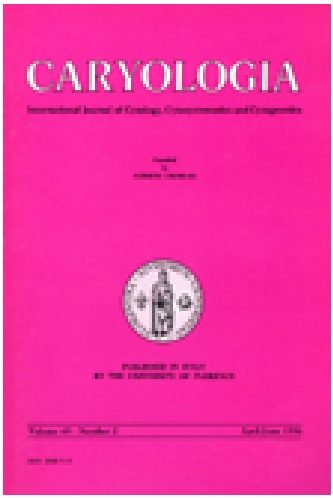


Caryologia: International Journal of Cytology, Cytosystematics and Cytogenetics
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tcar20

Stylar peroxidases and heteromorphic incompatibility reactions in Primula acaulis Hill («thrum» morph)

Luisa Carraro[a], Giuliana Lombardo[a] & Paolo Gerola[a]
[a] Dipartimento di Biologia, Sezione di Botanica Sistematica, Università degli Studi di Milano, 20133 Milano, Italy.
Published online: 31 Jan 2014.

CARYOLOGIA Vol. 49, n. 2: 101-112, 1996

Stylar peroxidases and heteromorphic incompatibility reactions in *Primula acaulis* Hill («thrum» morph)

LUISA CARRARO, GIULIANA LOMBARDO and PAOLO GEROLA

Dipartimento di Biologia, Sezione di Botanica Sistematica, Università degli Studi di Milano, 20133 Milano, Italy.

SUMMARY — In non-, self- and cross-pollinated styles of *Primula acaulis* «thrum» morph, the distribution of apoplastic peroxidase activity has been investigated, by means of the cytochemical treatment with diaminobenzidine. Apoplastic peroxidase activity was detected throughout the transmitting tract (at the «neck» region level) in non- and self-pollinated styles. The enzyme has been related to the predisposition of incompatible pollen tube rejection, no appreciable differences in enzyme distribution having been found between non- and self-pollinated styles since, in this morph, incompatible pollen tubes hardly ever reach the stylar neck. Compatible intermorph pollination caused the disappearance of apoplastic peroxidase activity in the central portion of the transmitting tract, where pollen tubes elongated, utilizing the reserves of the stylar cells, which appeared highly degenerate. Stylar apoplastic peroxidases seem then to play a role in heteromorphic incompatibility responses.

INTRODUCTION

The genus *Primula* is characterized by heterostilous heteromorphy linked with a self-incompatibility system, that allows only between-morph pollinations (GANDERS 1979). Little is known about the biochemistry of the incompatibility reaction but the sites of pollen germination or tube growth inhibition have been more investigated: blockage may occur either on the stigma surface through the failure of germination or of pollen tube penetration after germination, or in the stigma head during growth of the pollen tube through the transmitting tissue, or in the stylar transmitting tract (SHIVANNA *et al.* 1981). Sites of pollen tube inhibition after illegitimate pollinations differ between within-thrum crosses and within-pin crosses and usually more than one site is responsible for the inhibition reaction, none of the barriers being complete (WEDDERBURN and RICHARDS 1990). In *Primula vulgaris*, following controlled

Corresponding author: Prof. Luisa Carraro, Dipartimento di Biologia, Sezione di Botanica Sistematica, Università degli Studi di Milano, Via Celoria 26, 20133 Milano, Italy.

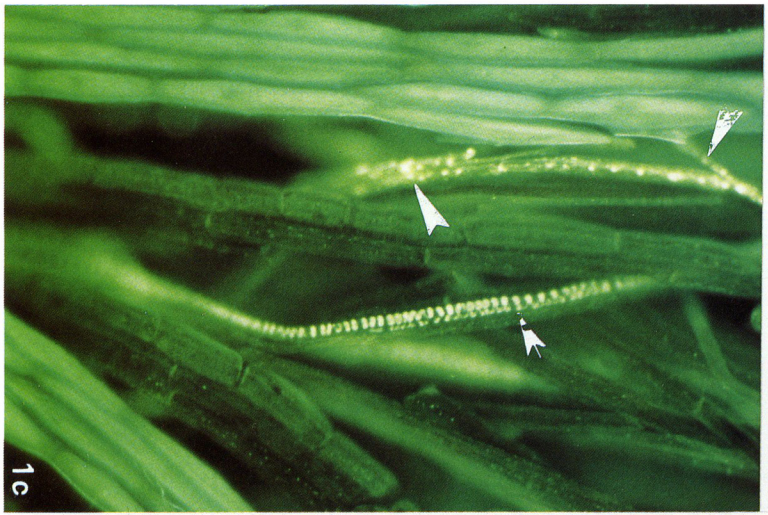
self-pollination, a large number of incompatible grains did not germinate at all; the tubes of self-pollen, germinated on the stigma, penetrated the papillae in different sites, depending upon the morph, afterwards in the « thrum» morph the few self-pollen tubes being blocked in the stigma head, whereas in the «pin» morph tubes were frequently observed in the stylar tract, site of their blockage (SHIVANNA *et al.* 1981). In *Primula obconica* the expression of incompatibility takes place after germination: selfing «thrum» flowers, pollen tubes grew randomly over the papillar cells and «pin» self-pollen tubes were inhibited while elongating in the style (STEVENS and MURRAY 1982). Furthermore STEVENS and MURRAY (1982) noticed that the proteinaceous material of sporophytical origin present in the pollen wall was removed by prolonged washing in aqueous media without modifying the incompatibility reaction. They concluded that «the incompatibility reaction is between pollen tubes and stigmatic tissue and differs from the homomorphic sporophytic system where pollen wall proteins elicit the incompatibility response».

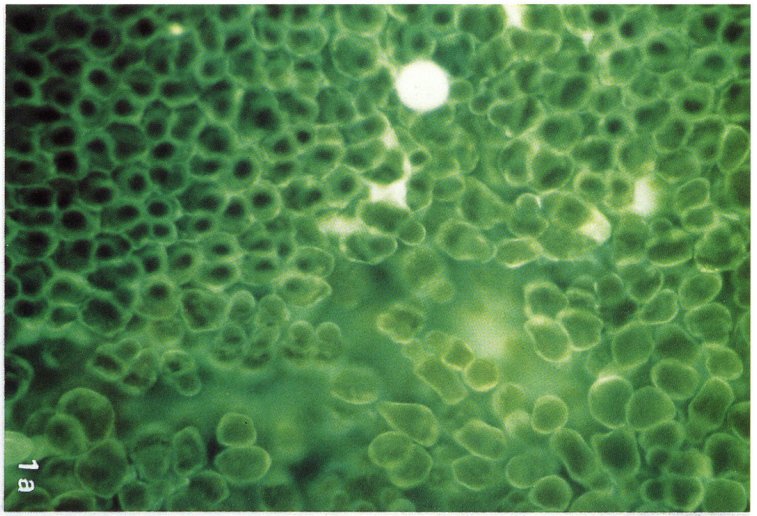
Intramorph self-incompatibility in «pin» flowers of *Primula acaulis* has been object of previous reports on the possible linking of peroxidases with incompatibility responses (CARRARO *et al.* 1985, 1990*a*): by means of cytochemical electron techniques apoplastic peroxidases have been observed in the stylar transmitting tissue of non-pollinated «pin» flowers; peroxidase activity being increased by self-pollination and disappearing from the central portion of the tissue after cross-pollination. The hypothesis that these apoplastic peroxidases play a role in the predisposition of the pistil to reject incompatible pollen tubes and in the rejection mechanism itself, has been further supported by peroxidases distribution in ultraviolet-irradiated «pin» plants of *Primula acaulis*, showing pseudo-self-compatibility (CARRARO *et al.* 1990*a*): the breakdown of self-incompatibility caused by irradiation was always coupled with the absence of peroxidase activity.

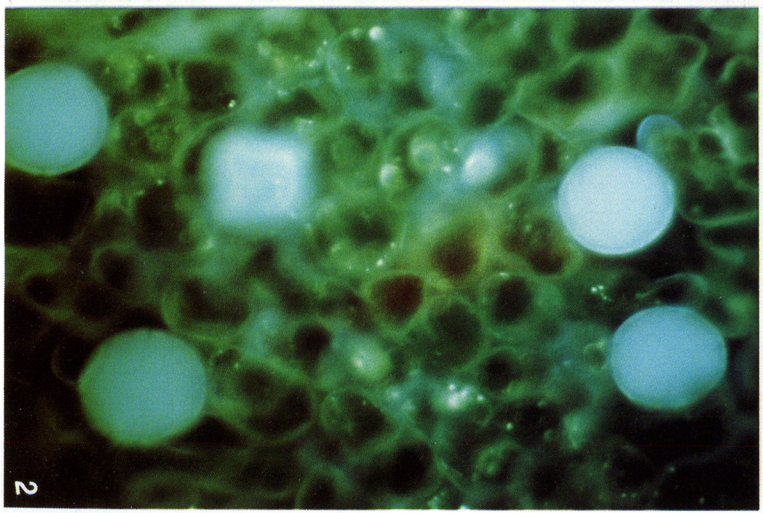
The role of peroxidase enzymes in incompatible pollen tube inhibition was first proposed by PANDEY (1967) and thoroughly investigated by BREDEMEIJER and BLAAS (1975) in *Nicotiana*.

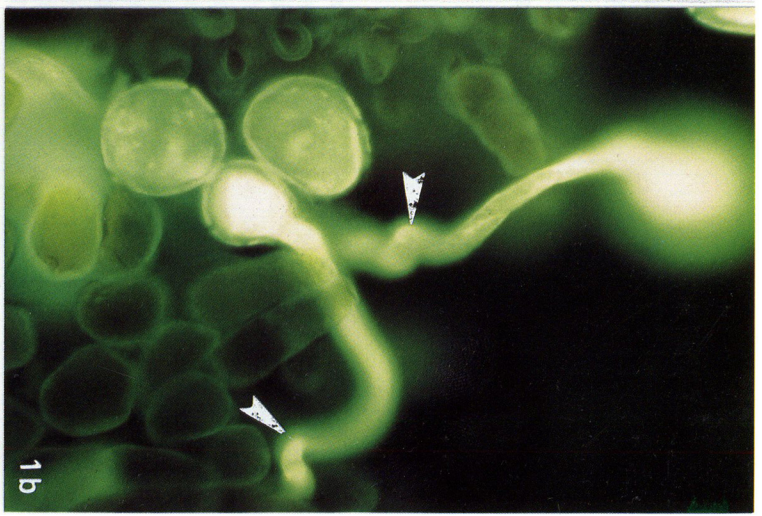
Fig. 1. — Fluorescence micrographs of «thrum» pistils not subjected to controlled pollination. a) Stigma head with a single, non germinated self-pollen grain adhering to the papillae (× 270). b) Stigma head with few adhering self-pollen grains: while some did not germinate, the pollen tubes of the germinated ones seem to coil around the stigmatic papillae (arrow-heads) (× 450). c) Wall thickenings of a vessel (arrow) and sieve elements (arrow-heads) in the stylar neck (× 270).

Fig. 2. — Fluorescence micrograph of «thrum» pistil following controlled self-pollination. 40 h after pollination, none of the «thrum» pollen grains adhering to the stigma head did germinate (× 450).









In the present report we investigated the presence and localization of apoplastic peroxidases in the stylar transmitting tissue of non-, self- and cross-pollinated «thrum» pistils of *Primula acaulis* and discussed the obtained data, taking into account the role of apoplastic stylar peroxidases previously proposed for non-, self- and cross-pollinated pistils of the «pin» morph of the same species.

MATERIALS AND METHODS

The observations were made on plants of *Primula acaulis* Hill (= *Primula vulgaris* Hudson, in conformity with Index Kewensis) from natural populations, transferred into cultivation, keeping carefully the «pin» plants isolated from the «thrum» ones.

Controlled pollinations were performed as previously described (CARRARO *et al.* 1985): «thrum» flowers were self-pollinated with «thrum» pollen and cross-pollinated with «pin» pollen. Non-pollinated «thrum» flowers having been also examined. The pistils were collected 2, 4 and 40 h after pollination, together with non-pollinated ones and treated for fluorescence, as well for electron and light microscopy.

Fluorescence microscopy. — The pistils were fixed in FAA and stained with water soluble aniline blue dye as described by MARTIN (1959).

Samples were observed using a Leitz Orthoplan with UV illumination and filter block Leitz B2 (exciting filter BP 340-410, beam-splitting mirror RKP 455, suppression filter LP 470).

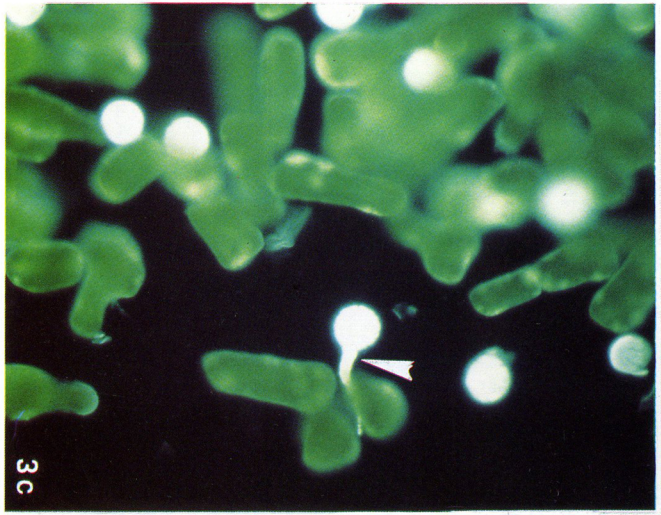
Electron and light microscopy. — Prefixation, fixation, histochemical determination of peroxidase activity with DAB (diaminobenzidine) reaction and embedding were performed as previously reported (CARRARO *et al.* 1985, 1986). Ultrathin cross-sections of the 'neck' region were obtained with an Ultracut E. Reichert-Jung ultramicrotome and examined with a Jeol JEM 100 transmission electron microscope. Semi-thin cross-sections of the same neck region were examined with a Leitz Orthoplan light microscope. The sections were not stained, to avoid any overlapping with reaction products.

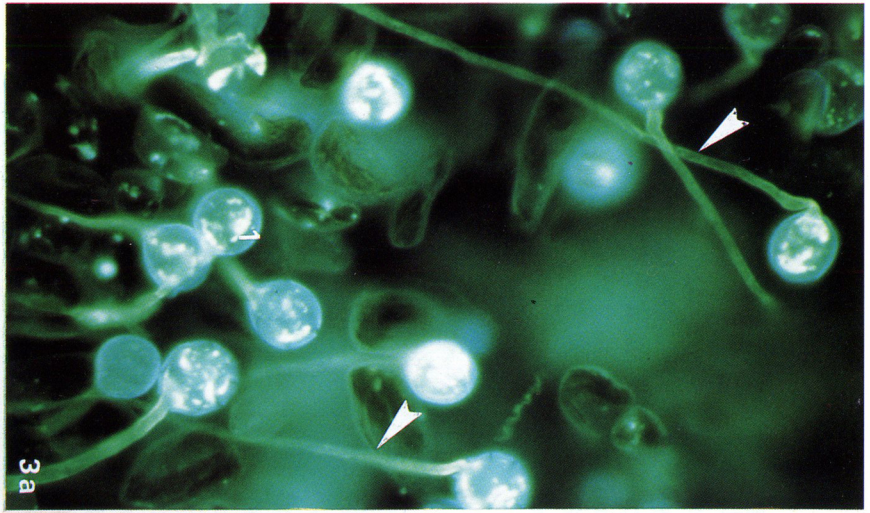
RESULTS AND DISCUSSION

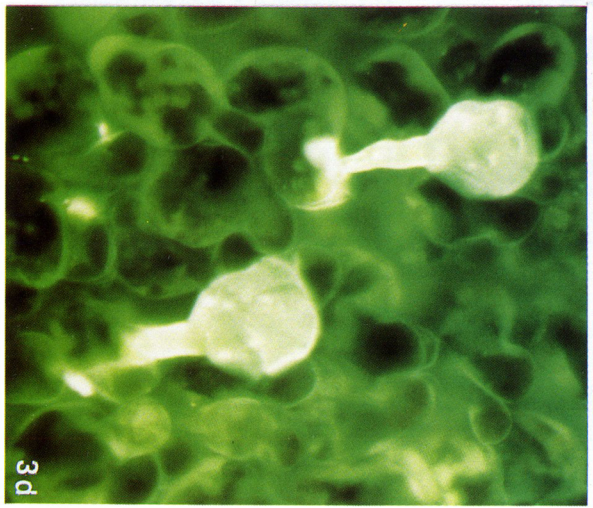
Non-pollinated pistils.

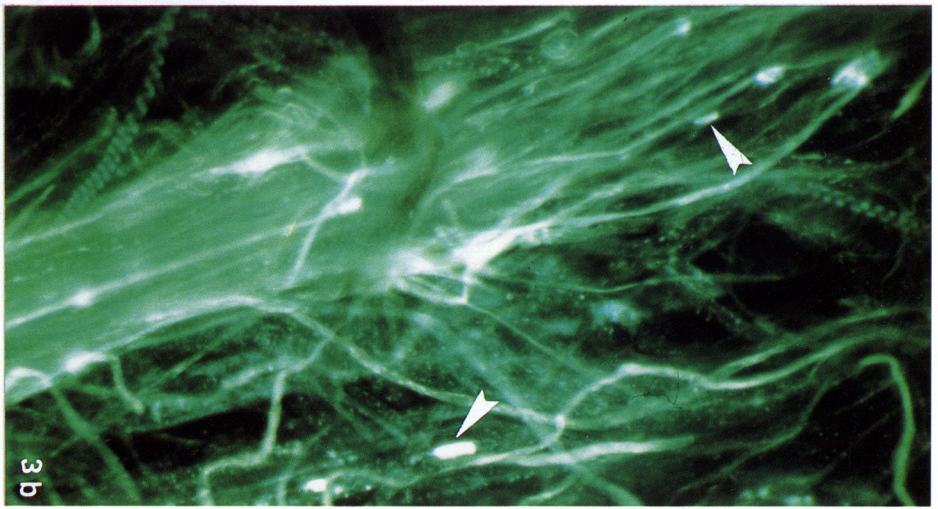
Because of the reciprocal position of anthers and style in the «thrum» morph flowers (high anthers and low stigmas), it is impossible to avoid natural

Fig. 3. — Fluorescence micrographs of «thrum» pistils following controlled cross-pollination. a) 40 h after pollination, the numerous pollen tubes (arrow-heads) of «pin» pollen penetrated the stigma head (× 450). b) Compatible pollen tubes with evident callose plugs (arrows-heads) elongating in the stylar neck, 40 h after cross-pollination (× 450). c) 2h after pollination very few pollen grains initiated to germinate (arrow-head) (× 270). d) 4 h after pollination many pollen tubes initiated to penetrate the stigma head (× 720).





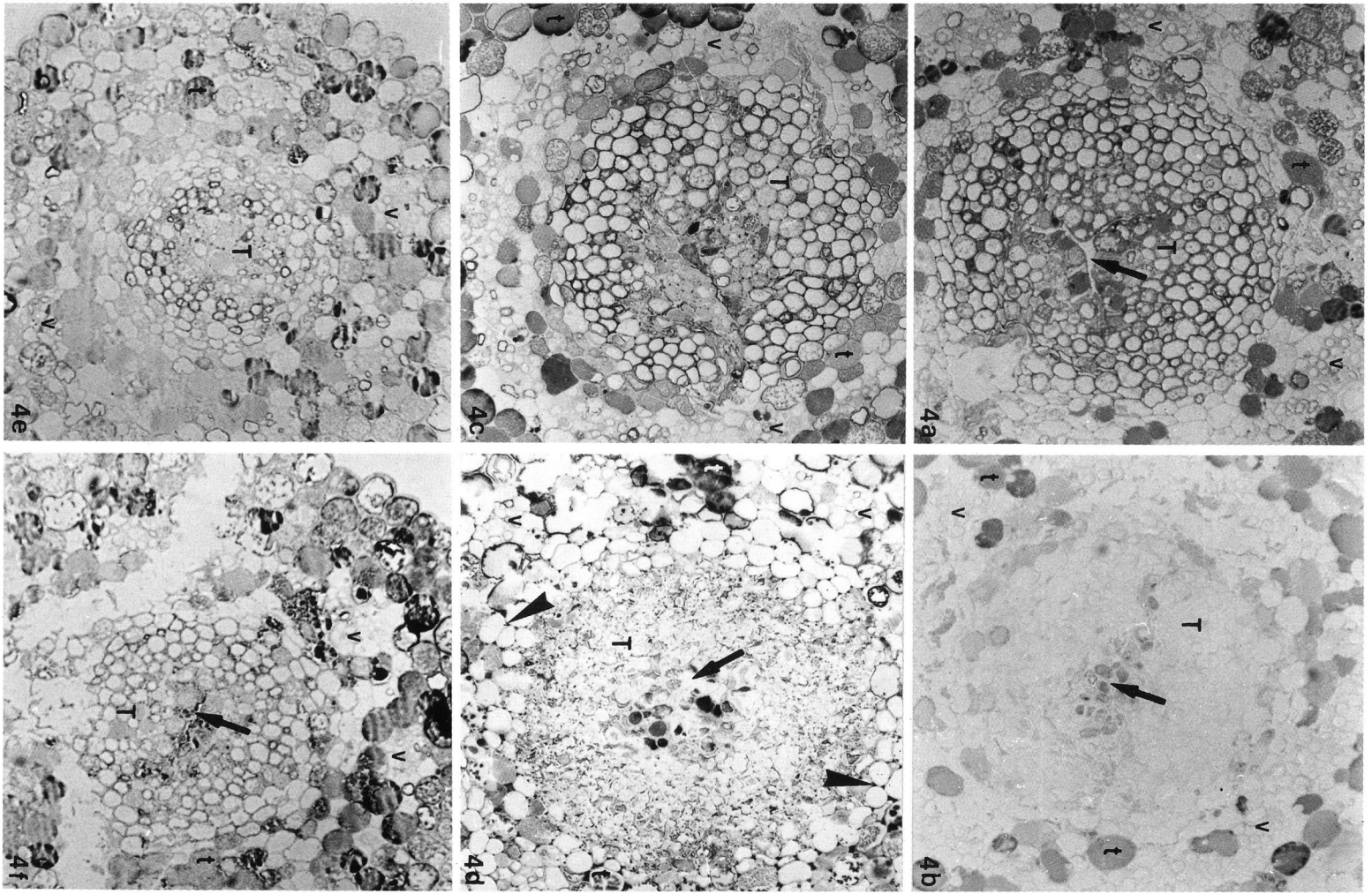




self-pollination, without emasculating the flowers. Moreover emasculation often damages the buds, which are still very small, when pollen grains ripen and anthers dehisce. Consequently «non-pollinated» styles are unachievable: indeed some pollen grains were nearly always detectable on the stigmatic papillae of flowers not subjected to controlled pollination (Fig. 1a and b). Most of such grains did not germinate; when germination occurred, the few pollen tubes grew randomly over the stigmatic papillae and sometimes seemed to coil around them (Fig. 1b), as already described by SHIVANNA *et al.* (1981) after self-pollination of the «pin» morph. However, while self «pin» tubes penetrate the stigma head and arrest along the style (SHIVANNA *et al.* 1981; STEVENS and MURRAY 1982), self «thrum» tubes never reached the stilar «neck», where the only fluorescing elements, after aniline blue staining, are the thin vessels and the sieve elements (Fig. 1c).

In semi-thin cross sections of the stylar neck region (Fig. 4a), the transmitting tissue consisted of small roundish cells, delimiting numerous intercellular spaces: the ovarian cavity appeared like a central, thin slit, and a parenchimatous cortical tissue with some vascular bundle surrounded the transmitting tract. Many cells of the cortical tissue contained vacuolar tannins, as already described by HESLOP-HARRISON *et al.* (1981). Tannin cells also occurred among the transmitting cells. After DAB treatment (Fig. 4a), the cell walls of the transmitting tissue appeared dark-coloured and osmiophylic, when compared with those of the non treated samples (Fig. 4b). This result suggests the presence of apoplastic peroxidase activity throughout the transmitting tissue of non-pollinated styles; as observed for the «pin» morph of the same species (CARRARO *et al.* 1990*a*) such activity being probably related to the predisposition of the style to reject incompatible pollen tubes (CARRARO *et al.* 1986). However self pollen tubes of the «thrum» morph penetrate the stilar neck quite seldom, being more frequently blocked on the stigma head; stylar peroxidases are therefore a second barrier to incompatible tube growth, in agreement with the above reported observations of WEDDERBURN and RICHARDS (1990).

Fig. 4. — Light micrographs of semithin cross sections through the «neck» region of «thrum» pistils. (× 320). a) DAB treated, non pollinated pistil: the transmitting tissue cells are roundish and their cell walls appear osmiophilic, indicating the presence of apoplastic peroxidase activity. b) Non treated, non-pollinated control sample: the cell walls of the transmitting tissue result poorly contrasted. c) DAB treated pistil, 40 h after self-pollination: the cell walls of the transmitting tissue appear dark-coloured and osmiophylic, indicating the presence of peroxidase activity. d) DAB treated pistil, 40 h after cross-pollination: the transmitting tissue is highly degenerate and peroxidase activity limited to the intercellular spaces of the peripheral transmitting tissue (arrow-heads). e) and f) DAB treated pistils, respectively 2 and 4 h after cross-pollination: the transmitting tissue appears well structured, the cell walls contrast progressively fainting, indicating a decrease in peroxidase activity. Arrows = ovarian cavities; v = vascular bundles; t = tannin cells; T = transmitting tissue.



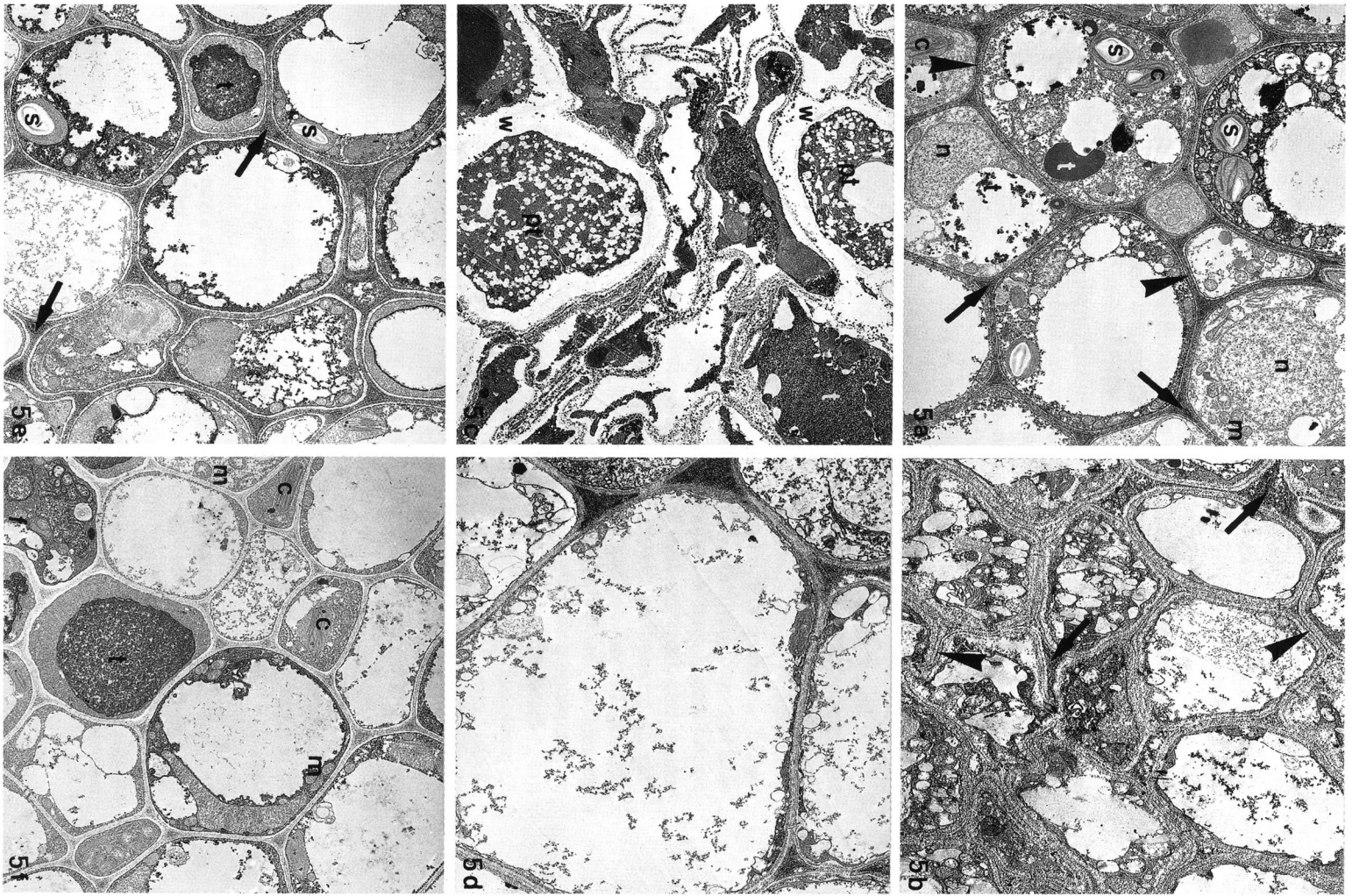
At the electron microscope the samples subjected to the DAB reaction showed a heavy electron-opaque precipitate, indicating peroxidase activity, localized in the intercellular spaces and outer cell wall layers of the transmitting tissue (Fig. 5a). Similar ultrastructural localization of peroxidase activity has been previously described for the «pin» morph of *Primula acaulis* (CARRARO *et al.* 1990*a*) and related to incompatibility responses. Cell ultrastructure described for the «pin» morph, is repeated also in the «thrum» one: as already noticed by HESLOP-HARRISON *et al* (1981) there is no ultrastructural dimorphism between the stilar tissues of the two morphs. The cells of the transmitting tract contain large vacuoles, often repleted with tannins, numerous small mitochondria and chloroplasts with well developed granal systems and primary starch grains; roundish well structured nuclei and plates of ribosomal endoplasmic reticulum are also frequently visible (Fig. 5a).

Self-pollinated pistils.

After controlled self-pollination and aniline blue staining, many pollen grains were seen adehering on the stigmatic papillae (Fig. 2); they never resulted germinated and visibly larger that «pin» pollen grains (compare Fig. 2 with Fig. 3a). Heterostylous species often show pollen size polymorphism, «thrum» pollen being larger. In the far 1877 DARWIN suggested that the pollen size difference was related to the necessity for larger energy reserves in thrum pollen, whose tubes must grow down the long styles of «pin» flowers. Also fluorescence observations of the stylar neck, demonstrated the complete absence of pollen tubes (data not shown), indicating that after «thrum» selfing incompatible pollen grains are mainly inhibited already in germination.

In semi-thin cross section of the stylar neck region of DAB treated samples (Fig. 4c) the cell walls of the transmitting tract appeared dark-coloured and osmiophylic like those of non pollinated flowers (compare with Fig. 4a). Also cell ultrastructure was similar and the electron-opaque precipitate observable on the cell walls and in the intercellular spaces of the transmitting tissue

Fig. 5. — TEM of transmitting tissue (neck region of DAB treated «thrum» pistils) (× 3800). a) Non pollinated sample: electron opaque precipitate, indicating peroxidase activity, is localized in the intercellular spaces (arrows) and on the outer cell wall layers (arrow-heads). b) 40 h after self-pollination, peroxidase activity results localized in the intercellular spaces (arrows) and on the outer cell wall layers (arrow-heads). c) 40 h after cross-pollination the transmitting tissue cells are highly degenerate and apoplastic peroxidase activity disappeared. Compatible pollen tubes (pt) show a thick callose wall (w) and highly vacuolated cytoplasm. d) 40 h after cross-pollination, an electron-opaque precipitate, indicating peroxidase activity, is limited to the peripheric intercellular spaces of the transmitting tissue. e) 2 h after cross-pollination, the transmitting tissue cells appear well structured and provided with starch reserves; apoplastic peroxidase activity is still observable (arrows). f) 4 h after cross-pollination, the transmitting tissue cells are well structured and apoplastic peroxidase activity has completely disappeared. c = chloroplasts; m = mitochondria; n = nuclei; s = starch grains; t = vacuolar tannins.



(Fig. 5b) suggests the presence of apoplastic peroxidase activity in the transmitting tract. No self-pollen tubes were recognizable among the transmitting tissue cells, since incompatible pollen tubes are mainly blocked on the stigma head; the absence of incompatible pollen tubes in the neck region justifies the absence of an increase in peroxidase activity, already described for self-pollinated «pin» styles (CARRARO *et al.* 1990*a*). In that case apoplastic peroxidase activity, present in non-pollinated pistils predisposed for pollen tube rejection, increased in the presence of incompatible pollen tubes, that were blocked along the style; on the contrary, in the «thrum» morph, where self-pollen tubes do not reach the style, peroxidase activity did not increase, supporting the pattern of peroxidase action in incompatibility responses already proposed in previous papers (CARRARO *et al.* 1985, 1986, 1990*a* and *b*).

Cross-pollinated pistils.

40 hours after controlled cross-pollination, at the fluorescence microscope, nearly all «pin» pollen grains had germinated (Fig. 3a); pollen tubes penetrated straightway the stigma head and were clearly recognizable in the neck region (Fig. 3b). Some of them had already reached the ovary (data not shown), according to the time course of events following intermorph pollinations reported by SHIVANNA *et al.* (1981).

In semi-thin cross section of the stylar neck of DAB treated samples, the transmitting tissue appeared highly degenerate (Fig. 4d) and the cell walls were poorly contrasted. At the electron microscope the pollen tubes, provided by a thick callose wall and containing a highly vacuolated cytoplasm, were easily recognizable among the degenerate transmitting tissue cells (Fig. 5c). Apoplastic peroxidase activity was lacking at the central portion of the transmitting tract, where pollen tube elongate, but persisted in its outer portion (Fig. 5d). As previously reported for the «pin» morph of *Primula acaulis* (CARRARO *et al.* 1990*a*), cross-pollination causes the disappearance of peroxidase activity, where pollen tubes elongate, allowing their heterotrophyc growth at transmitting tissue's expense. The maintenance of apoplastic peroxidase activity at the periphery of the transmitting tissue has already been observed in cross-pollinated «pin» styles and it has been proposed that such peroxidases are not involved in the incompatibility process (CARRARO *et al.* 1990*a*).

2 hours after cross-pollination only few pollen grains appeared germinated (Fig. 3c) and the pollen tubes did not jet reach the stylar neck (data not shown); 4 hours after cross-pollination the number of germinated pollen grains was larger (Fig. 3d), but pollen tubes still did not reach the stylar neck (data not shown). In both samples, at the light microscope, the stylar transmitting tissue in the neck region appeared well structured and the cell wall contrast progressively fainter than in the non-pollinated samples (compare Figs. 4a, e and f), suggesting a peroxidase activity decrease. Electron microscopic observations in fact demonstrated that apoplastic peroxidase activity, still present in

the central portion of the transmitting tissue 2 hours after compatible cross-pollination (Fig. 5e), became completely absent from the same region 4 hours after pollination (Fig. 5f). No pollen tube was detectable among the transmitting cells (in agreement with fluorescence microscopic data), that appeared well structured and provided with starch reserves. It might be proposed that removal of the peroxidase barries anticipates pollen tube elongation, probably caused by molecular signals originating from the germinating compatible pollen grains on the stigma head. The few incompatible self-pollen grains occasionally germinating on the stigma, seem not able to send such signals and the peroxidase barrier is always present throughout the transmitting tract of styles not subjected to controlled cross-pollination (Figs. 4a and 5a).

CONCLUSIONS

In the heteromorphic species *Primula acaulis*, stylar peroxidases seem to cover an important role in incompatibility responses and their distribution in non-, self- and cross-pollinated styles of both morphs is coincident with our previous data on *Petunia hybriba* (CARRARO *et al.* 1986).

Non-pollinated styles show apoplastic peroxidase activity as «predisposition» to pollen tube rejection; controlled incompatible intramorph pollination causing an increase in peroxidase activity in the «pin» morph, where pollen tube inhibition occurs along the style, while such increase lacks in the «thrum» morph, since most pollen grains are blocked on the stigma and do not reach the style. In both morphs, compatible intermorph pollination causes a progressive weakening of the peroxidase barrier, that disappears completely 4 hours after pollination, thus allowing pollen tubes to elongate, utilizing the reserves of the transmitting tissue cells.

Acknowledgements. — This work was supported by fund of M.U.R.S.T., Italy. The authors are indebted to Prof. M. Orsenigo for his critical reading and correction of the manuscript. Moreover the authors gratefully acknowledge the technical assistance of Mr. R. Cavatorta.

REFERENCES

BREDEMEIJER G.M.M. and BLAAS J., 1975. — *A possible role of a stylar peroxidase gradient in the rejection of incompatible growing pollen tubes.* Acta Bot. Neerl., 24: 37-48.

CARRARO L., GEROLA P.D., LOMBARDO G. and GEROLA F.M., 1990*a*. — *Pseudo-self-compatibility in ultraviolet-irradiated plants of Primula acaulis ('pin' morph).* J. Cell Sci., 95: 659-665.

—, 1990*b*. — *Peroxidase activity and gametophytic incompatibility: bud-pollination in Petunia hybrida.* Caryologia, 42: 225-234.

CARRARO L., LOMBARDO G. and GEROLA F.M., 1985. — *Electron-cytochemical localization of peroxidase in self- and cross-pollinated styles of Primula acaulis.* Caryologia, 38: 83-94.

—, 1986. — *Stylar peroxidase and incompatibility reactions in Petunia hybrida.* J. Cell Sci., 82: 1-10.

DARWIN C., 1877. — *The different forms of the plants of the same species* . John Murray, London.

GANDERS F.R., 1979. — *The biology of heterostyly*. New Zealand J. Bot., 17: 607-635.

HESLOP-HARRISON Y., HESLOP-HARRISON J., SHIVANNA K.R., 1981. — *Heterostyly in Primula. 1. Fine-structural and cytochemical features of the stigma and style in Primula vulgaris Huds.* Protoplasma, 107: 171-187.

MARTIN F.W., 1959. — *Staining and observing pollen tubes in style by means of fluorescence*. Stain Techn., 34: 125:128.

PANDEY K.K., 1967. — *Origin of genetic variability: combination of peroxidase isoenzymes determine multiple allelism of the S-gene.* Nature, 18: 669-672.

SHIVANNA K.R., HESLOP-HARRISON J. and HESLOP-HARRISON Y., 1981. — *Heterostyly in Primula. 2. Sites of pollen inhibition, and effects of pistil constituents on compatible and incompatible pollen-tube growth.* Protoplasma, 107: 319-337.

STEVENS V.A.M. and MURRAY B.G., 1982. — *Studies on heteromorphic self-incompatibility systems: physiological aspects of the incompatibility system of Primula obconica.* Theor. Appl. Genet., 61: 245-256.

WEDDERBURN F. and RICHARDS A.J., 1990. — *Variation in within-morph incompatibility inhibition sites in heteromorphic Primula L.* New Phytol., 116: 149-162.

Received 7 May 1996; accepted 26 July 1996