patterns of the style, ovary and callus tissue.

On separation of the stylar extracts in the second dimension a population of these proteins was observed in the acidic region (A, B and C). It was not possible to designate precisely the S2- and S3-related proteins to the patterns obtained. Callus tissue (E) exhibited proteins in the basic region (Box B) not detected in either stylar or ovarian tissue.

A. Styles collected from buds of less than 20mm length
B. " " " " of between 20-30mm
C. " " " " of more than 40mm
D. Mature ovaries
E. Callus tissue


A
Mr x 10⁻³
94
67
43
30
20
B
Mr x 10⁻³
94
67
43
30
20
C
Mr x 10⁻³
94
67
43
30
20
D
Mr x 10⁻³
94
67
43
30
20
E
A
B
Mr x 10⁻³
94
67
43
30
20
9.0
5.0
3.5
pH

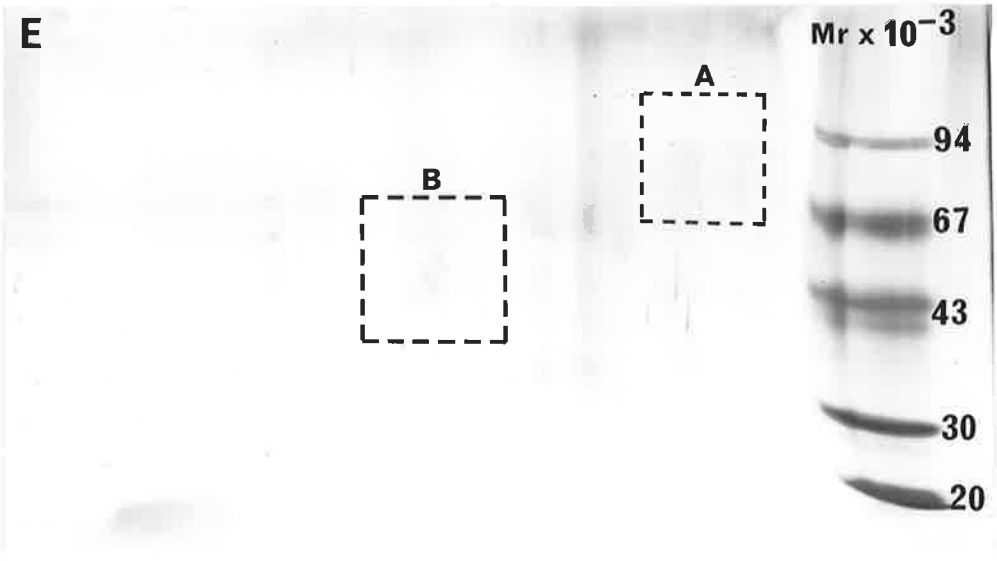

E
A
B
Mr x 10⁻³
94
67
43
30
20

Fig. 6 Induction of callus from stylar tissue.

Sections of styles were excised and cultured in an MDAUC medium (See Appendix) containing 3.0 mg/L kinetin and 0.2 mg/L NAA with 16h daylength. The first sign of callus growth appeared as dedifferentiated tissue was detected after a 4 week lag period

Table 1 Effect of different concentrations of growth factors on callus development.

A Control with only MDAUC medium

B MDAUC +2.0 mg/L kinetin

C MDAUC + 2.0 mg/L kinetin + 0.3 mg/l NAA

D MDAUC + 2.0 mg/L kinetin + 2.0 mg/L NAA

Treatment	Week				
	1	3	5	7	9
A(i)	-	-	-	-	-
(ii)	-	-	-	-	-
(iii)	-	-	-	-	-
(iv)	-	-	-	-	-
B(i)	-	-	-	-	-
(ii)	-	-	-	-	-
(iii)	-	-	-	-	-
(iv)	-	-	-	-	-
C(i)	-	-	+	+	+
(ii)	-	-	-	+	+
(iii)	-	-	-	+	+
(iv)	-	-	-	-	-
D(i)	-	-	-	-	-
(ii)	-	-	-	-	-
(iii)	-	-	-	+	+
(iv)	-	-	-	-	+

for either self- or cross-pollination (Tables 2 and 3). The self-incompatibility effect was not apparent even in the almost mature buds with approximately 17% of self-pollen grains germinating through the transmitting tissue to the lower portion of the style (Figs. 7 and 8).

However when stigmas exhibiting surface secretions were selected (the flowers having undergone anthesis), marked differences were observed between self- and cross-pollination after a 72h interval (Table 5;Fig. 9). Callose plugs occurring at regular intervals along the lengths of the tubes were observed in both the self- and cross-pollinated stigmas. However, in the self-pollinated samples rupturing of pollen tubes near the ovary-style junction was evident (Fig. 11c). Less than 27% of self-pollinated compared with almost 100% of cross-pollinated stigmas had reached the ovary-style junction by the end of germination (Fig. 10).

3.1.5 Cloning of cDNA for a stylar glycoprotein associated with the expression of the S-allele.

Approximately 20 mg of total RNA was obtained from 2 gm (fresh weight) *Petunia* styles (referring to stigma and styles). A sample was then electrophoresed on a 1.5%(w/v) agarose gel to ensure that the total RNA was intact and free of contaminants (Fig.12). Messenger RNA was obtained using an oligo dT affinity column which yielded approximately 5 µg from an initial concentration of 0.5mg total RNA. The synthesis of first strand of cDNA could be visualized on exposure to X-ray film after separation on a 5% (w/v) polyacrylamide gel (Fig.13). Recombinant colonies were picked at random and screened for the presence of the insert by subjecting the isolated plasmid to Eco R1 digestion. As shown in Fig.14,it was not possible to excise the cDNA fragment from the vector due possibly to some modification of the restriction site on the Eco R1 linkers. The restriction endonuclease Pvu II however successfully excised the 528 bp fragment from recombinant number (d).

600 recombinants were screened using the synthetic 30-

Table 2 No. of self-incompatible and cross-compatible pollen tubes detected in lower style region of *P. hybrida* after 3h.

Pollen from freshly dehised anther sacs was gently brushed across the stigma surfaces and germination allowed to proceed for the required time. The pistils were then fixed and stained with aniline blue and examined by fluorescence microscopy.

Bud length (mm)	No. grains on stigmas	Pollen tubes detected in upper and lower regions of the style	
		Upper	Lower
S 0-20	150	150	0
"	120	120	0
C 0-20	350	350	0
"	200	200	0
S 20-30	130	130	0
"	300	300	0
C 20-30	320	320	0
"	170	170	0
S 30-40	200	200	0
"	150	150	0
C 30-40	600	600	0
"	400	400	0

S self-pollinated pistils
C cross-pollinated pistils

Table 3 No. of self-incompatible and cross-compatible pollen tubes detected in lower style region of *P. hybrida* after 7h.

Pollen from freshly dehised anther sacs was gently brushed across the stigma surfaces and germination allowed to proceed for the required time. The pistils were then fixed and stained in aniline blue and examined by fluorescence microscopy.

Bud length (mm)	No. grains on stigmas	Pollen tubes detected in upper and lower regions of the style	
		Upper	Lower
S 0-20	160	160	0
"	210	210	0
C 0-20	180	180	60
"	120	120	35
S 20-30	300	300	0
"	130	130	0
C 20-30	350	350	0
"	220	220	0
S 30-40	300	300	0
"	500	500	0
C 30-40	350	350	0
"	120	120	0

S self-pollinated pistils
C cross-pollinated pistils

Table 4 No. of self-incompatible and cross-compatible pollen tubes detected in lower style region of *P. hybrida* after 24h.

Pollen from freshly dehised anther sacs was gently brushed across the stigma surfaces and germination allowed to proceed for the required time. The pistils were then fixed and stained in aniline blue and examined by fluorescence microscopy.

Bud length (mm)	No. grains on stigmas	Pollen tubes detected in upper and lower regions of the style	
		Upper	Lower
S 0-20	102	102	32
"	120	120	33
C 0-20	85	85	10
"	100	100	30
S 20-30	400	400	30
"	130	130	25
C 20-30	100	100	15
"	80	80	0
S 30-40	120	120	20
"	400	400	9
C 30-40	300	300	2
"	280	280	20

S self-pollinated pistils
C cross-pollinated pistils

Fig. 7 The effect of self-pollination after a 24h period on pistils at different stages of development.

Germination of the pollen tubes into the lower region of the styles was observed even for styles from almost mature (30-40mm bud length) flowers.

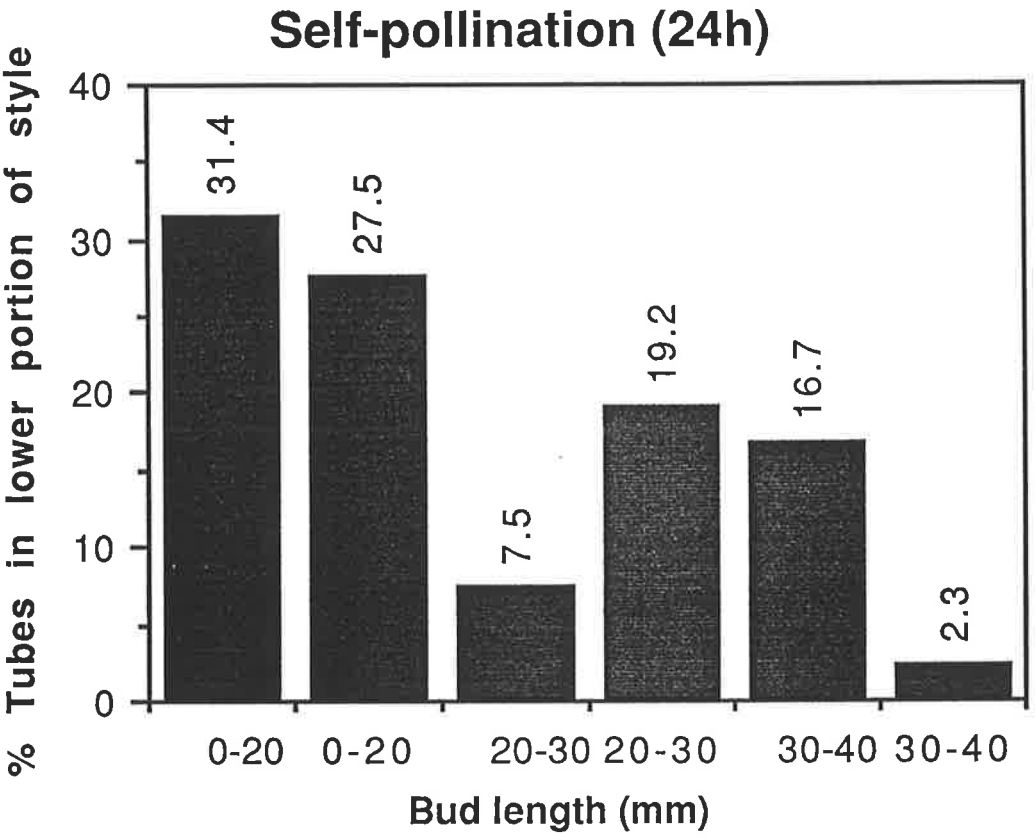

Self-pollination (24h)
% Tubes in lower portion of style
40
30
20
10
0
31.4
27.5
7.5
19.2
16.7
2.3
0-20
0-20
20-30
20-30
30-40
30-40
Bud length (mm)

Fig. 8 The effect of cross-pollination after a 24h period on pistils at different stages of development.

A comparatively fewer number of cross-compatible pollen tubes were observed to traverse the lower portion of even immature styles (cf. Fig.7).

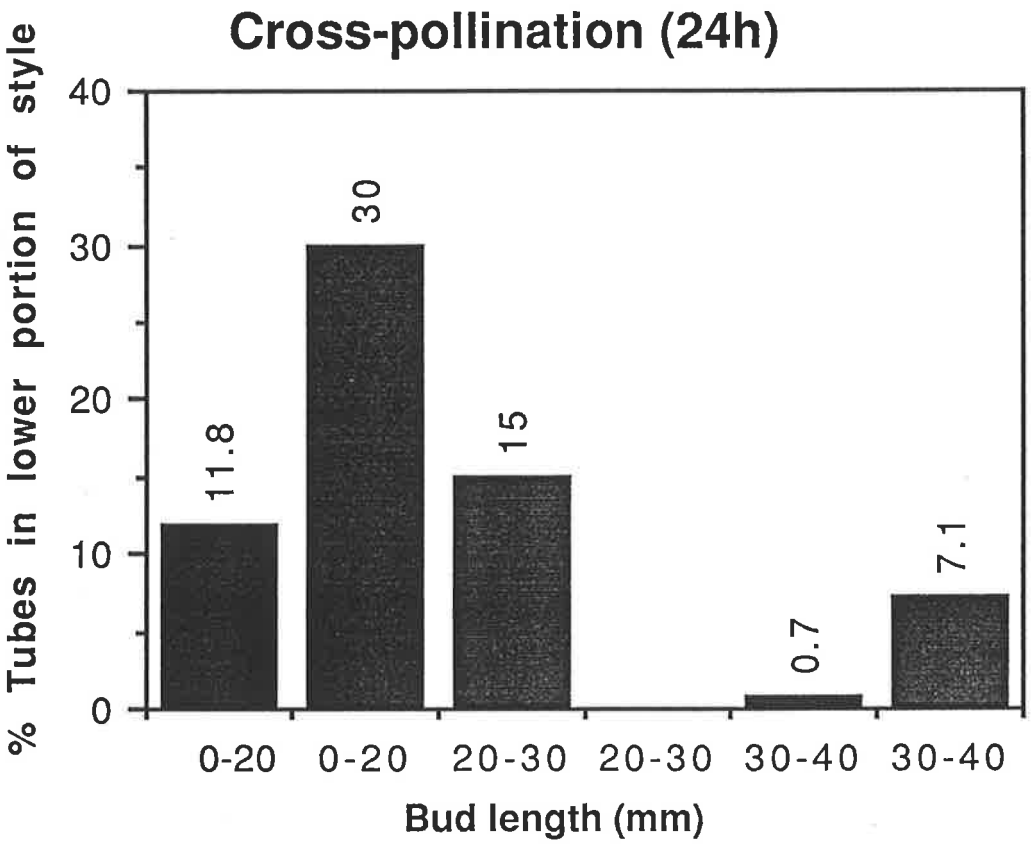

Cross-pollination (24h)
% Tubes in lower portion of style
40
30
20
10
0
11.8
30
15
0.7
7.1
0-20
0-20
20-30
20-30
30-40
30-40
Bud length (mm)

Table 5 No. of self-incompatible and cross-compatible pollen tubes detected in lower style region of mature *P. hybrida* pistils with stigma surface secretions after 72h.

Pollen from freshly dehised anther sacs was gently brushed across the stigma surfaces and germination allowed to proceed for the required time. The pistils were then fixed and stained in aniline blue and examined by fluorescence microscopy.

Mature pistils	No. grains on stigmas	Pollen tubes detected in upper and lower regions of the style	
		Upper	Lower
Selfed	500	500	130
"	600	600	100
"	400	400	50
Crossed	600	600	600
"	700	700	700
"	400	400	400

Fig. 9 The effect of self- and cross-pollination after a 72h period on mature styles exhibiting surface secretions.

Only a small proportion of self-incompatible (<27%) in contrast to cross-compatible (100%) tubes were observed to germinate through the transmitting tissue to the lower region of the styles.

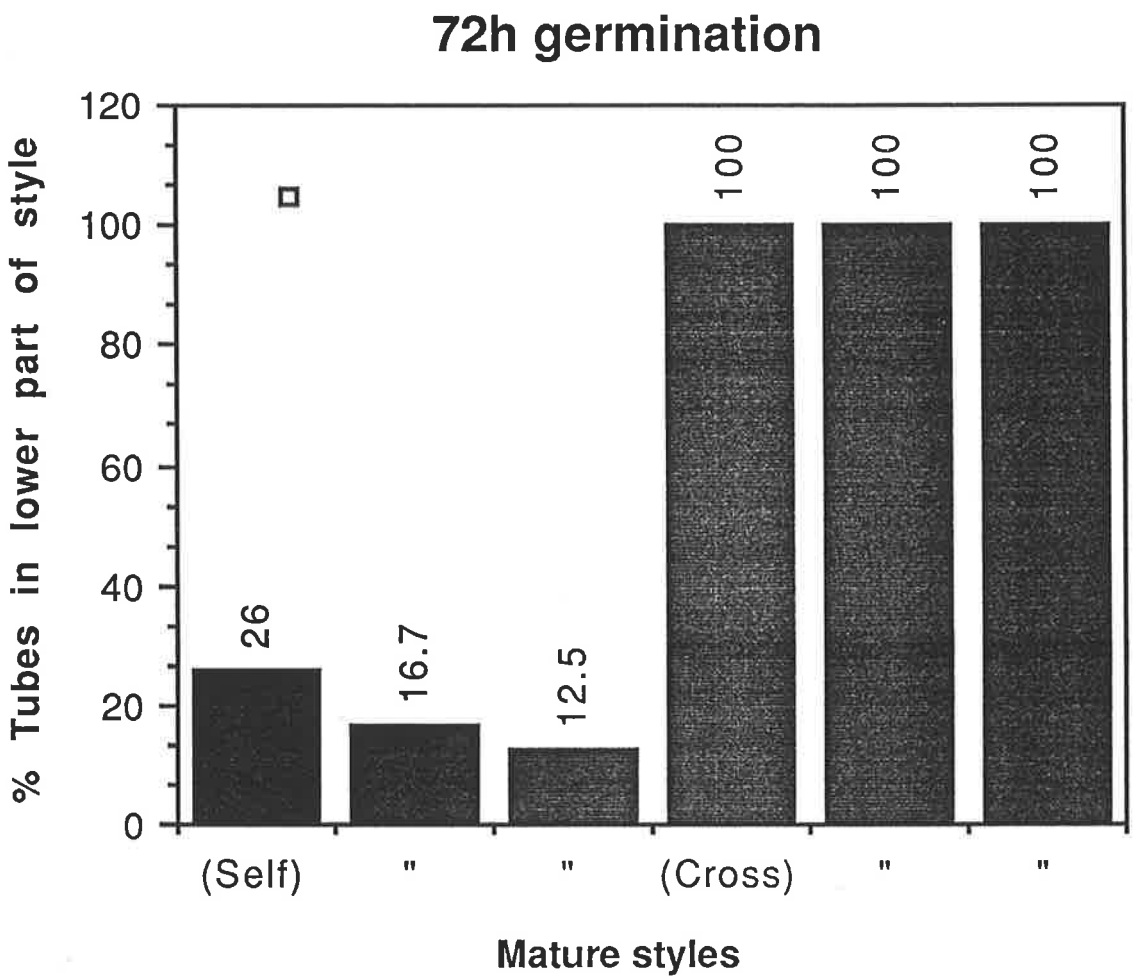
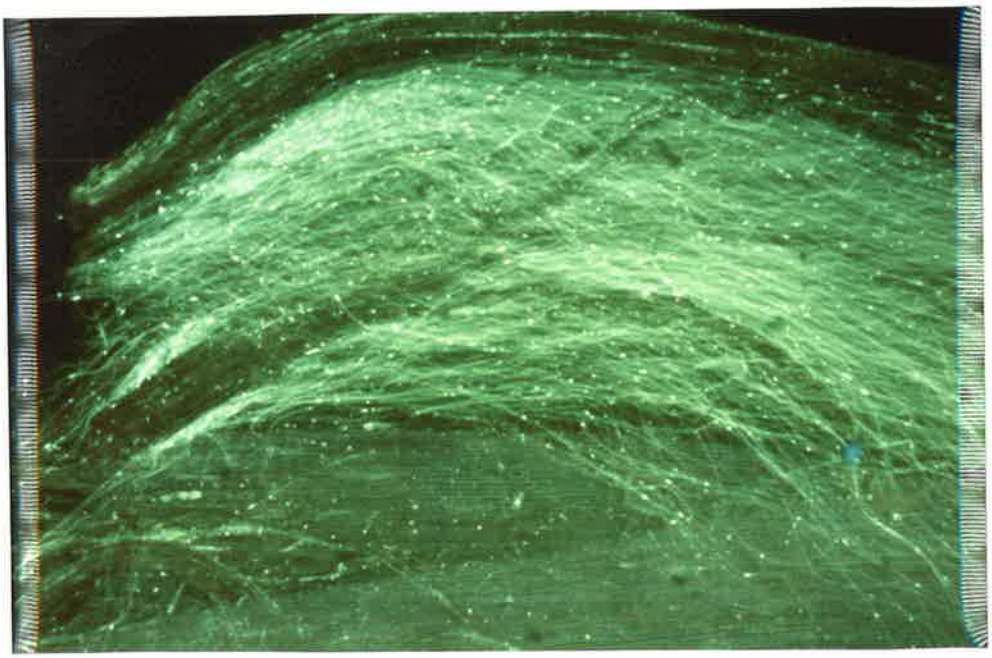

72h germination
% Tubes in lower part of style
120
100
80
60
40
20
0
26
16.7
12.5
100
100
100
(Self)
"
"
(Cross)
"
"
Mature styles

Fig. 10 Detection of cross-compatible pollen tubes by fluorescence microscopy on mature stigmas exhibiting surface secretions

a. Stigmas were cross-pollinated and germination allowed to proceed for 72h before examining for signs of tube penetration (25 x magnification).
b. Almost 100% of the tubes grew through the transmitting tissue to reach the ovary-style junction (50 x magnification).
c. Callose plugs were detected at regular intervals along the pollen tubes (100 x magnification).

a

b



c

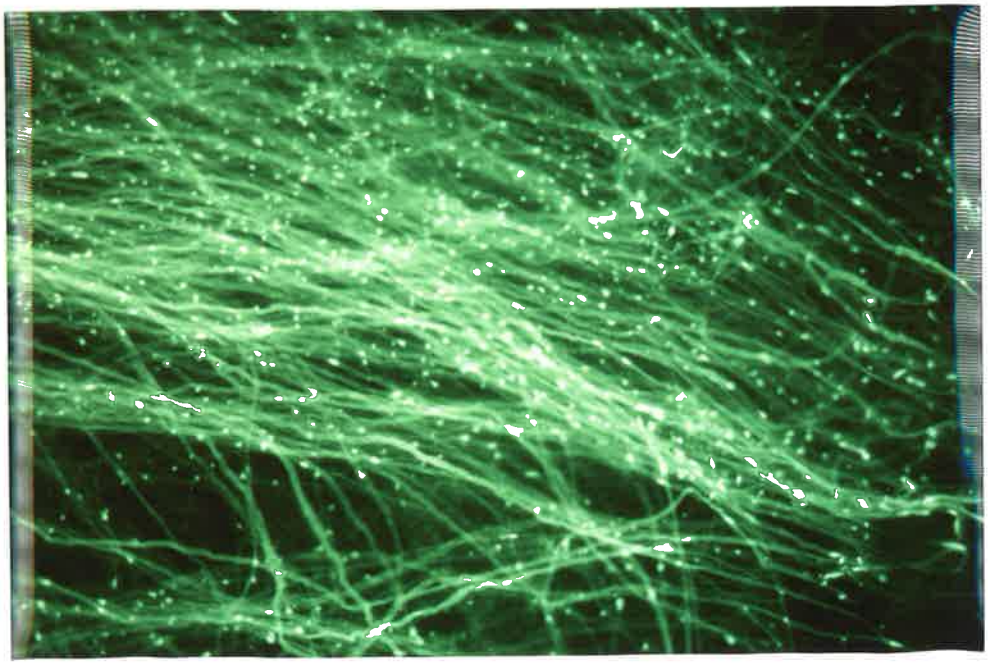
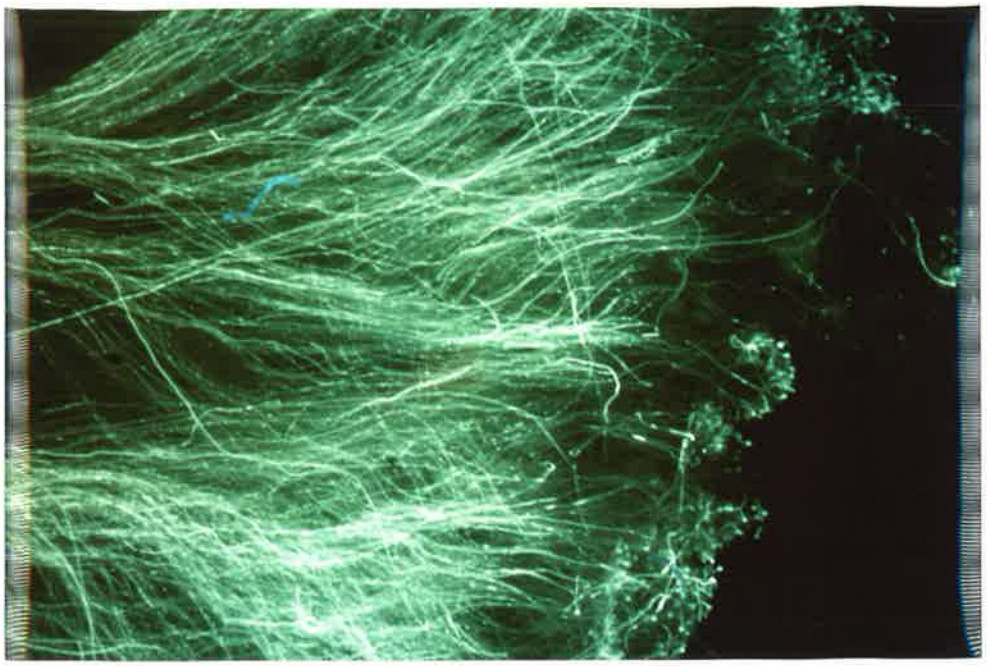


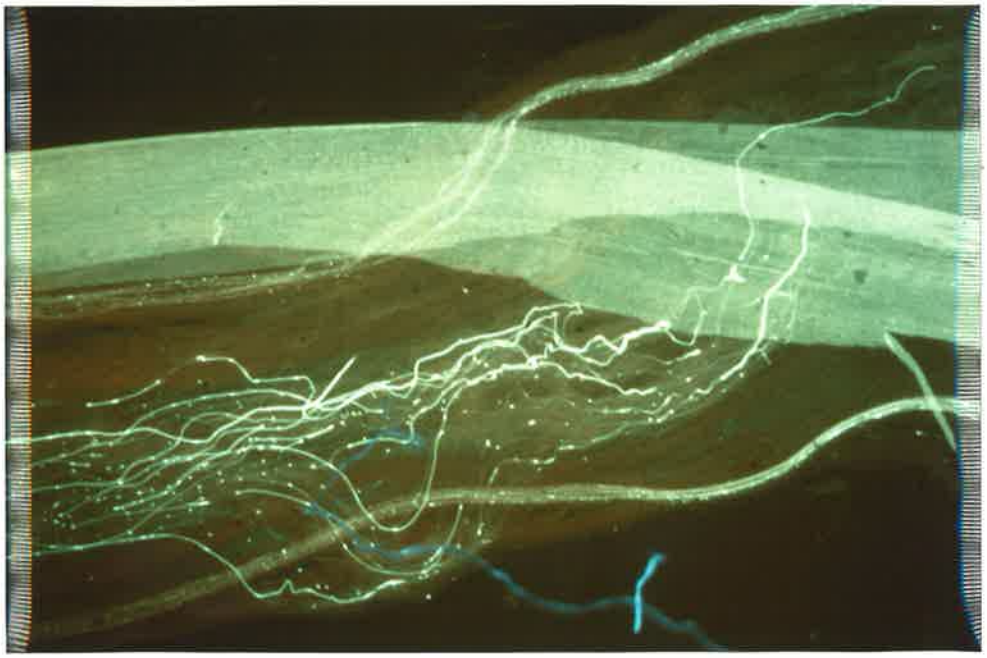
Fig. 11 Detection of self-incompatible pollen tubes by fluorescence microscopy on mature stigmas exhibiting surface secretions

a. As with the cross-compatible pollination, no differences could be detected in either the appearance or number of self-incompatible pollen grains germinating on the stigma surface (25 x magnification).
b. Only a small number (<25%) of the pollen tubes was found in the lower region of the style (50 x magnification).
c. Most of the tubes in the lower style region had ruptured and had lost their cytoplasmic contents leaving only a faint outline of the tube walls visible (100 x magnification).

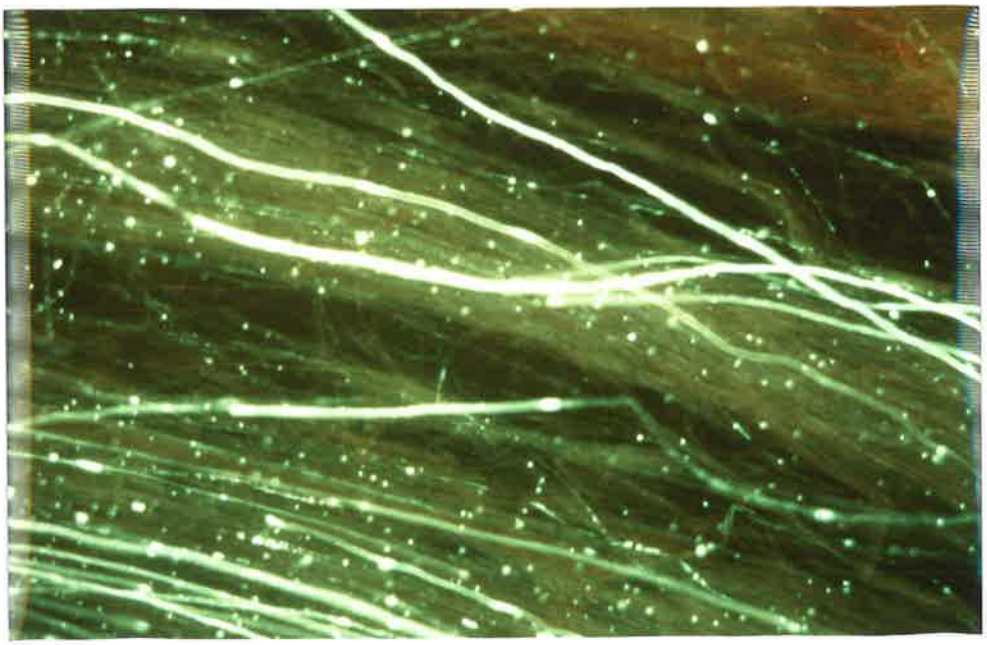
a



b



c



mer probe, none of which exhibited a hybridization signal strong enough to positively determine whether the insert was related to the gene responsible for the expression of the S-associated glycoprotein in *N alata.*

3.1.6 Comparison of mature and immature styles as a source of mRNA for cDNA synthesis

Both the oligo dT12-18mer and the 30-mer oligonucleotide sequence were used as primers for first strand cDNA synthesis. Messenger RNA from immature styles was the more active source of templates. The results illustrated in Fig.13 show that using oligo dT 12-18mer, the a high incorporation of radioactive dCTP occurred both in the mature and immature samples. In contrast, with the 30-mer sequence as a primer, cDNA synthesis was detected only when mRNA was used from immature stigmas.

3.2 *Phalaris coerulescens*

3.2.1 Comparison between stigma and leaf proteins of the two loci of the self-incompatible grass *Phalaris coerulescens* by SDS-polyacrylamide gel electrophoresis.

The leaf extracts of all four clones exhibited very similar protein patterns (Fig.15b). In contrast, variations in stigma protein patterns can be observed in the region extending from 43-97 kda. Expression of these proteins in stigmas does not however correlate with the alleles assigned to each individual clone as in the case of *Petunia* (Kamboj and Jackson,1986). For example, expression of a protein band at 50 kda (arrow in Fig.15a) present in the two S-locus homozygotes (lanes 3 and 4) cannot be detected in the heterozygote in lane 1 (S12 Z12). Similarly, the Z-locus homozygote (lane 2) possesses an 80 kda protein band which is not evident in the S12 Z12 sample. However, since the S12 Z22 genotype was not available, no further comment can be made at this stage. An additional factor which has to be considered is the existence of two loci for self-

Fig. 12 Detection of total RNA extracted from mature and immature pistils of *P. hybrida* visualized on an agarose (1.5%) gel.

The two prominent ribosomal RNA bands present in both samples indicated that no degradation by RNAses had occurred.
Lane 1 Total RNA extracted from immature pistils.
Lane 2 Total RNA extracted from mature pistils.

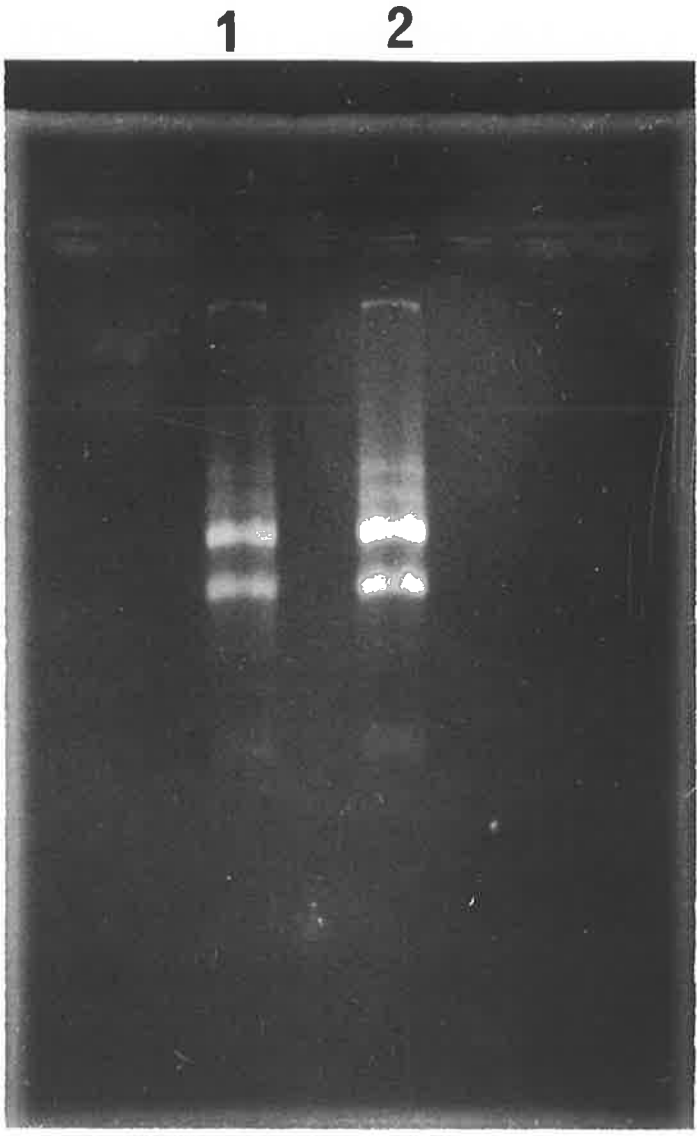

1
2

Fig. 13 Autoradiogram of single stranded cDNA of mature and immature pistils.

Lane 1 mRNA from mature pistils (oligo dT primer).
Lane 2 " " " (30-mer primer)
Lane 3 mRNA from immature pistils (oligo dT primer)
Lane 4 " " " " (30-mer primer)

When primed with oligo dT 12-18 mer, mRNA extracted from immature pistils were shown to have the highest rate of incorporation of radioactive dCTP (Lane 3). Some degree of activity was detected when the 30 base nucleotide sequence was the source of the primer (Lane 4). In contrast for the same amount of mRNA from mature pistils (1 μg) used for cDNA synthesis the activity was shown to be lower (Lane 1) whilst using the 30-mer sequence as the primer showed no detectable cDNA synthesis(Lane 2).

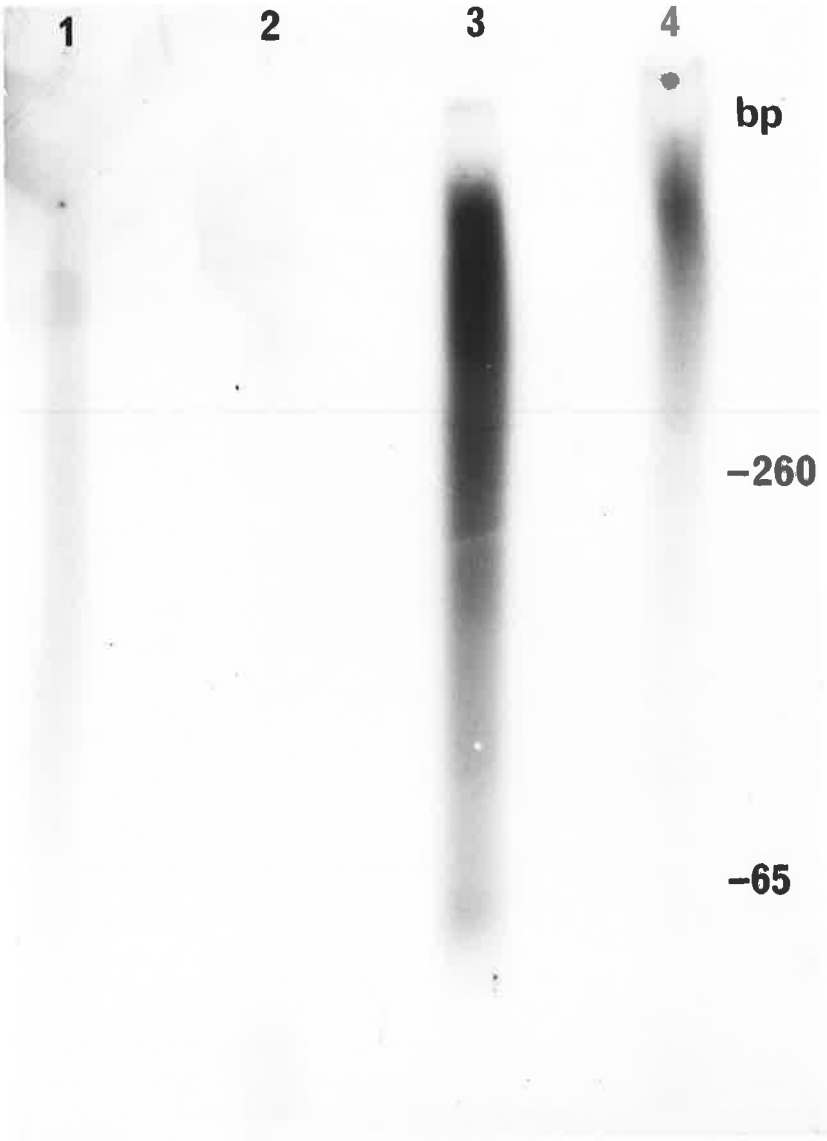
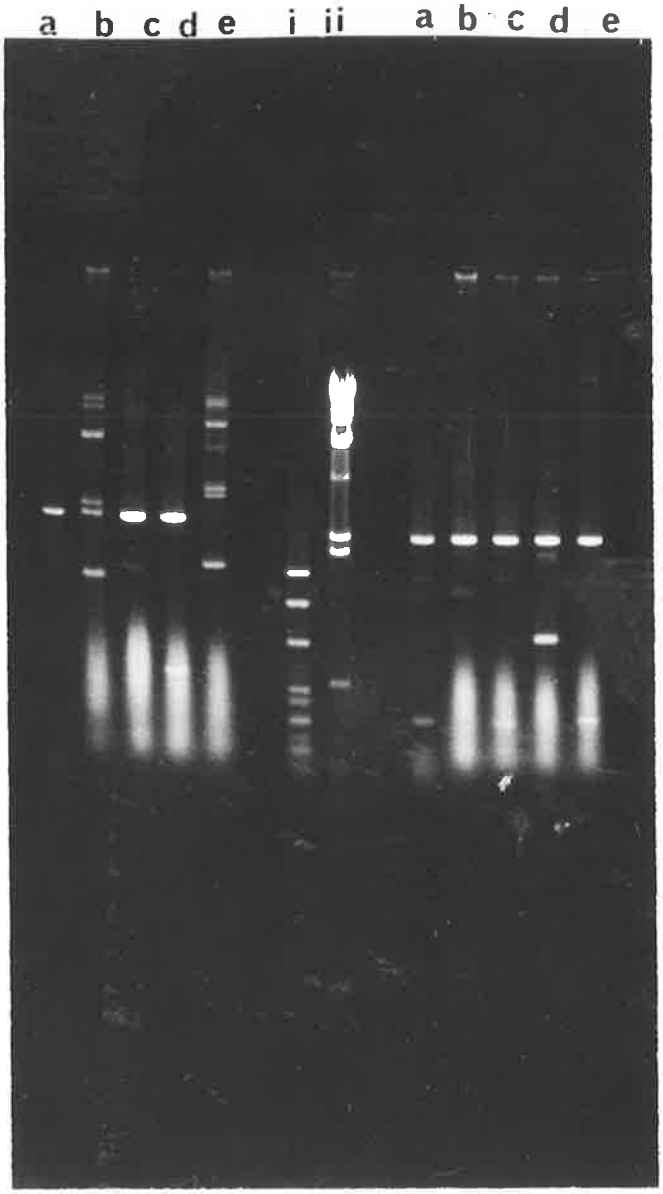

1
2
3
4
bp
–260
–65

Fig. 14 Recombinant colonies bearing the putative S-gene insert.

The 5 recombinant clones (on the left of the marker fragments) treated with the restriction endonuclease Eco R1 were incompletely digested. The process was repeated with the enzyme Pvu II (right hand side of the markers) which yielded a fragment of 528 bp from the clone in Lane (d).

Lane (i) λdv1/Hae III marker (bp)	Lane (ii) λ HindIII marker (bp)
1 713	23 644
1 310	9 588
890	6 742
534	4 467
460	2 298
362	1 974
352	580
272	


a b c d e i ii a b c d e

in compatibility. S- and Z-allele products may well interact to bring about changes in their molecular weights and hence electrophoretic mobility.

3.2.2 Ultra-thin Isoelectric focusing patterns of stigma proteins.

The best resolution of stigma proteins was achieved over the pH range 5-9 (Tan and Jackson,1987). Differences in banding patterns between the clones were observed over a pI range from approximately 5 to 7 (Fig.16). Clones in lanes 1, 2 and 3 were selected because they share a common Z-locus (Z12) thus simplifying the interpretation of the data. The heterozygote exhibited a number of bands, some of which were distinct from the homozygotes S11 and S22 (lanes 2 and 3 respectively) as well as expressing protein bands common to all three clones.

Fig. 15(a, b) Silver-stained SDS-PAGE (12.5%) protein patterns of stigma and leaf of *P. coerulescens*.

Very little variation was detected in the leaf protein patterns of the four clones (Fig 15 b). In contrast the stigmas displayed differences in their protein patterns in the region extending from 43-97 kda (Fig. 15a)(arrows).

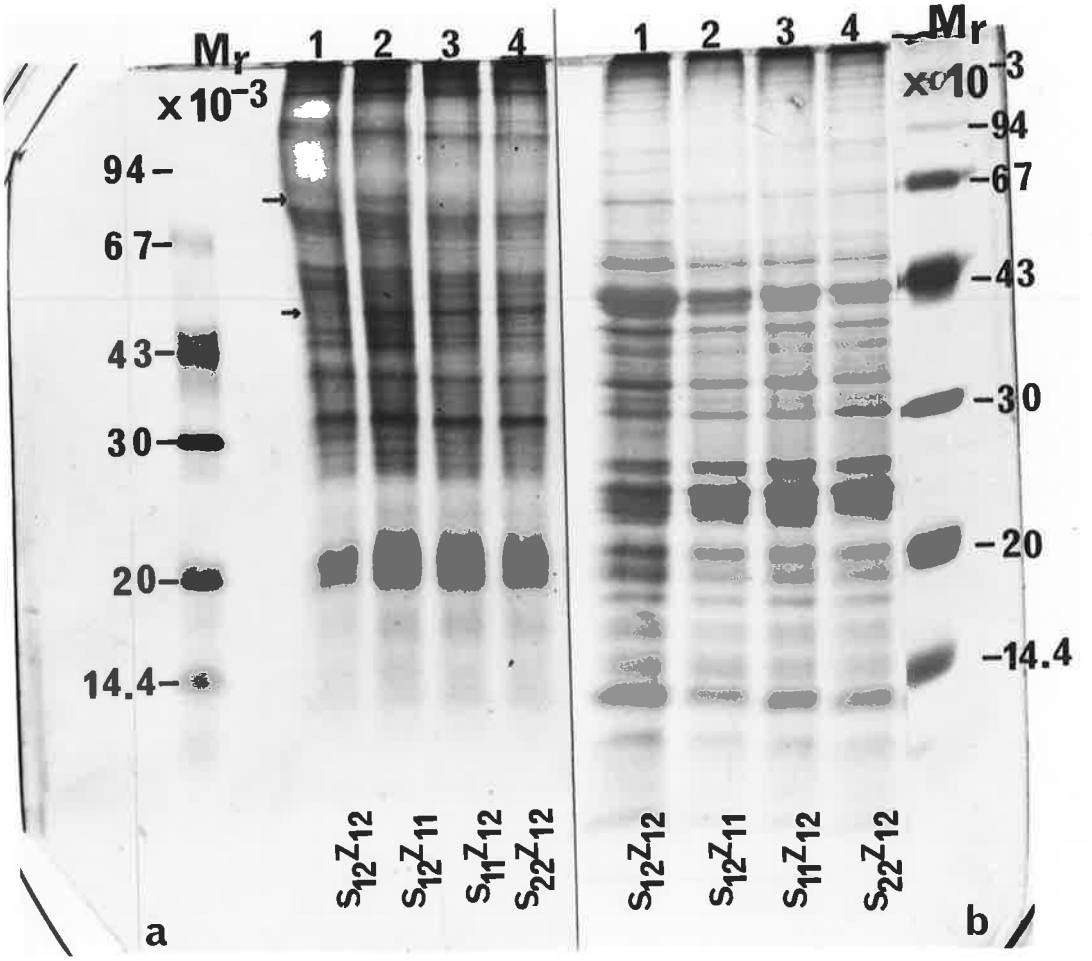
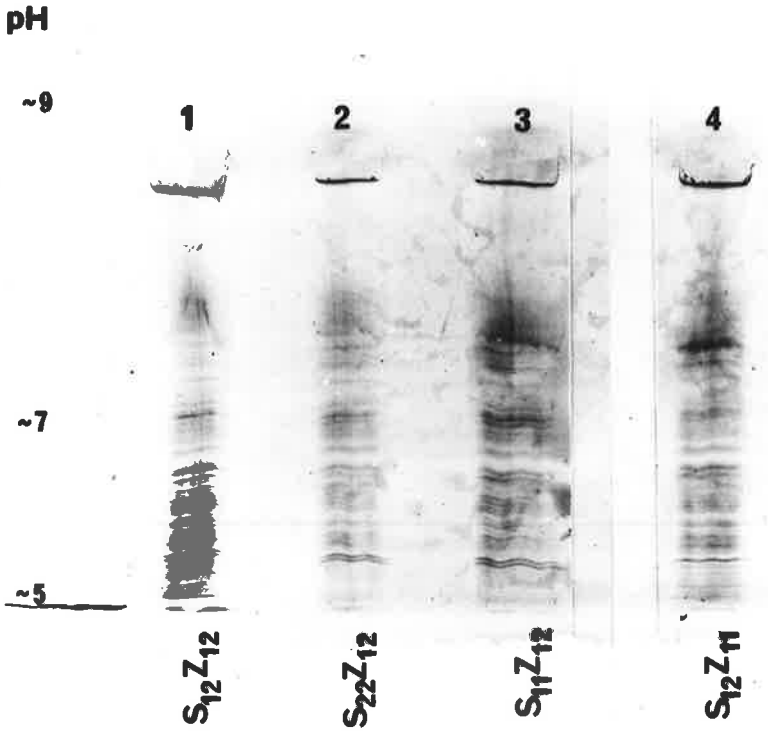
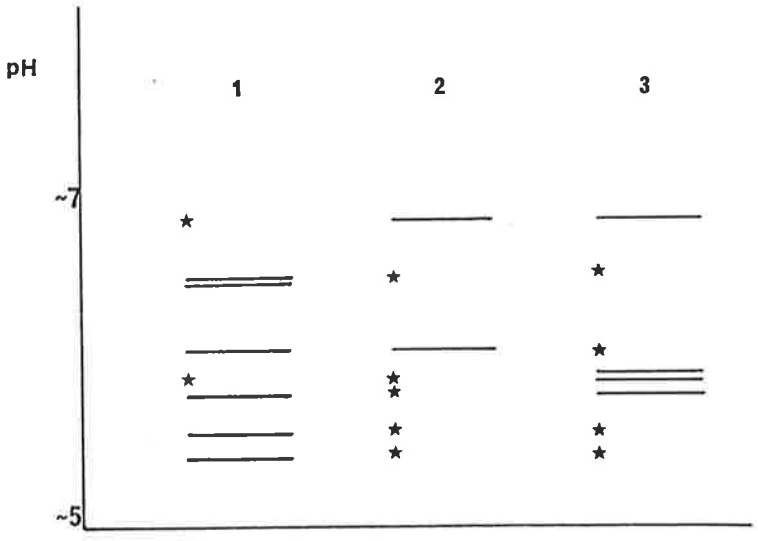

Mr
x10⁻³
94
67
43
30
20
14.4
1
2
3
4
S12Z12
S12Z11
S11Z12
S22Z12
a
b

Fig. 16 Ultra-thin IEF of stigma proteins of *P. coerulescens*.

Differences in the pI region of approximately 5.0-7.0 for the clones S12Z12, S22Z12 and S11Z12 (Lanes 1, 2 and 3 respectively) have been represented schematically. (* Denotes band absent from the sample)





* Not present in the sample

4. DISCUSSION

4.1 *Petunia hybrida* W166H (S2S3)

4.1.1 Comparison of S-allele related proteins expressed in stigma, ovary and callus.

The S2- and to a lesser degree the S-3 related proteins are expressed along the entire length of the mature style as shown in Fig.1. The spatial distribution of these proteins together with the number of self-pollination tubes which traversed half the length of the stigma (Table5) indicate that the site of the self-incompatible reaction is located at the lower half of the style. Similarly Raff *etal.*, (1981) have found an S-antigen distributed in both the stigma and lower portions of the mature styles of *Prunus avium* . In direct contrast Heslop-Harrison (1983) observed that the site of tube inhibition to be located in the upper zone of the stylar transmitting tract. Clarke *etal.*, (1986) have also reported that the S2-gylcoprotein to be concentrated in the stigma and upper segments of the style of *Nicotiana alata.*

The S-related proteins were detected in stigmas of immature buds (< 20mm in length), flower buds of between 20-30mm and those which were on the brink of flowering (> 50mm) (Fig.2). The intensity and distribution of the proteins on separation in SDS-polyacrylamide gel electrophoresis and their IEF patterns were shown to be very similar. In comparison Harris *etal.*, (1984) have postulated the occurrence of the S-gene product in the developing style to be coincident with the expression of self-incompatibility so that the S-gene product was not present during the early stages of development.

The lack of inhibition of the self-pollination tube even after a 24h germination period (Table4;Fig.7) suggests that the time at which the stigmas express the S-related recognition factor correlates well with the presence of a surface secretion detected in *Petunia* pistils at anthesis. Self-pollination of stigmas exhibiting

this surface secretion, have a marked reduction in the number of tubes reaching the lower half of the style as compared to 100% of cross-pollinated tubes detected at the ovary-style junction (Table5;Fig.9). In addition a large proportion of the self-pollination tubes which reached the lower half of the style had lost their cytoplasmic contents leaving only a faint outline of their walls visible.

The successful generation of callus from stigmas by tissue culture prompted closer examination of its protein composition. The resulting protein profile by SDS-polyacrylamide gel electrophoresis (Fig.3) was very similar to those of stigma and mature ovaries. This is to be expected since stigmas are developed from ovarian tissue. The presence of the S2- and S3-related proteins confirm that the genes responsible for the expression of these proteins are still functional in the dedifferentiated tissues. The detection of the S3-related proteins only in immature ovaries is related to the temporal relationship between the the expression of the S genes and the development of the tissue. The absence of a 20 kda protein in callus which was also expressed in all tissues was the only notable difference observed.

In comparison with stigmas and ovaries, the IEF pattern of callus tissue showed differences in the pI regions over the ranges 4.0-6.0 and 7.0-8.0 (Fig.4). A number of callus proteins focused in the first dimension corresponding to the basic region when separated in the second dimension were observed to be callus-specific i.e., this group of proteins were not detected in either the second-dimensional gels of stigma or ovarian tissue(Fig.5E). Similarly the lack of callus proteins focused at the highly acidic range contrasts sharply with the abundance of proteins of the stigmas and ovaries in this region.

Previous work on other gametophytic self-incompatibility systems including *Nicotiana alata* (Bredermeijer and Blaas,1981; Clarke *etal.*, 1985;1986) and *Prunus avium* (Mau *etal.*, 1982) have indicated a relationship between the S-genotype and the expression of a specific class of proteins. Their characteristics include a high

pI value (approximately 8.0-9.5) and molecular weights of between 3.5-4.0 kda. Kamboj and Jackson (1986) have reported a similar result for *Petunia hybrida* where they have attempted to correlate the S-genotype with glycoproteins expressed by the pistils. These glycoproteins have a range of molecular weights of between 27-33 kda and isoelectric points in the basic region of 8.3-8.5. Differences are evident however in the second-dimension gel electrophoresis pattern of the clone W166H (an S2S3 heterozygote in the S-locus) studied in this investigation and those of Kamboj and Jackson. Whilst these authors have stated the 'S proteins' were the only ones visible on the gel " by virtue of their abundance" after staining with Coomassie Blue, the present investigation revealed a population of proteins in the acidic region (Fig.5A, B and C). Factors which may account for this difference include the type of extraction buffer used by these authors (10mM Tris-HCl pH 7.5) compared with that used in this study i.e., 37.5 mM Tris-HCl pH 8.8 containing 1% (w/v) SDS and 4% (w/w) β-mercaptoethanol. The latter buffer was adopted for all protein extractions since it removed membrane and other bound proteins. In addition, the gels were silver stained using the method of Morrisey (1981) which is a very sensitive method of protein detection. No evidence presented in this thesis indicates that the S2- and S3-related products (on separation in the second dimension) were present in the basic region in contrast to Kamboj and Jackson's claim that these were the only detectable proteins at the designated basic region of pI 8.3-8.5.

Nasrallah and Nasrallah (1986) reported on the polymorphic nature of the S-locus specific glycoproteins (SLSG's) present in sporophytic self-incompatibility types eg. *Brassica* and *Raphanus*.. In contrast, with gametophytic self-incompatibile types, S-related products have been characterized as glycoproteins with their isoelectric points in the basic region only. Based on the results of the present study where the S-related proteins in *Petunia hybrida* W166H have been shown to be acidic, the possibility of other gametophytic self-incompatible species possessing similar characteristics cannot be excluded.

4.1.2 cDNA cloning using *P. hybrida* pistils as a source of mRNA .

From the data presented in Section 4.1.1, it is apparent that the S2- and S3-related proteins are expressed during the early stages of pistil development. The time at which pistils are harvested is of vital importance in obtaining an mRNA population containing transcripts of the S-locus. The data in Fig.13(lanes 3 and4) show that immature pistils (< 40mm bud length) are the most active source of mRNA templates for cDNA synthesis. A small degree of sequence homology is also shared with the 30-mer synthetic primer derived from the N-terminus of the *N. alata* S2-glycoprotein (Clarke *etal.*,). The weak mRNA:cDNA signal observed when using mRNA from mature pistils may be linked to the synthesis of S-related proteins at an earlier stage of bud formation (Fig.13 lane 1). In comparison both Nasrallah *etal.*, (1985) and Clarke *etal.*, have elected to use mature self-incompatible stigmas from *B. oleracea* and *N. alata* respectively in constructing their cDNA libraries.

When considering the lack of self-pollination tube inhibition in non surface secreting mature pistils (Table4;Fig. 7), the S-related proteins although present may be extruded onto the papillar surface only at anthesis. The time at which the surface secretion was detected on the stigmas coincided with the release of self-pollen from the anthers, an event designed to prevent self-fertilization. The observation of differential RNA synthesis in self- and cross-pollinated styles of *P. hybrida* may be a consequence of the initial pistil-pollen recognition event on the stigma surface (van der Donk,1974).

The use of the same 30-mer sequence as a radioactive probe did not yield a positive hybridization signal. About 60 of the 600 recombinants would be a conservative estimate of those containing fragments of the S-gene as inserts. The lack of a strong signal may be attributed to the lack of homology between the S-genes coding for the acidic S-related proteins in *P. hybrida* and the oligonucleotide sequence derived from the very basic (pI~9.8) *N. alata* S2-glycoprotein.

The future work of cDNA cloning of the S-related proteins would involve constructing cDNA libraries from stigmas obtained from known self-compatible and self-incompatible clones of *P. hybrida*.. Based on the premise that the S-locus would not be present in self-compatible stigmas, the technique of differential hybridization could then be employed to screen for the S-genes as inserts in the self-incompatible library.

4.1.3 *In vitro* tissue culturing of styles of *Petunia hybrida* W166H.

The successful production of callus from styles of *P. hybrida* carried out in this present study is a significant development. Styles were selected as a source of material in order to examine the effect of dedifferentiation on the expression of the S-related genes. The results in Fig.3(Lane 2) shows clearly the presence of the S2- and S3-related proteins in callus tissue.

The 4 week lag period prior to callus formation is not uncommon since other workers using reproductive tissue have noted a similar time interval. These range from two weeks for ovules of *Gossypium hirsutum* (Hsu and Stewart,1976) and styles and anthers of *Prunus avium* (Raff and Clarke,1981) to over 4 weeks for anthers of *P. hybrida* and *P. axillaris* (Mitchell *etal.*, 1980). The concentrations of growth factors used in this study to initiate callus formation (3.0 mg/L kinetin;0.3-2.0 mg/L NAA) were similar to those used by Raff and Clarke (1.0 mg/L kinetin;2.0 mg/L NAA). Differences in their technique included the use of Schenk and Hildebrant's medium instead of the MDAUC basal culture used in this study and they cultured styles and anthers in the dark for a two week period before transferring to liquid suspension cultures.

An extension of this study would involve the isolation of proteins secreted from calli derived from other tissues of the ovary,

pollen and leaves of *P. hybrida*.. A comparative study of the types and tissue-specificity of these proteins could then be used as markers to identify S-related gene expression of explants regenerated from style derived callus. The possibility of using callus from a compatible *P. hybrida* clone as a medium of gene expression in gene transfer experiments with the Ti plasmid from Agrobacteria would allow for the testing of any putative self-incompatible gene.

4.1.4 Compatible and incompatible pollinations of *P. hybrida* W166H.

No difference in the degree of germination of either self- or cross-pollination tubes was detected in stigmas at three stages of development (Tables 2, 3 and 4). After a 7h germination period, the number of self-pollination tubes present in the upper region of the stigma and style were similar to those of cross-pollination tubes (Table 3) These self-pollination tubes also exhibited more vigourous growth with a higher percentage of the tubes reaching the lower portion of the style. The converse was observed when stigmas exuding a surface secretion were selected (Table5;Fig.9). Although the percentage of intact incompatible pollen tubes present in the lower half of the style was considerably fewer, the number observed in the upper region was comparable to that of compatible pollinations. Callose was detected in both types of pollen tubes (Figs.10 and 11). Herrero and Dickinson (1979) reported a depletion of starch reserves in Ka3 and T2 u stigmas of *P. hybrida* (S2S2 and S3S3 genotype respectively) only during compatible reactions. This may be due to the starch reserves being utilised in maintaining pollen tube growth and reducing the number of tubes disintegrating in the lower region of the style.

Contrary to the observations of corolla wilting within 24h of self-pollinated stigmas of of *P. hybrida* (Gillisen 1976, 1977; Gillisen and Hoekstra, 1984) there were no signs of the flowers becoming less turgid either in self- or cross-pollination experiments. Injury to the stigma during the pollination procedure may account for this phenomenon.

4.2 *Phalaris coerulescens*

4.2.1 Expression of stigma-specific proteins of *P. coerulescens* .

Stigma proteins of all four clones showed differences in their protein profiles within the range of 43-94 kda. The lack of any such differences in their leaf patterns suggests a relationship between the genotype and protein expression in stigmas (Fig.15 a and b). The IEF patterns are also indicative of differences in stigma-specific proteins in the pI region of 5.0-7.0 (Fig.16). The existence of stylar proteins specific to a particular genotype has been demonstrated for both the single S-locus gametophytic and sporophytic self-incompatible types (Linskens,1975; Bredermeijer and Blaas,1981 and Clarke *etal.*, 1986). However in *P. coerulescens* where self-incompatibility is controlled by the two, polyallelic S- and Z-loci no simple correlation is evident between the genotype and the expression of stigma-specific proteins.

It has been assumed that in the single S-locus system the alleles produce monomers which remain unaggregated or are co-polymers with a polypeptide from another silent homozygous gene (Lewis,1979). This hypothesis for the two loci of Gramineae involves the aggregation of co-polymers between two different genes *in vivo* . These proteins appear as monomers in a denaturing SDS-polyacrylamide gel. It is apparent from Fig.15a that the self-incompatibility associated proteins are not the major components of the stigma extracts and therefore cannot be assigned specifically to each allele. Based on this result it may be necessary to revise Lewis's hypothesis regarding the two loci in Gramineae where it is postulated that one allele produces one protein. Moreover these proteins could have the same molecular weights and consequently would co-migrate when electrophoresed. Although the clones $S_{12}Z_{12}$, $S_{11}Z_{12}$ and $S_{22}Z_{12}$ share a common Z-locus, their IEF patterns (Fig.16 lanes1, 2 and 3) are too complex to simply assign these proteins to a specific genotype as is possible in the one S-

locus system.

Nasrallah and Nasrallah (1986) have suggested that in both sporophytic and gametophytic systems, the S-alleles are responsible for the expression of the specific proteins which are glycosylated. In the current study it has not been possible to determine whether these findings apply to *P. coerulescens*.. The limited quantity of stigmas available has limited the detection of proteins bearing sugar moieties. However, it appears that both the S- and Z-alleles control the expression of certain stigma proteins but not those of leaves.

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APPENDIX

Composition of culture medium MDAUC
(All figures are either in mg/L or percentages of w/v)

Macroelements

$CaCl_2.2H_2O$	440.0
$FeSO_4.7H_2O$	27.8
KH_2PO_4	170.0
KNO_3	1 900.0
$MgSO_4.7H_2O$	370.0
Na_2 EDTA	37.3
NH_4NO_3	1 650.0

Microelements

$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
H_3BO_3	6.2
KI	0.83
$MnSO_4.4H_2O$	22.3
$Na_2MoO_4.2H_2O$	0.25
$ZnSO_4.7H_2O$	8.6

Sucrose	2.0%
Nicotinic Acid	0.50
Pyridoxine	0.10
Thiamine HCl	0.10
Glycine	3.0
Agar	0.8%
pH	5.8