POLLEN–STIGMA INTERACTIONS IN *BRASSICA OLERACEA*

I. ULTRASTRUCTURE AND PHYSIOLOGY OF THE STIGMATIC PAPILLAR CELLS

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SUMMARY

The osmotic potential ($\psi_0$) of the stigmatic papillar cells of *Brassica oleracea* is $-14.8$ bars. In laboratory conditions each cell transpires water at rates within the range from $3 \times 10^{-5}$ to $5 \times 10^{-5} \text{mm}^3\text{h}^{-1}$. A small increase in transpiration rate is detected following cross-(compatible) but not self-(incompatible)pollination. No significant changes in $\psi_0$ occur following pollinations of either compatibility.

Electron microscopy reveals an active papillar cytoplasm apparently secreting proteins into the cell wall via small vesicles. The cuticle is discontinuous and freeze-fracture techniques indicate that channels transverse the cell wall, suggesting a possible pathway for the movement of protein molecules of high molecular weight from the cytoplasm to the stigma surface.

Analysis of electron-microscopic autoradiographs of mature, self-incompatible papillae following pulse-chase experiments with L-[3H]leucine and treatment with cycloheximide shows that protein molecules secreted into the cell wall may return to the cytoplasm at a later stage. The significance of these results is discussed in terms of current models of the pollen–stigma interaction in *Brassica*.

INTRODUCTION

Although the passage of water through the surface of the stigmatic papillar cells and into the pollen grain is critical for pollen germination in species with stigmas of the 'dry' type (Heslop-Harrison & Shivanna, 1977; Heslop-Harrison, 1979) and, indeed, a major factor in the self-incompatibility (S.I.) response in *Brassica* appears to be the denial of stigmatic water to self pollen (Roberts, Stead, Ockendon & Dickinson, 1980; Ferrari et al. 1983), little is known about the osmotic or transpiratory characteristics of these cells. Further, since the discovery of the superficial nature of the S.I. rejection response in *Brassica* (Kroh, 1964; Ockendon, 1972), and the identification of a proteinaceous pellicle investing the stigma surface in species with dry stigmas (Mattsson, Knox, Heslop-Harrison & Heslop-Harrison, 1974), it has tacitly been assumed that the stigmatic molecules responsible for the recognition of self pollen simply pass from sites of synthesis in the cytoplasm out on to the stigma surface, remaining there until pollination, whereupon they perform their cognitive function. Some evidence that a more dynamic situation obtains at the stigma surface was presented by Stead,

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Roberts & Dickinson (1980). During investigations into the rôle of stigma surface proteins in pollen grain adhesion it was found that the loss of adhesion detected after protease treatment could rapidly be recovered. Experiments with cycloheximide indicated that surface proteins were cycled in the pellicle over a period of approximately 2 h. Confirmation of this finding would be of considerable significance to any hypothesis of the mechanism of S.I.

There is also strong evidence that the stigmatic molecules involved in pollen adhesion and S.I. are glycoproteins of 60 000 to 70 000 $M_r$ (Stead et al. 1980; Ferrari, Bruns & Wallace, 1981a; Nishio & Hinata, 1982) and it is important to establish the manner of secretion of these macromolecules through the cellulosic stigmatic cell wall. Clearly, molecules of much higher $M_r$ do pass freely through the cell wall in a number of secretory systems (e.g. the secretion of extracellular polysaccharides of $M_r > 100 000$ by sycamore cells in culture (Bauer, Talmadge, Keegstra & Albersheim, 1973), the secretion of enzymes by leaf glands of insectivorous plants (Heslop-Harrison & Knox, 1971), and the secretion of high $M_r$ glycoproteins by nectaries (Kristen, 1978)), but it has not been shown whether in *Brassica* this results from a generally larger pore size, or from a small number of specialized channels.

More information is needed about the ultrastructure and physiology of the stigmatic papillae, and the secretion of the recognition molecules, before a complete picture of their rôle in the S.I. response can be obtained. In this paper we present the results of attempts to understand more fully the osmotic and transpiratory characteristics of these cells. We also report ultrastructural observations of stigmatic papillae, including an electron-microscope autoradiographic study in which stigmas were supplied with L-[3H]leucine and the movement of proteins was examined following treatment with cycloheximide to inhibit synthesis and, hence, secretion of the pellicle proteins.

**Materials and Methods**

**Plant material**

Seeds of *Brassica oleracea* of known S-genotype and of a variety not requiring vernalization for flowering were kindly supplied by Dr D. J. Ockenden (N. V. R. S., Wellesbourne, Warwicks, U. K.). Plants were grown in a heated greenhouse until the first flower buds were produced, whereupon they were transferred to a growth chamber at 15°C and 70% relative humidity (R.H.) for the duration of the flowering period.

**Osmotic potential and transpiration experiments**

The osmotic potential of the papillar cells was estimated for unpollinated, cross-pollinated and self-pollinated stigmas by a simple density gradient method (Ross, 1974) and also by the method of incipient plasmolysis (Sutcliffe, 1974). In the former method excised stigmas were incubated for 1 h at 20°C in sucrose solutions of various molarities (0-1, 0-2, 0-3, ..., 1-0 m) and then placed at the top of a graduated cylinder containing a sucrose density gradient and released. After 60 s the position in the column to which the stigmas had fallen was recorded and plotted against the molarity of the original incubating solution. The point of intersection of the two curves thus produced was read off the graph and used to estimate $\psi_0$ values of the stigmatic papillar cells. In the latter method entire excised stigmas, and segments and slices of stigmas cut using a botanical razor, were incubated for various times at various molarities of sucrose, sorbitol and polyethylene glycol. Incipient plasmolysis was defined as the point at which 50% of the papillar cells exhibited plasmolysis when scored under the light microscope.
For the transpiration experiments the ovary, style and stigma were excised from freshly cut flowers by cutting through the base of the ovary using a new razor blade. The excised pistils were then inserted into a transpirometer assembly made by drawing a capillary tube from a Pasteur pipette. The position of the water meniscus in the capillary was recorded at half-hour intervals and the effect of applying cross and self pollen to the stigma was investigated. Pollen was loaded onto the stigma direct from freshly dehisced anthers and brushed gently to produce an even layer of pollen over the entire stigma surface, as judged by viewing under a stereomicroscope (Wild M8). Crossed, selfed and unpollinated pistils were left side-by-side on a laboratory bench at 20–22°C and protected from excessive movement of air during the course of the experiment. The volume of water transpired by each pistil was measured and plotted against time, and the rate of transpiration was determined. As a control lanolin was applied to the papillae of transpiring stigmas in place of cross or self pollen. Care was taken not to disturb the position of the meniscus during the application of pollen or lanolin.

**Electron microscopy (EM)**

Excised stigmas were fixed for 4 h at 4°C in Karnovsky’s fixative and rinsed overnight with three changes of phosphate buffer (0.03 M, pH 7.2) at 4°C. Following osmication for 3 h in 1% aqueous osmium tetroxide the material was dehydrated through an acetone series and embedded in Epon 812, using epoxypropane as carrier. Gold sections were stained with uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Jeol 100B TEM operating at 60 kV.

**Preparation for freeze-fracture experiments**

Freshly excised stigmas were rapidly frozen in liquid nitrogen ‘slush’ and fractured in a Polaron freeze-fracture device. The fracture face was coated with a carbon film and shadowed with platinum. The replicas thus obtained were floated off onto water and cleaned using increasing concentrations of chromic acid. After 24 h in 100% chromic acid, replicas were gradually returned to water via serial dilutions of the acid. Replicas were picked up on 200 mesh gold grids and examined in the TEM.

**EM autoradiography**

For feeding with L-[3H]leucine the ovary, style and stigma were excised by cutting through the base of the ovary using a new razor blade. The cut end was immersed in modified White’s medium by insertion through a hole in parafilm covering a small plastic vial, which contained the medium. L-[3H]leucine (Amersham, U.K.) was added to the medium. This amino acid was chosen because it has been shown to be a major component of stigmatic glycoproteins involved in SI (Ferrari et al. 1981a; Nishio & Hinata, 1982) and was therefore particularly suitable for the present study. Feeding rates of between 35 and 350 μCi/ml for 4–8 h were tested. After feeding pistils were chased for 16 h in modified White’s medium containing 10 μg/ml cold leucine and then cross and self-pollinated. All the stigmas tested were found to be capable of rejecting self pollen and accepting cross pollen.

Pistils fed for 8 h and chased for 16 h were transferred to vials containing chase medium to which had been added cycloheximide (2 × 10⁻⁴ M, Sigma U.K.) and maintained for 2 h and 4 h alongside controls, which were simply left for a further 2 h and 4 h in the original chase medium, and not exposed to cycloheximide. The stigmas from each treatment were then excised, fixed and prepared for electron microscopy as described previously. Gold sections on 200 mesh gold grids were coated with emulsion using Ilford L4 nuclear emulsion as described by Porter, Bird & Dickinson (1982). After exposure for 10 weeks at 4°C the autoradiographs were developed in Kodak D19 and unstained sections were examined in the TEM.

**Analysis of EM autoradiographs**

Silver grains were scored as associated with vacuole, cytoplasm or cell wall. In excess of 400 grains were scored for each treatment over a number of different sections. Relative areas occupied by vacuole, cytoplasm and cell wall were estimated from the photographs. Background radioactivity was found to be negligible. The observed number of grains relative to the expected number assuming a random distribution of radioactivity in the cells gave an estimate of the relative specific activity (R.S.A.) of each part of the cell. The difference in R.S.A. between treatment and control was
plotted as a percentage of the control R.S.A. to give a measure of the effect of cycloheximide on the distribution of labelled proteins.

The statistical significance of the results was tested using $\chi^2$ as applied to autoradiographs by Callow & Evans (1978) and also using the $2 \times 2$ contingency table (Bishop, 1966).

RESULTS

Osmotic potential and rate of transpiration of the stigmatic papillae

The osmotic potential ($\psi_m$) of the stigmatic papillae was found to be $-14.8$ bars ($1 \text{ bar} = 10^{5}\text{Pa}$) in all stigmas examined, independent of the method used. No change in $\psi_m$ was detected following cross and self-pollination. The rate of transpiration in laboratory conditions was estimated to be between $0.18$ and $0.32 \text{ mm}^3\text{ h}^{-1}\text{ stigma}^{-1}$. Since there are approximately 6000 papillae per stigma (Roberts, unpublished), and assuming no differences in transpiration between individual papillae, this gives a range of $3 \times 10^{-5}\text{ mm}^3$ to $5 \times 10^{-5}\text{ mm}^3$ of water transpired by each papilla per hour. Following cross-pollination a very slight increase in transpiration rate was detected but no differences were found after self-pollination (Fig. 1A, B). Lanolin applied to stigmatic papillae prevented further transpiration (Fig. 1c).

![Fig. 1](chart.png)

Fig. 1. The effect on stigma transpiration of: A, cross-pollination; B, self-pollination; and C, smearing the stigmatic papillae with lanolin.

![Fig. 2](image.png)

Fig. 2. A. Papillar cell cytoplasm showing aggregations of rough endoplasmic reticulum (rer). A mitochondrion (m) is also visible. $\times 42320$. B. A dictyosome (d) and associated vesicles (arrows) in papillar cell cytoplasm of Brassica. $\times 76500$. C. Periphery of the papillar cell cytoplasm showing the highly active plasma membrane (pm) and numerous cytoplasmic vesicles (arrows). $\times 50000$. 

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Stigmatic papillae of *B. oleracea*
Fig. 3
Stigmatic papillae of *B. oleracea*

Fig. 4. A. Autoradiograph of papillar cell previously labelled with L-[3H]leucine. Silver grains are visible over the cytoplasm (cy). ×37320. B. Autoradiograph of papillar cell previously labelled with L-[3H]leucine. Silver grains are visible associated both with the cytoplasm (cy) and cell wall (w). ×21200. C. Material as depicted in B, but following a 2 h exposure to cycloheximide. Silver grains are visibly associated with the cytoplasm (cy) and vacuole (v). ×20120.

Fig. 3. A. Vertical section through the outer surface of the papillar cell wall (w). The discontinuous cuticle (c) is clearly evident as is a thin superficial layer (arrows), probably the pellicle. ×71250. B. Tangential section through the papillar wall displaying the particulate nature of the cuticle (arrows). The surface of the papillar cytoplasm (cy) is just discernible. ×11750. C. Freeze-fracture replica of papillar cell showing the cell wall (w), cytoplasmic organelles (arrows), the tonoplast (t) and vacuole (v). ×2760. D. Freeze-fracture replica of papillary cell wall showing a series of channels (arrows) passing from the cytoplasm (cy) to the cell surface (s). ×20800.
**Ultrastructure of the stigmatic papillae**

Electron microscopy reveals an active cytoplasm with dictyosomes budding off small vesicles apparently destined for secretion into the cell wall (Fig. 2A, B, C). The discontinuous nature of the cuticle is shown particularly well in tangential section (Fig. 3A); a thin outer pellicle that stains for esterase activity (unpublished results) is also visible (Fig. 3A). Freeze-fracture replicas of the papillar cells indicate the existence of channels from 50 nm in diameter traversing the cell wall (Fig. 3A, D).

**The effect of cycloheximide on protein distribution in radioactively labelled stigmatic papillae**

Electron-microscope autoradiography of papillae fed with L-[³H]leucine resulted in

![Graph showing the effect of cycloheximide](image)

Fig. 5. The effect of exposure to cycloheximide for 2 h and 4 h on the distribution of radioactively labelled proteins in stigmatic papillae. Open histograms represent the percentage change in R.S.A. of the vacuole relative to untreated controls; stippled histograms represent changes in R.S.A. of the cytoplasm; and shaded histograms represent changes in R.S.A. of the cell wall. The reduction in R.S.A. of controls left for 4 h in chase medium in the absence of cycloheximide relative to similar controls left for 2 h is also presented. In each case the results were statistically significant at \( P < 0.001 \).
Stigmatic papillae of B. oleracea

the formation of silver grains clearly associated with either vacuole, cytoplasm or cell wall (Fig. 4A, b, c). After 2 h exposure to cycloheximide the R.S.A. of the cell wall was reduced by some 33 % relative to the control (Fig. 5). Over the same period the R.S.A. of the vacuole decreased by 5 %, while that of the cytoplasm increased by 4 % relative to controls. A similar pattern of response was detected after 4 h exposure to cycloheximide although the decrease in the R.S.A. of the cell wall was less pronounced, being only some 12 %. The R.S.A. of untreated stigmas maintained in chase medium was less after 2 h than the corresponding controls left for 2 h in chase medium. The R.S.A. of vacuole, cytoplasm and cell wall decreased by 3 %, 8 % and 10 %, respectively, during this time.

DISCUSSION

Osmotic and transpiratory characteristics of the stigmatic papillae

The value obtained for the osmotic potential of papillar cells of −14.8 bars is fairly low (i.e. a high ψw value) but well within the range found in plant cells. Such a value may well be important in regulating the flow of water to the pollen, since a slow rate of water uptake has been shown to be critical for the germination of trinucleate pollens (Bar Shalom & Mattsson, 1977). The fact that no difference in osmotic potential was detected following cross and self-pollination, or indeed between stigmas of different S-genotypes (unpublished results), suggests that the S.I. system in Brassica does not operate via osmotic differences, as it may do in the heterostylic S.I. system of Linum grandiflorum (Lewis, 1943). Neither were we able to detect any difference in the rate of plasmolysis of stigmas treated with extracts of cross and self pollen, indicating that whole pollen is necessary to stimulate the barrier to water passage observed following self-pollination (Roberts et al. 1980).

The rate of transpiration as determined by our very simple technique is approximately equivalent to each cell losing its own volume of water every hour. This may be thought to be a very high rate of transpiration and may be due to the fact that excised pistils were used in the experiments and left completely exposed to the atmosphere without the protection normally afforded by the rest of the flower. The ready supply of water from the transpirometer would also serve to keep the papillae fully turgid and result in a low cuticle resistance to water passage, according to the hypothesis of Mattsson et al. (1974). Nevertheless, we believe that the results obtained give a reasonable estimate of the hydraulic conductivity of stigmatic papillar cells. Experiments to measure the water potential difference between pollen and stigma, and hence to compare the hydraulic conductivity of papillar cells with the values determined for the hydraulic conductivity of epidermal cells of Tradescantia using a pressure probe technique (Tomos, Steudle, Zimmerman & Schulze, 1981), are currently in progress, as are experiments to determine whether water flow to the grain is mainly apoplastic or mainly symplastic. We have yet to find evidence of Casparian bands in papillar cell walls, and should water move to the grain via an apoplastic pathway the papillar plasmamembrane would be eliminated as a possible
site for regulation of flow and the hydraulic conductivity of the symplasm would of course be irrelevant to the study of pollen--stigma interactions.

It has also been estimated that pollen grains of *Brassica* increase in volume by some $2 \times 10^{-6}$ mm$^3$ during re-hydration (Stead, Roberts & Dickinson, 1979). If we assume that the pollen grain is in contact with the papilla over one tenth of the papillar surface, and that water may move into the pollen grain at the same rate that water evaporates from the surface of an unpollinated papillae, then this gives a value of $3 \times 10^{-6}$ to $5 \times 10^{-6}$ mm$^3$ of water passing to the grain every hour. This is sufficient to account for the observed increase in pollen volume in less than 1 h. However, pollen on the stigma normally takes between 1 h and 6 h to achieve full rehydration (Roberts *et al.* 1980; Ferrari *et al.* 1983). Allowing for evaporation from the pollen (*Brassica* pollen can both take up and lose water from the atmosphere very rapidly; Stead *et al.* 1979; Ferrari *et al.* 1983), and possibly a smaller area of contact than the one tenth of papillar surface assumed, our estimates of transpiration accord well with the published rates of grain rehydration on the stigma. This suggests that sufficient water is available at that stigma surface for pollen to hydrate without any prior modification of the stigma surface being necessary.

Our results do, however, indicate that some modification of the stigma surface takes place immediately following cross-pollination as a new, slightly higher rate of transpiration is detected, whereas no changes in rate occur following self-pollination. At first sight these results might be thought to be completely opposite to that predicted by our model (Dickinson & Roberts, 1983). This can be explained in two ways. Either the barrier preventing water flow to self pollen is very localized and does not significantly effect transpiration or, alternatively, it may be that self pollen is taking up water from the stigma but simultaneously transpiring at the same rate because it is rendered incapable of using this water for rehydration. Cross pollen is undoubtedly taking up water from the stigma and this rapid uptake, coupled with increased evaporatory loss from the pollen (the pollen effectively increases the area on the stigma available for water loss), would account for the sudden increase in transpiration rate. Digestion of the stigmatic cuticle could result in a similar effect but would not be expected to be immediate. We may conclude that since an adequate supply of water is potentially available for pollen of each compatibility, and since water is the sole activator of germination (Ferrari *et al.* 1983), the hypothesis that self pollen is positively recognized and actively rejected, rather than the S.I. system acting to favour cross-pollen positively, is strengthened by these findings.

**Pathway of secretion of pellicle proteins**

Although proven only for animal cells all our data are in agreement with the hypothesis that secretion of proteins proceeds via a route beginning with synthesis in the endoplasmic reticulum, followed by processing in the dictyosomes, which produce small vesicles destined for fusion with the plasma membrane and secretion of their contents into the cell wall. A possible pathway for passage of high $M_r$ molecules from the plasma membrane to the superficial pellicle is indicated by our discovery of small channels traversing the cell wall. Similar channels have been
Stigmatic papillae of B. oleracea observed in the stigmatic papillae of *Gladiolus* (Clarke, Abbot, Mandel & Pettitt, 1980). Such structures may therefore be common to all stigmas of the dry type, and essential for the production and maintenance of an active superficial proteinaceous pellicle (Mattsson et al. 1974). The discontinuous nature of the cuticle suggests that it would not be a major barrier to the passage of proteins and indeed pollen proteins pass readily through such stigmatic cuticles in the Gramineae (Vithanage & Heslop-Harrison, 1979).

Cycling of surface proteins

The large reduction in protein levels in the cell wall relative to other smaller changes in protein distribution in vacuole and cytoplasm after 2 h exposure to cycloheximide indicates that proteins secreted into the wall may return to the cytoplasm at a later stage. This finding apparently confirms previous results suggesting that secreted proteins may cycle between cell surface and cytoplasm (Stead et al. 1980). The alternative hypothesis, that secreted proteins denature on the surface and are sloughed off every few hours, would be extremely wasteful and seems somewhat unlikely. It is also unlikely that the levels of radioactivity used have in any way affected protein distribution as stigmas were still functionally active in terms of their ability to distinguish between cross and self pollen.

The time-scale of protein loss from the wall is also in good agreement with the time taken for pollen adhesion to be lost following cycloheximide treatment (Stead et al. 1980). Curiously, the flagellar adhesion proteins of *Chlamydomonas* take an almost identical period of time to turn over (Snell & Moore, 1980). After 4 h of cycloheximide treatment a similar pattern of response to that observed after 2 h is revealed. The fact that the reduction in cell wall R.S.A. is not as large after 4 h as after 2 h (12% compared to 33%) could be taken to indicate that protein is being added to the wall from a presynthesized pool not normally available. It is perhaps more likely that after 4 h exposure to cycloheximide the cells have become irreversibly damaged and a loss of membrane integrity has resulted in a net leakage of protein into the wall.

The existence of a pathway for the return of secreted proteins from cell to cytoplasm is unexpected as plant cells do not generally seem to endocytose macromolecules. It may be that proteases in the cell wall are able to break down the secreted macromolecules for resorption at the papillar plasma membrane. Indeed, it is tempting to speculate that proteases released from the pollen grain significantly affect cell wall dynamics and add another dimension to the pollen–stigma interaction. In any event endocytosis of high *M*<sub>c</sub> molecules has also been shown to occur in response to challenge by fungal pathogens (Albersheim et al. 1981). The host–pathogen interaction is in many ways similar to the pollen–stigma interaction (Ferrari, Lee & Wallace, 1981b; Hogenboom, 1983) and thus our results point to another comparable feature; that is to say, the dynamic rôle played by the cell wall. The rôle of the cell wall in such intercellular communication systems will be discussed further in a subsequent publication.

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REFERENCES


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