European Journal of Plant Pathology **104:** 545–551, 1998.

Formation of wall openings in root cells of *Arabidopsis thaliana* following infection by the plant-parasitic nematode *Heterodera schachtii*

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Accepted 6 April 1998

Key words: Arabidopsis thaliana, cyst nematodes, development, histology, syncytium, ultrastructure

Abstract

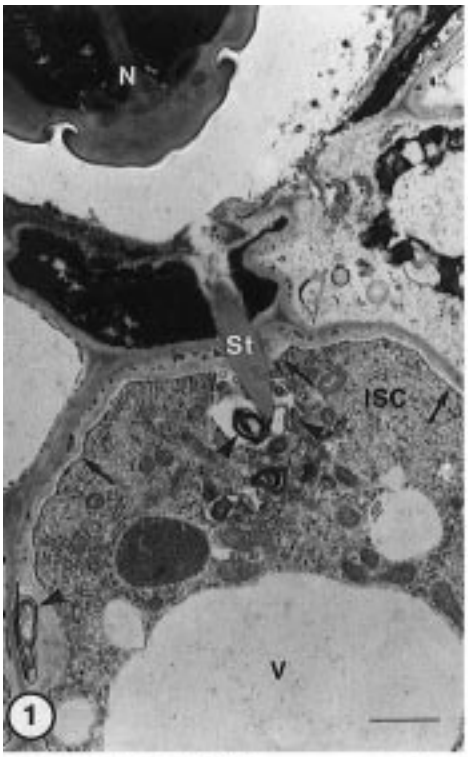
The induction and differentiation of feeding structures (syncytia) of the cyst nematode *Heterodera schachtii* in roots of *Arabidopsis thaliana* is accompanied by drastic cellular modifications. We investigated the formation of cell wall openings which occurred during syncytium differentiation. At the beginning of syncytium induction, a callose-like layer was deposited inside of the wall of the initial syncytial cell (ISC). First wall dissolutions developed by gradual widening of plasmodesmata between the ISC and neighbouring cells. As a general thickening of syncytial cell walls blocked existing plasmodesmata, other large openings were formed by enzymatic dissolution of intact walls by putative cellulase activity.

Abbreviations: ER – endoplasmic reticulum; ISC – initial syncytial cell; J2 – second stage juvenile; J3 = third stage juvenile.

Introduction

Arabidopsis thaliana has proven its suitability for the genetic, molecular biological and physiological analyses of host-parasite interactions (e.g. Dangl, 1993; Meyerowitz and Somerville, 1994). Since *A. thaliana* has been shown to be a good host of some plant-parasitic nematodes (Sijmons et al., 1991), host-parasite interactions with sedentary nematodes were studied. Cyst nematodes invade plant roots and induce a complex feeding structure in the vascular cylinder, which is a syncytium composed of fused cells. The syncytium is the only nutrient source used throughout nematode development which extends of about 8 weeks for females and about 2 weeks for males. Golinowski et al. (1996) analysed the structure of syncytia associated with female juveniles. Starting from an initial syncytial cell (ISC), the syncytium expands along the vascular tissue by continuous integration of new cells by the partial dissolution of their walls. During nematode development, the syncytia assume a specific phenotype such as the absence of a central vacuole, the proliferation of ribosomes, plastids, smooth endoplasmic reticulum and the formation of wall ingrowths at their interface with adjacent xylem vessels. Syncytium induction commences when a juvenile inserts its stylet into an ISC (Wyss, 1992). Secretions from esophageal glands of still unknown composition are thought to contain factors that trigger syncytium formation.

Syncytia associated with females were usually induced in procambial or cambial cells (Golinowski et al., 1996) while syncytia initiated in the pericycle of secondary roots were associated with male development (Sobczak et al., 1997). The development of male juveniles is short because it is only during the first two parasitic stages that nutrients are drawn from the syncytium. The syncytium degrades after active feeding is completed. Therefore, with male development induction and maintenance of the syncytium, its final degradation can be observed under optimal conditions. In this study we have analysed in detail the pathogenesis of root infection and related changes of the cell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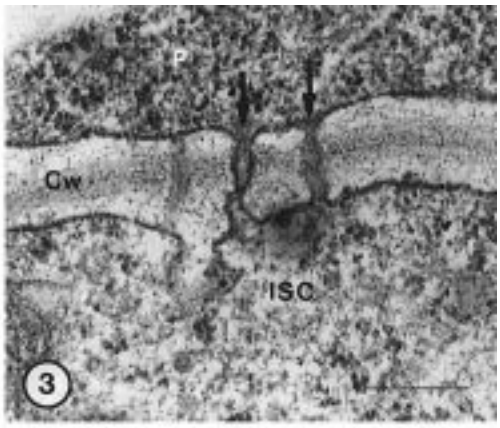
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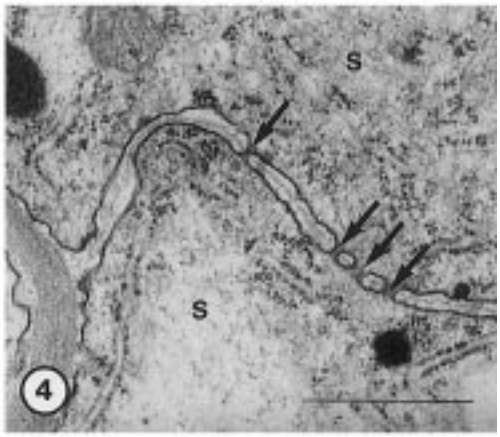
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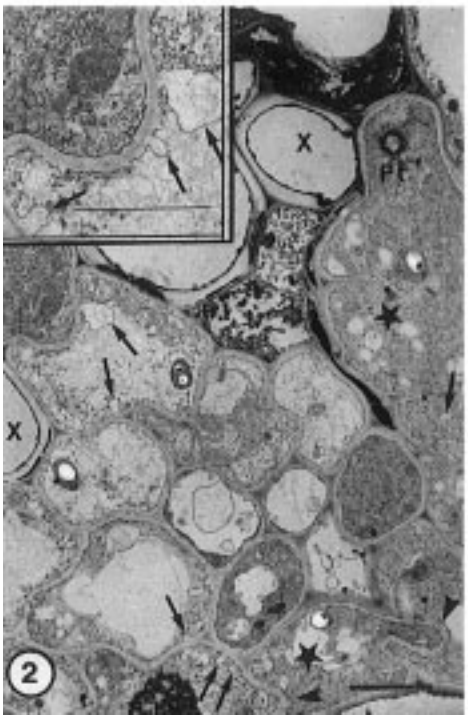
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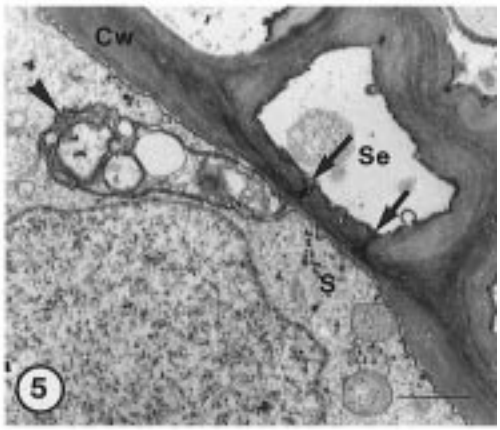
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Figures 1–5.
Figure 1. Second stage juvenile (N) of *Heterodera schachtii* during preparation phase at an initial syncytial cell (ISC) in the pericycle. The nematode stylet (St) is inserted in the ISC. Translucent callose-like material (arrow) is deposited around the tip. Similar depositions are formed at other parts of walls of the selected cell and in neighbouring cells (not shown). Myelin bodies (arrow heads) consisting of membrane fragments are visible at the stylet tip. Bar 1 μm.
Figure 2. A syncytium induced in the pericycle 18h after selection of the ISC. The syncytium is composed of former pericyclic cells (asterisks) connected by cell wall openings (bold arrows). The cell walls of some syncytial elements are bent (curved arrows). Numerous paramural bodies (enlarged insert: arrows) are formed at cell walls of syncytium and neighbouring cells. A feeding tube (Ft) of the associated juvenile is visible in the ISC. Bars 3 μm.
Figure 3. Widened plasmodesmata (arrows) in a cell wall (Cw) of an ISC selected among pericyclic cells (P) 6h after syncytium induction. Bar 0.25 μm.
Figure 4. Cell wall openings (arrows) created by widened plasmodesmata between two distal syncytial elements (S) 24 h after syncytium induction. Bar 1 μm.
Figure 5. Plasmodesmata (arrows) in a syncytial cell wall (Cw) between syncytium (S) of a third stage juvenile (2d after second moult) and a sieve element (Se). Plasmodesmata are closed by deposition of cell wall material originating from the syncytial protoplast. Adjacent to one of them a large paramural body (arrow head) containing internal vesicles appears to bud from the syncytial plasmalemma. Bar 2 μm.

walls and the series of events leading to the formation of the wall openings which are an essential feature of syncytia.

Materials and methods

Plant and nematode culture

Plants of *Arabidopsis thaliana*, ecotype Landsberg erecta, were cultured under conditions as described by Golinowski et al. (1996) and Sobczak et al. (1997) on the medium described by Sijmons et al. (1991). Three-week-old *A. thaliana* plants were inoculated with batches of about 100 freshly hatched *Heterodera schachtii* second-stage juveniles, obtained from sterile agar stock cultures (Grundler 1989). Plants were grown on a thin layer of agar on a glass coverslip and, after inoculation, transferred into an observation chamber as described by Wyss (1992) for *in vivo* inspection.

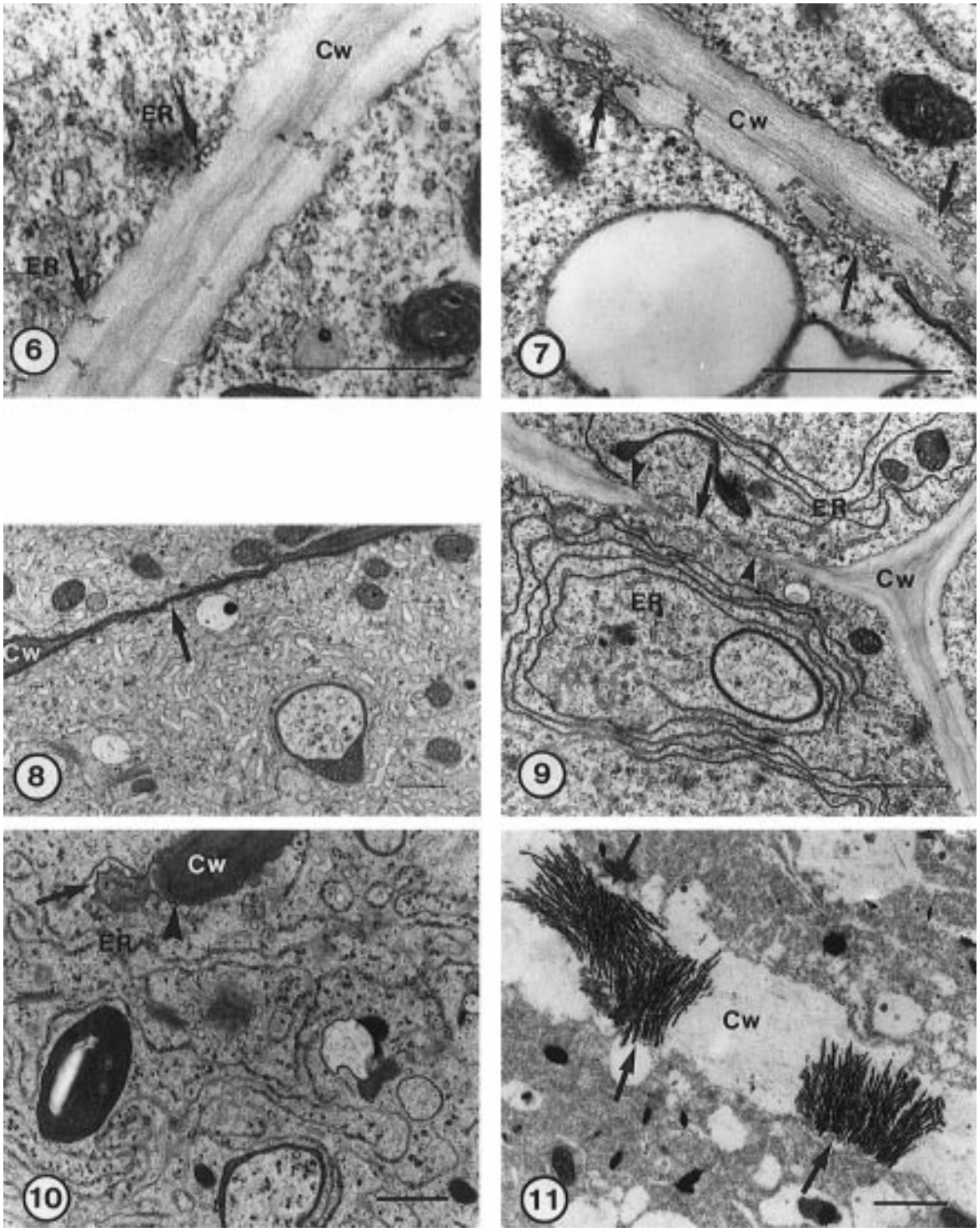
Analysis of fixed material

For ultrastructural analyses, root segments with feeding sites and associated nematodes were dissected at developmental stages which were determined individually for each juvenile by *in vivo* microscopy (Wyss, 1992). Samples were taken at 6 h, 12 h, 18 h, 24 h, and 48 h after feeding site induction and 2 d after the second moult with 10-20 specimens for each time interval. The specimens were fixed in modified Karnowsky's fixative and embedded in Spurr's low viscosity resin as described previously (Golinowski et al., 1996; Sobczak et al., 1997). Ultra-thin (60 nm) sections were made with a Reichert (Leica) Ultracut S microtome. Examinations were made on a Siemens 101 transmission electron microscope operating at 80 kV.

Enzymatic localisation of cellulase activity

The cellulase activity was localised at the ultrastructural level with the formation of electron dense, usually fibrillar, deposits produced by the reaction of reducing sugars liberated by the hydrolysis of exogenously applied carboxymethylcellulose. Samples were prepared according to a method described by Nessler and Mahlberg (1981). A fixative containing 2% paraformaldehyde and 2% glutaraldehyde in 50 mm phosphate buffer (pH 7.2) was used. The samples were fixed for 1 h at 4 °C and washed thoroughly at least 20 times in 10 ml of 50 mm phosphate buffer (pH 7.2) at 4 °C. They were incubated for 30 min at room temperature in 50 mm citrate buffer containing 0.01% sodium carboxymethylcellulose (Roth, FRG). After incubation, the samples were transferred for 10 minutes to boiling Benedict's reagent (Römpp, 1966) and then washed four times in distilled water for 10 min. The samples were post-fixed for 2 h at room temperature in 1% osmium tetroxide in 50 mm cacodylate buffer (pH 7.2) and subsequently washed in four 10 min changes in the same buffer. Dehydration, embedding, sectioning and staining was done as described above. Unstained sections and sections stained with uranyl acetate and lead citrate were examined with an electron microscope.

Control samples were incubated in 50 mM citrate buffer alone and then transferred to the boiling Benedict's reagent.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Figures 6–11. Sections of syncytia associated with male J3 juveniles of *Heterodera schachtii* 2 days after moulting, showing the sequence of events leading to the formation of openings in syncytial cell walls (Cw) without the involvement of plasmodesmata. Bars 1 μm.
Figure 6. Membranes of endoplasmic reticulum (ER) accumulate adjacent to one or both sides of the cell wall where first indications of wall lesions (arrows) begin to form.
Figure 7. Lesions in the cell wall (arrows) become more extensive.
Figure 8. Undissolved middle lamella (arrow) still separates the two adjacent syncytial elements.
Figure 9. The middle lamella is dissolved and a cell wall opening (arrow) is formed. The opening is surrounded by sharp and thin cell wall stubs (arrow heads). The continued separation of the two systems of endoplasmic reticulum (ER) during fusing of the syncytium with an adjacent cell is clearly visible.
Figure 10. At the final phase of wall dissolution cell wall stubs are rounded (arrow head) and are sometimes associated with paramural bodies (arrow). The integration of the ER system is established.
Figure 11. Formation of a cell wall opening, comparable with Figure 7. Precipitations of reducing sugars (arrows) inside the syncytial cell wall indicate cellulase activity.

Results

In the roots of plants inoculated at the age of 3 weeks the infective second stage juveniles (J2) of *H. schachtii* induced ISCs usually in pericyclic cells of the thin lateral roots and only males developed on these syncytia. Under these conditions the invasion and early development of the juveniles was easy to observe in the light microscope until the infection sites were dissected for the ultrastructural investigation.

Cells selected as ISCs were found to undergo a fixed sequence of events leading to the development of syncytia. During the initial phase of feeding site formation, there is a so-called preparation phase (Wyss, 1992), namely, when the juvenile was motionless with its stylet inserted in a selected ISC, electron translucent cell wall material was deposited around the stylet tip and at the affected area of the cell wall (Figure 1). Similar depositions occurred in neighbouring cells. Frequently, fragments of membranes condensed in myelin bodies which appeared in the cytoplasm and were sometimes embedded into the newly deposited wall layer.

At the end of preparation phase, the few plasmodesmata that very rarely occurred between pericycle cells were modified to form the first cell wall openings (Figure 3). During this early stage of syncytium development, the first openings were formed by the gradual widening of the plasmodesmata and the fusing of protoplasts of two adjacent cells. Fully developed openings with fused protoplasts were observed for the first time in 18 h old syncytia (Figure 2).

After 18 h a syncytium was composed of several pericyclic cells that had cytoplasmic continuity caused by wide wall openings (Figure 2). The outer syncytial walls were slightly thickened and the layer of newly deposited electron translucent material was still observable. During further differentiation, the electron translucent cell wall layer changed its appearance, such that in a 6-day-old syncytium it had acquired the same staining pattern as other parts of the thickened syncytial walls (Figure 5). The wall between syncytial elements and pre-conditioned neighbouring cells was often bent and paramural bodies were frequently found at these sites (Figure 2). The terminal cells at the leading edges of a syncytium fused progressively with neighbouring cells thus expanding the syncytium along the vascular cylinder. These fusions started also with the widening of plasmodesmata that often occurred in groups (Figure 4).

Functional plasmodesmata were never found in the outer wall of the more developed syncytia of J3. Frequently, plasmodesmata appeared in the walls of sieve elements (Figure 5), but they were always closed from the syncytium side by wall material. However, the syncytial walls at these places were usually thinner than elsewhere and often associated with large paramural bodies containing numerous vesicles.

The formation of wall openings at plasmodesmata occurred only in syncytia induced by nematodes during the early J2 stage. However, in syncytia of older J2 and later stages wall openings formed exclusively in syncytial cell walls without the involvement of plasmodesmata. The sequence of events leading to the formation of such openings is documented in Figures 6-11: initially membranes of ER accumulated adjacent to the syncytial walls and first fine wall lesions appeared (Figure 6). Later, the lesions expanded and the walls acquired a ‘corrosion-like’ pattern (Figure 7). The cell walls were usually degraded from both sides at separate but adjacent sites. During this process neighbouring syncytial elements were separated by un-dissolved pectinous middle lamella (Figure 8). Later, the middle lamella was also dissolved and the plasmalemma fused at the junction of both neighbouring protoplasts, whereas the ER remained

separated (Figure 9). The ER systems of adjacent cells first extended and then fused across the cell wall openings. After the formation of an opening, the wall stubs were spiked but became later rounded, while the plasmalemma often formed paramural bodies at these sites (Figure 10).

In the cellulase-specific detection assay, precipitations of liberated sugars formed locally during the initial stages of lesion formation (Figure 11) thus indicating that cellulase activity is involved in the cell wall dissolution. After an opening was formed no further cellulase activity was observed.

Discussion

Cell walls of *A. thaliana* show specific changes during syncytium formation. The wall of affected cells thicken and are composed principally of hemicellulose, pectins and cellulose. Except for one report (Jones and Northcote, 1972), lignin or other phenolics were never found to occur in syncytial cell walls (Sobczak, 1996).

The nature of the thickened walls formed during ISC selection is not clear. It is possible that the deposited material is callose, which is known to be one of the basic plant defence mechanism induced during pathogen infection (e.g. Kobayashi et al., 1995). As cellulose, callose is supposed to be formed *in situ* by a glucan synthase, which is bound to the plasmalemma (Delmer, 1987). In a study on feeding sites of the sedentary nematode *Nacobbus aberrans* callose was detected with aniline blue treatment of Spurr's embedded sections (Jones and Payne, 1977). In syncytia this method gave no positive result. Using polyclonal antibodies, specific against 1,3-ß-glucose, Hussey et al. (1992) showed that callose is deposited as an electron-translucent layer on walls of root cells parasitised by *Criconemella xenoplax*, an ectoparasitic nematode.

The function of the observed paramural bodies is not clear. In general, paramural bodies, multivesicular bodies and other similar boundary formations are referred to transport activities of the cell. However, it is still a matter of debate, whether they are related to cell wall depositions (Marchant and Robards, 1968), exocytosis (Kronestedt-Robards and Robards, 1991) or endocytosis (Tanchak and Fowke, 1987).

The formation of cell wall openings was reported to occur very early after syncytium induction (Gipson et al., 1971; Stender et al., 1982; Golinowski et al., 1996). In our study samples were taken 6 h, 12 h, and 18 h after ISC selection and 2 d after the second moult. We found first large wall openings within 18h after ISC selection in curved walls. Endo (1991) reports on similar results in observations in roots of susceptible soybean infested with a compatible race of *H. glycines*. Plasmodesmata and pit fields are suggested to be the primary sites of cell wall opening by Jones (1981) and Endo (1986). However, according to our observations there are two different sites and types of wall dissolution: 1) during the early phases of syncytium development, wall openings were exclusively formed at plasmodesmata; 2) during later stages of syncytium development the large openings were usually formed in areas without the involvement of plasmodesmata. At later stages the outer syncytial walls were greatly thickened and open plasmodesmata were found neither in serial nor in single sections.

The findings about precipitation of liberated reducing sugars indicate that formation of the cell wall openings was temporally and locally controlled. When the wall opening had reached the proper size, the mechanism was turned off as no more liberated sugars could be detected. The method used for the assessment of cellulase activity is indirect and detects the presence of reducing sugars liberated by the action of enzymes. This cytochemical method has several weaknesses (Sexton and Hall, 1991): 1) the products of hydrolysis can diffuse from the site of origin, 2) high concentrations of reducing sugars may be present endogenously, or 3) the penetration of exogenously applied substrates may be slow and restricted by e.g. cell walls. However, in our study these difficulties did not occur as the site where enzymes were detected resemble the sites of cell wall degradation.

Acknowledgements

The authors thank Petra Sedlag and Maria Gagyi for technical assistance. We also acknowledge the support received from our Universities, the DAAD (Deutscher Akademischer Austauschdienst) and the EU (AIR1-CT92-0695) to establish and maintain the scientific contact. M. Sobczak is indebted to the DAAD for fellowship support. This work was partially supported by the Polish Committee for Scientific Research (KBN) grant no. 6P 204 006 05. We also would like to express our thanks to the Institute of Botany, Christian-Albrechts-Universität Kiel, for kindly giving access to the transmission electron microscope.

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