PEROXIDASES IN THE CELL WALLS AND INTERCELLULAR SUBSTANCE OF POLLINATED NICOTIANA ALATA STYLES

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SUMMARY

In unpollinated *Nicotiana alata* styles peroxidase activity was relatively high in the epidermis, the vascular system and the transmitting tissue. Especially the cell walls of these tissues contained considerable amounts of peroxidase. In the transmitting tissue the peroxidase activity was also high in the cytoplasm and in the intercellular substance. Extraction of the cell walls by means of a vacuum infiltration method revealed the presence of four peroxidase isoenzymes in the walls of cortex including vascular traces.

After pollination the activity of several cortical peroxidase isoenzymes increased in both cytoplasmic and wall fractions. Generally, the amounts of the peroxidase isoenzymes extracted from walls of cross-pollinated styles exceeded those extracted from self-pollinated styles. In pollinated styles the peroxidase positive intercellular substance of the transmitting tissue was replaced by the pollen tubes. The possible role of peroxidase in the intercellular substance is discussed in relation to the physiological function of the transmitting tissue.

1. INTRODUCTION

In pistils with a solid style, the pollen tubes grow through the intercellular substance of the transmitting tissue by dissolving the pectins enzymatically (for review see LINSKENS & KROH 1970). The release and action of pollen enzymes in the style is well known (STANLEY 1964; STANLEY & LINSKENS 1965; MAEKINEN & BREWBAKER 1967; KROH & LOEWUS 1978). These enzymes may serve to metabolize external substrates for nutrition of the elongating tubes. Sugars and amino acids are taken up from the style by the pollen tubes and are possibly metabolized by the tubes (LINSKENS & ESSER 1959; KROH et al. 1970).

The release of enzymes from stylar cells starts already prior to anthesis independent of pollination. HERRERO & DICKINSON (1979) demonstrated the presence of acid phosphatase, peroxidase and esterase in the intercellular substance of the transmitting tissue of *Petunia hybrida* styles by a histochemical method. *In situ* extraction of cell walls by vacuum infiltration revealed the presence of a number of peroxidase isoenzymes in the walls and/or intercellular spaces of aged *Nicotiana alata* styles (BREDEMEIJER 1977).

Thus, the enzymes and substrates released from both pollen and style can interact in the intercellular substance giving rise to an exogenous metabolism which may directly influence the pollen tube growth (LINSKENS 1968). The pre-
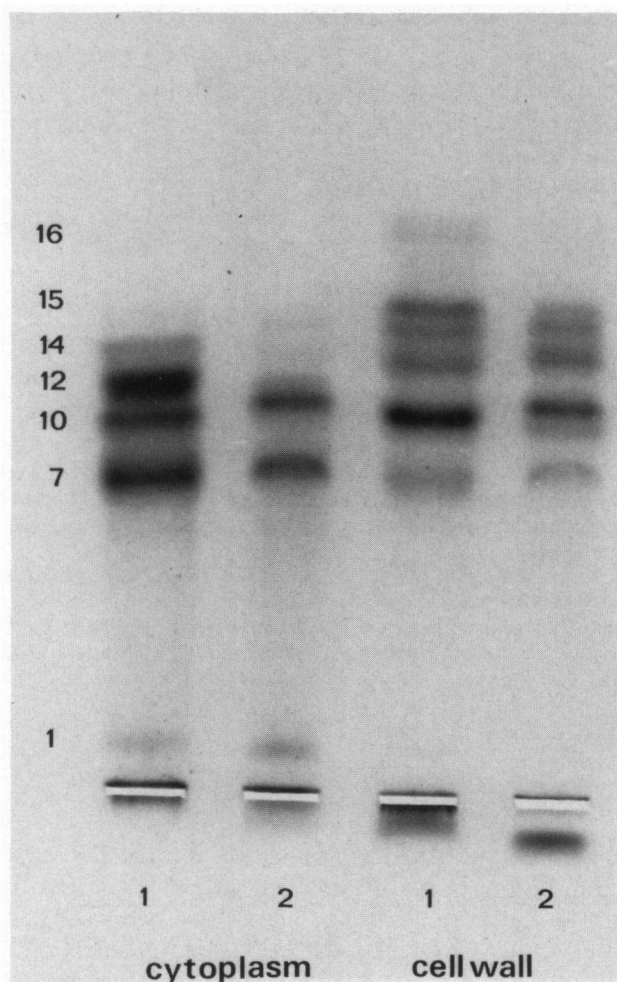
Fig. 1. Peroxidase isoenzymes of the cytoplasmic and wall fractions in styles 4 days after cross-pollination. The wall peroxidases were obtained by centrifugation of stylar segments after vacuum infiltration with 4% NaCl (1) or with 0.02 M phosphate buffer, pH 7.0 (2). The remaining tissue was used to extract the cytoplasmic isoenzymes.

sent study was undertaken with self-incompatible *N. alata* in the frame work on the role of peroxidases in the style, particularly with respect to the exogenous metabolism in the transmitting tissue. This article presents data on the release of stylar peroxidases after pollination and explains the possible role of the peroxidase in the intercellular substance.

2. MATERIAL AND METHODS

The self-incompatible clone OWL (S_2S_3) of *Nicotiana alata* Link and Otto was grown under controlled conditions as described previously (BREDEMEIJER 1974). Flowers were collected at anthesis, emasculated and selfed, or cross-pollinated with clone OB-2 (S_6S_7). After incubating the flowers at 15 °C (16 h illumination at 8,000 lux) the styles were collected and used for *in situ* extraction of wall and intercellular peroxidases (BREDEMEIJER 1977).

The peroxidase isoenzymes were separated by means of starch gel electrophoresis and stained with benzidine in the presence of hydrogen peroxide (BREDEMEIJER 1974). The staining intensity of peroxidase bands was measured by scanning photographs of the gels with a Vitatron Manual TLD 100 densitometer. Pollen tube length was determined as described previously (BREDEMEIJER & BLAAS 1975).

The cytochemical localization of peroxidase was carried out according to the procedure reported by RAA (1973). The tissues were prefixed in glutaraldehyde, stained with p-phenylene diamine and hydrogen peroxide and embedded in Epon before slicing.

3. RESULTS

3.1. Free and ionically bound wall peroxidase isoenzymes

The *in situ* extraction of the stylar cell walls has been carried out with a low molarity infiltration fluid (0.02 M phosphate buffer, pH 7.0) and with a high molarity fluid (4% NaCl). With the former infiltration solution the free peroxidases were extracted from cell walls and intercellular spaces, while with the latter the ionically bound peroxidases were also extracted. Comparison of the results obtained with the two infiltration fluids revealed mainly quantitative differences (*fig. 1*). The amounts of the peroxidase isoenzymes P-12 and P-14 in the low molarity fluid were the same as those in the high molarity fluid, while the amounts of P-10, P-15 and P-16 were greater in the latter. Apparently, the wall P-12 and P-14 occurred as freee enzymes, whereas certain proportions of P-10, P-15 and P-16 were ionically bound in addition to their occurrence as free enzymes. In control series only traces of peroxidase isoenzymes were detected in the fluid obtained after centrifugation of noninfiltrated styles.

The cytoplasmic fractions of P-12 and P-14 were ionically bound, while the other isoenzymes occurred as free enzymes (*fig. 1*).

3.2. Wall localized peroxidase isoenzymes in self- and cross-pollinated styles

The intercellular fluid obtained by vacuum infiltration of unpollinated styles with the high molarity fluid (4% NaCl) contained small amounts of P-6, P-7, P-12 and P-15 and a great amount of P-16. After pollination the activity of P-7, P-12, P-15 and of a new isoenzyme P-10 extracted from the walls increased, while the activity of P-16 decreased. The peroxidase isoenzyme patterns 4 days
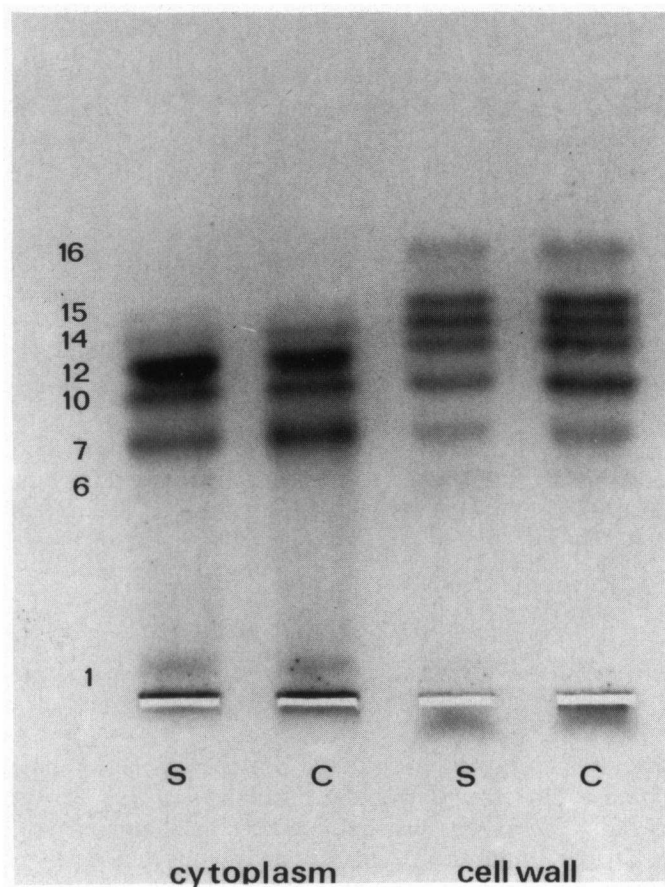
Fig. 2. Peroxidase isoenzymes of the cytoplasmic and wall fractions in self- (S) and cross-pollinated (C) styles 4 days after pollination.

after pollination show that the wall fraction contained almost all the isoenzymes detected in the cytoplasmic fraction (fig. 2). Further, the relative amounts of the various peroxidase isoenzymes extracted from the walls differed from one isoenzyme to the other and were generally higher in cross-pollinated styles than in selfed styles.

The induction of P-10 and its release from cytoplasm to cell walls was studied in detail. Unpollinated styles did not contain P-10. In pollinated styles this isoenzyme was induced just below the stigma within one day after pollination and thereafter extended to the basal end of the style (BREDEMEIJER & BLAAS 1975). The release of P-10 to the walls also started within one day after pollination in the topmost part of the style. The increase in total P-10 activity in self-pollinated styles continued during the period of 6 days investigated (table 1). However,

Table 1. Relative amounts of peroxidase isoenzyme P-10 extracted by vacuum infiltration with 4% NaCl from cell walls in segment 15–30 mm of self-pollinated styles. Data are averages of 3 experiments.

Days after pollination	Total P-10 activity	Cell wall P-10 activity	% of total P-10	Pollen tube length (mm)
2	23370	3435	14.7	9.8
3	44360	4040	9.1	14.0
4	85020	7060	8.3	21.0
6	107310	5795	5.4	28.6

the maximum P-10 activity extracted from the walls was already reached after 4 days. At 2 days after pollination the wall P-10 activity amounted to 14.7% of the total P-10 activity in stylar part 15–30 mm from the stigma. During this period pollen tubes have not yet entered this part of the style (*table 1*). One day after pollination P-10 activity was still too low to have a reliable estimation.

3.3. Histochemical localization of peroxidase
In mature styles peroxidase activity was high in the epidermis, the vascular system and the transmitting tissue, but was low in the cortex. Generally, the cell walls of all these tissues exhibited a high peroxidase activity, especially in the
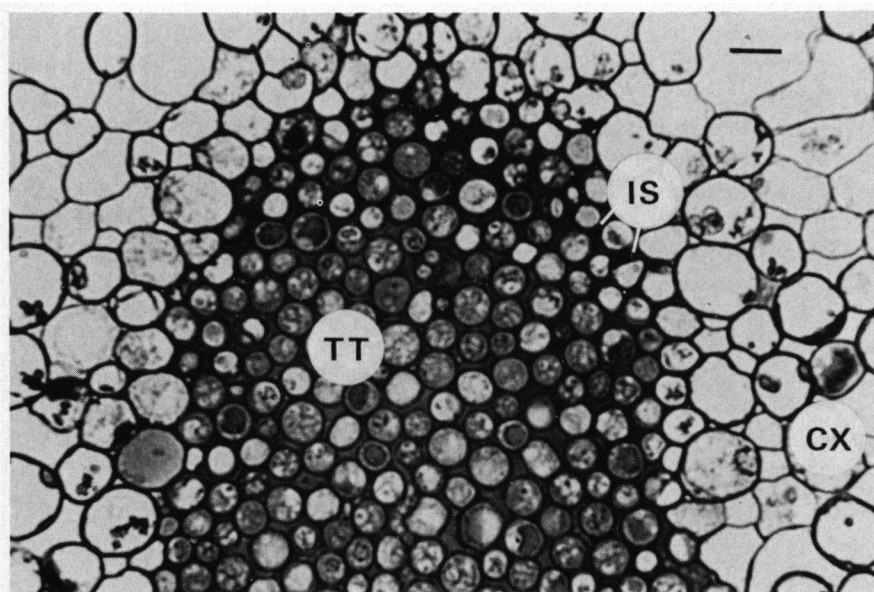

Fig. 3. Peroxidase localization in the transmitting tissue (TT) and enveloping cortex (CX) of an unpollinated style. Note strong peroxidase activity in the intercellular substance (IS) of the transmitting tissue. Transverse section was made of the lower region of the style. Scale bar = 10 μm in each picture. Unstained control sections were hardly visible under the light microscope.
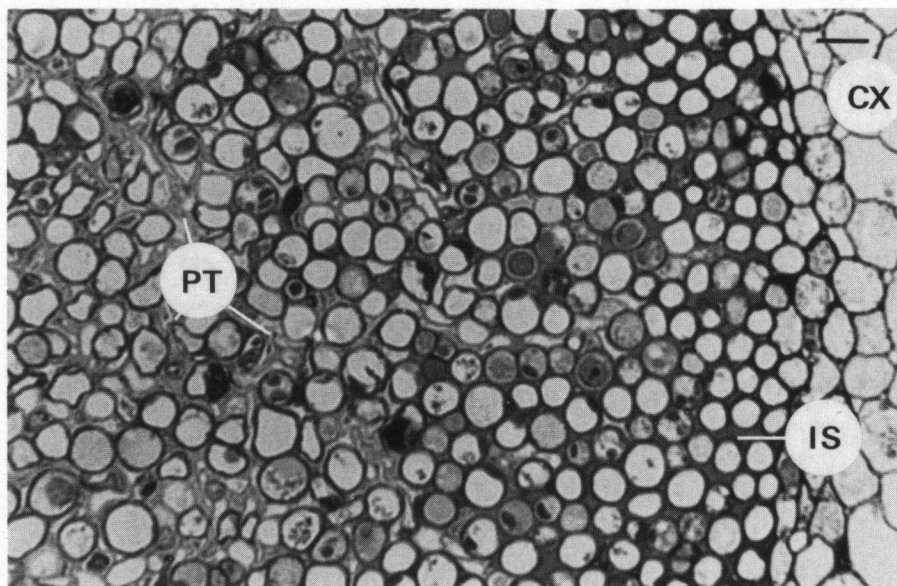
Fig. 4. Distribution of peroxidase in the transmitting tissue 4 days after compatible pollination. Transverse section of the upper region of the style. Many strongly flattened pollen tubes (PT) had almost completely replaced the peroxidase-positive intercellular substance (IS).

transition zone between cortex and transmitting tissue. In the latter tissue cytoplasm, cell walls and intercellular substance showed intense peroxidase staining (*fig. 3*).

The sites of peroxidase localization 4 days after pollination were similar to those in unpollinated styles. However, the peroxidase activity in cell walls and cytoplasm of the cortex and vascular traces was higher in pollinated styles. Moreover, the transmitting tissue contained darkly stained spots, which seem to occur at higher frequency after selfing than after cross-pollination. At sites with a high number of pollen tubes the peroxidase positive intercellular substance was almost completely replaced by the tubes (*fig. 4*). The sections of the pollen tubes revealed cylindrical appearance or flattening for apical region and remaining portion (see DE NETTANCOURT et al. 1973). The data presented in *fig. 5* clearly demonstrate that the wall of the pollen tube is bipartite with a peroxidase positive outer layer (pecto-cellulosic wall) and a peroxidase negative inner layer (callosic wall). The cytoplasm of the tubes exhibited intense peroxidase staining which is, however, hardly visible in strongly flattened tubes.

4. DISCUSSION

4.1. Peroxidase in the cortex
The *in situ* wall extraction of mature styles revealed the presence of one major peroxidase isoenzyme (P-16) and four minor ones (P-6, P-7, P-12 and P-15) in

the cell walls. These isoenzymes except P-12 were localized in the cortex and/or vascular traces (BREDEMEIJER 1979). After pollination, the activity of P-16 in cytoplasm and wall fraction decreased, while the activities of P-7 and P-15 as well as that of pollination-induced P-10 increased. At 4 days after pollination the activity of the peroxidase isoenzymes extracted from cell walls was higher in cross-pollinated styles than that in self-pollinated styles. Possibly, the increased degeneration of parenchymatous cells following the passage of compatible pollen tubes (HERRERO & DICKINSON 1979) was accompanied by an increase in permeability of the cell membranes and a consequent increase in peroxidase leakage.

The release of P-10 from cytoplasm to cell walls started immediately after its induction during the first day after pollination. It is therefore very well possible that pollination-induced ethylene not only controls the induction of P-10 (BREDEMEIJER 1982) but also its release. Ethylene control of enzyme secretion has been proposed earlier by others (JONES 1968; ABELES & LEATHER 1971). BIRECKA et al. (1976) reported an increase in the amount of ionically and covalently bound wall peroxidase isoenzymes after ethylene treatment.

It has been proposed that cell wall peroxidases are involved in lignification, hydrogen peroxide formation (MAEDER et al. 1980), tannin formation (see MAEDER et al. 1975a), IAA oxidation and disease resistance (see Maeder et al. 1975b). As concerned the wall peroxidases in the cortex and vascular traces of the style more information on hydrogen donor specificity and localization is needed to establish whether these isoenzymes are involved in one or more of the reactions.

4.2. Peroxidase in the transmitting tissue

It is unlikely that the *in situ* wall extraction technique has given a good indication of the wall associated fraction of the transmitting tissue. Probably, only a very small proportion of the peroxidase was extracted from the walls because the intercellular spaces, in contrast to those of the cortex, contained a viscous substance with only a few "empty spaces" (BELL & HICKS 1976).

The histochemical localization of peroxidase in the transmitting tissue of mature styles revealed a strong activity in cytoplasm, cell walls and intercellular substance (*fig. 3*). This means that the release of peroxidase from cytoplasm to walls and intercellular substance started already in immature styles which is in agreement with the results of HERRERO & DICKINSON (1979). CRESTI et al. (1976) have also reported the release of proteins into the intercellular substance during the last stage of stylar development.

The peroxidase activity in the transmitting tissue can be ascribed mainly to P-12 (BREDEMEIJER 1979). Probably, the pectins in the intercellular substance (KROH 1973; SASSEN 1974) bind the P-12 molecules forming a pectin-peroxidase complex which may be involved in maintaining active enzyme configuration (DE JONG 1967).

Since P-12 could be detected only in the transmitting tissue and not in any other part of the plant (BREDEMEIJER 1979) its role is probably closely correlated
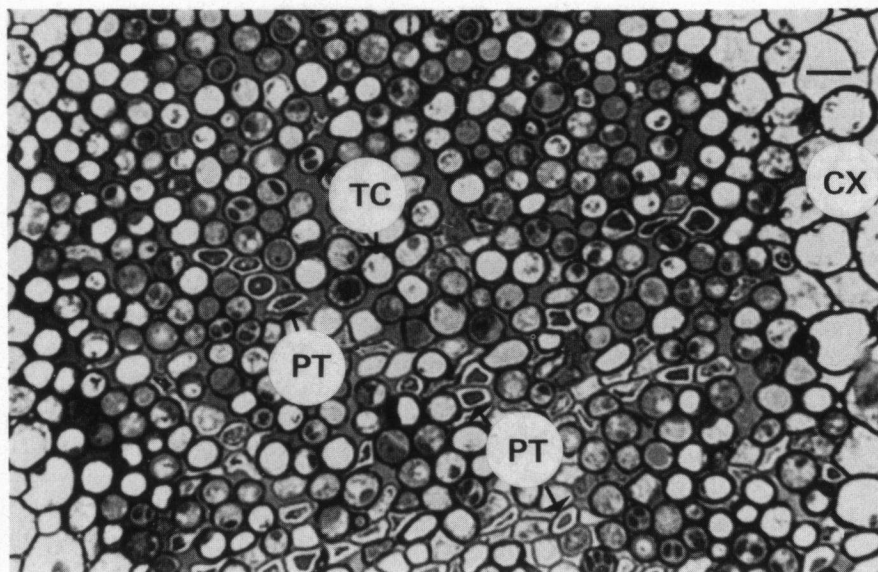
Fig. 5. Distribution of peroxidase in the transmitting tissue 4 days after compatible pollination. Transverse section approximately one third down the stigma. Note the pollen tube wall (PT) with a peroxidase positive outer layer and a peroxidase negative inner layer. TC, transmitting cell; CX, cortex.

with the specific physiological functions of this tissue, i.e. nutrition of the pollen tubes and incompatibility reaction (VASIL 1974; review LINSKENS & KROH 1970). On the one hand, the occurrence of P-12 in the walls and intercellular substance might be incidental and be related to membrane functions as suggested for peroxidases in other plant tissues (DE JONG 1966, 1967). On the other hand, P-12 might have been secreted to perform a specific role in the intercellular substance. Apart from the question whether the presence of P-12 in the intercellular substance is incidental or not, it is clear that this peroxidase isoenzyme might affect pollen tubes very directly. The possibility of a peroxidase-mediated regulation of pollen tube growth has been demonstrated earlier by using *in vitro* cultures (BREDEMEIJER 1975). P-12 in the style might regulate pollen tube growth by modification of nutrients, inactivation of enzymes secreted by the pollen tubes (see also MAEKINEN & BREWBAKER 1967) and oxidation of phenolic compounds which are known to diffuse from the pollen (STANLEY & LINSKENS 1965). A more direct effect of P-12 is possible *via* modification of structural proteins in the pollen tube walls (DASHEK et al. 1970) or *via* increasing the permeability of the tube walls similar to the peroxidase-induced permeability in yeasts (NIEDERMEYER 1975).

The fact that the total amount of P-12 in self- and cross-pollinated styles approximately was the same (BREDEMEIJER 1974; BREDEMEIJER & BLAAS 1975) does not rule out its involvement in rejection of incompatible pollen tubes or nutrition

of compatible tubes. Differences in concentrations of its substrate hydrogen per-oxide, hydrogen donors, activators or inhibitors between self- and cross-pollin-ated styles might cause differences in P-12 activity *in vivo*.

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