Cytochemical Localization of Pectinase Activity in Pollen Mother Cells of Tobacco During Meiotic Prophase I and Its Relation to the Formation of Secondary Plasmodesmata and Cytoplasmic Channels

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Abstract: Pectinase activity was localized at the ultrastructural level in pollen mother cells of tobacco (Nicotiana tabacum L.) during meiotic prophase to elucidate its role in the biogenesis of secondary plasmodesma (sPD) and cytoplasmic channel (CC). At the leptotene stage the enzyme was mainly present in the cisternae of smooth endoplasmic reticulum (SER) and their derived vesicles, but absent in the Golgi body and Golgi vesicles. Later at the zygotene stage, when sPDs and CCs were actively formed, strong pectinase activity was observed not only in the SER cisternae and their derived vesicles but also in the cell wall, especially in the vicinity of or within both simple and branched plasmodesmata, notably along the middle lamellae, which also characterized the sites of CCs being formed. The presence of exocytotic vesicles containing reaction products suggests that pectinase shares the same excretive pathway as that used by cellulase for its delivery into the wall, i.e. in active form via smooth endoplasmic reticulum (ER) and its derived vesicles by exocytosis. In combination with cellulase, pectinase also promotes the secondary formation of plasmodesmata and CCs by specifically digesting the pectin in middle lamella.

Key words: cytochemical localization; secondary plasmodesmata; cytoplasmic channel; pectinase; pollen mother cells; tobacco

In plants, plasmodesmata are classified as “primary” and “secondary” according to their origin. The former are formed during cytokinesis, while the latter generate de novo across the established cell wall (Jones, 1976; Kollmann et al., 1985; Kollmann and Glockmann, 1985; 1991; Robers and Lucas, 1990; Ding et al., 1992; Lucas et al., 1993). Cytoplasmic channel is another form of intercellular connection much larger than plasmodesma (PD) (Gates, 1908; Weiling, 1965; Bisalpufra and Stein, 1966; Heslop-Harrison, 1966; Baquar and Husain, 1969; Owen and Makaroff, 1995; Wang et al., 2002). While the mechanism responsible for primary PD formation is generally clear, those for the secondary PD (sPD) and the cytoplasmic channel (CC) still remain largely as enigmas, for which a few hypotheses that lack solid experimental support have been presented (Jones, 1976; Kollmann and Glockmann, 1991; Lucas et al., 1993; Ding and Lucas, 1996; Kragler et al., 1998; Ehler and Kollmann, 2001). In Jones’ model (1976), sPD formation is quite direct and simple, arising from wall perforation by enzymatic hydrolysis. However, as Jones (1976) noted: “the mechanism by which a site for secondary perforation of the wall is selected by the cell(s), and how the wall digesting enzymes are localized there is unknown.” The model proposed by Kollmann and Glockmann (1991) involves more complex processes of wall thinning and removal followed by its rebuilding through concerted activities of Golgi vesicles and endoplasmic reticulum (ER) branches, which is especially suitable to explain the sPD with complex branching morphology in some cases.

The formation of sPDs and CCs between developing PMCs has been studied in great detail (Cheng et al., 1987; Wang et al., 1998). They begin to form at leptotene, peak at synizesis (especially for CCs), and are sharply occluded at pachytene (Nie et al., 1984). Early investigations based on ultrastructural observations and acid phosphatase localization favor Jone’s model and suggest that the hydrolases needed for this process are likely moved to the wall either after their release out of ER cisternae into the cytoplasm or by ER-derived lysosomal-like vesicles via exocytosis (Cheng et al., 1987). The second picture was directly substantiated by the subsequent localization of cellulase activity during sPD and CC formation (Wang et al., 1998). The whole process appeared to consist of several successive steps in time: the enzyme was possibly synthesized on the rough ER and stored in active form in the smooth endoplasmic reticulum (SER), which often enlarged in some parts, giving rise to SER-vesicles that delivered their cellulase content into the cell wall by exocytosis, leading to the formation of sPD and CC by perforating the cell wall through hydrolysis, which also requires the co-action of pectinase to digest the...
middle lamellae (Jones, 1976; Lucas et al., 1993; Wang et al., 1998). Here we report cytochemical localization of pectinase activity in pollen mother cells of tobacco (Nicotiana tabacum) during meiotic prophase I and discuss its relation to the formation of secondary plasmodesmata and cytoplasmic channels.

1 Materials and Methods

Cytochemical localization of pectinase activity was performed according to the procedure of Allen and Nessler (1984). Tobacco (Nicotiana tabacum L.) was grown in the Botanic Garden at Lanzhou University. Anthers were collected and grouped according to their size (Table 1). The exact developmental stage of each group of anthers was determined by light microscopic examination. Anthers at different developmental stages were cut into 0.5-1.0 mm sized pieces and fixed in Karnovsky’s (1965) fixative (a mixture of 1% glutaraldehyde and 4% paraformaldehyde in 0.05 mol/L phosphate buffer, pH 7.2) at 0 °C for 5 h. Specimens were then rinsed with more than 10 changes of phosphate buffer and stored in the same buffer overnight at 0 °C. They were incubated at 25 °C for 30 min in 0.1 mol/L sodium acetate buffer (pH 5.0) containing 0.5% pectin and transferred to hot Benedict’s solution with 1.73% cupric sulfate, 17.3% sodium citrate and 10% sodium carbonate, and boiled for 10 min. Control tissues were incubated in acetate buffer without pectin, or were boiled for 10 min prior to incubation with pectin. Omission of Benedict’s reagent from the reaction mixture was used as further controls to check any artifacts arising from this reagent. After incubation, tissues were washed several times with distilled water and post-fixed in 1% OsO₄ overnight at 0 °C, followed by another wash with distilled water. Subsequent dehydration, Epon812 embedding, ultrathin sectioning, and double staining with lead and uranium were carried out as before (Cheng et al., 1987). The stained sections were examined with the Phillip-400T transmission electron microscope. Semi-thin sections (2 µm thick) cut with microtome were stained with Toluidine blue O and observed under OLYMPUS microscope.

2 Results

2.1 Characteristics of pectinase activity reaction products

In accordance with previous reports (Allen and Nessler, 1984; Wang, 1998), the deposits of pectinase reaction product (RP) (cuprous oxide) seen in this experiment appeared as small (ca. 20 nm) electron dense crystalline deposits in linear form (Figs.1-5, 7-10, 12-14, 16-18 and 23-28) and could not be detected in the controls (Figs.11, 15). In the present experiment, pectinase activity and its pattern of localization within endothecium, middle layer, tapetum and pollen mother cells (PMCs) changed during development (Table 2). The following is a detailed description of pectinase activity during meiotic prophase I.

2.2 Dynamic distribution of pectinase activity in pollen mother cells

At the leptotene stage, a pollen mother cell has a big central nucleus and rich cytoplasm with abundant cell organelles, such as mitochondria, ER and Golgi bodies (Figs.1-6, 9-10). The PMCs at this stage were separated...
Pollen mother cells of tobacco at leptotene stage: pectin and Benedict’s solution staining. 1-5. Pectinase reaction products (arrows) are present in the cisternae of smooth endoplasmic reticulum and their derived vesicles. The cisternae with pectinase reaction products (RPs) are often distended and sometimes oriented to the cell wall where a few deposits of RPs (arrow) are present (Fig.5). Note the absence of reaction product in Golgi body and Golgi vesicles (Figs.2, 3, 6). Bar = 0.5 µm (1-3, 5, 6), 1 µm (Fig.4). Abbreviations: GB, Golgi body; GV, Golgi vesicle; SER, smooth endoplasmic reticulum.
Figs. 7-10. 7-9. ER-derived vesicles with pectinase reaction products (arrows) are near cell wall (Fig. 7), being fused with the plasmalemma (Fig. 8) or are releasing their pectinase into the wall (Fig. 9) by exocytosis. 10. Pectinase RPs (arrow) are present in the cell wall. Abbreviations: CW, cell wall; ER, endoplasmic reticulum; ERV, endoplasmic reticulum-derived vesicle; N, nucleus; RPs, reaction products. Bar = 0.5 µm (Figs. 1-3, 5-10), 1 µm (Fig. 4).

Figs. 11-16. Pollen mother cells of tobacco at zygotene stage: pectin and Benedict’s solution staining. 11, 15. Negative controls, showing H- (Fig. 11) and Y-shaped (Fig. 15) plasmodesmata. No reaction products are observed. 12. Large amount of reaction products (RPs) is localized in the cell wall. Note the small RP deposit at the site between the two neighboring plasmodesmata (arrows). 13. Strong pectinase activity (arrow) is concentrated in the middle lamellae between two adjacent plasmodesmata, presumably responsible for H-shaped plasmodesma formation. 14. Pectinase reaction products (arrow) are present in the two simple plasmodesmata that appear to be in the process of their formation. 16. A forming Y-shaped plasmodesmata with strong pectinase activity (arrows) and closely associated smooth endoplasmic reticulum. Abbreviations: N, nucleus; PD, plasmodesmata; RP, reaction product; SER, smooth endoplasmic reticulum. Bar = 0.33 µm (Figs. 11, 15), 0.5 µm (Figs. 12-14, 16).
YU Chun-Hong et al.: Cytochemical Localization of Pectinase Activity in Pollen Mother Cells of Tobacco During Meiotic Prophase and Its Relation to the Formation of Secondary Plasmodesmata and Cytoplasmic Channels
Figs. 17-22. 17, 18. Tapetal cells: pectin and Benedict’s solution staining, the reaction products (RPs) (arrows) are mainly located in the middle lamellae at the sites of cytoplasmic channels in their process of formation. 19. Semi-thin section showing cytomixis (long arrow) between tapetal cells when pollen mother cells are at pachytene stage. Note that the cell wall (short arrow) and nuclei of tapetal cells (two nuclei within a cell) are different from those of pollen mother cells. 20. Intercellular migration of nuclear materials via cytoplasmic channel between tapetal cells (arrow). Little RP is found. 21-22. Cytomixis between pollen mother cells at synizesis; little RP is present. Abbreviations: CC, cytoplasmic channel; CH, chromatin; CW, cell wall; N, nuclei; Nu, nucleolus; PD, plasmodesmata; PMC, pollen mother cells; RP, reaction product; TC, tapetal cells. Bar = 0.5 µm (Figs. 17, 18, 20-22), 2.5 µm (Fig. 19).
Figs. 23-28. Cytochemical localization of pectinase activity in the cells of middle layers (Figs. 23-27) and endothecium (Fig. 28) of tobacco anther, which is similar to that observed in pollen mother cells at the lepotene-zygotene stage (Figs. 1-10). Abbreviations: CW, cell wall; ERV, endoplasmic reticulum-derived vesicle; GB, Golgi body; GV, Golgi vesicle; N, nucleus; PD, plasmodesmata; SER, smooth endoplasmic reticulum; V, vacuole.

by thin cell walls (Fig. 10). As reported in the previous study on cytochemical localization of cellulase (Wang et al., 1998), some parts of SER cisternae contained strong pectinase activity and distended (Figs. 1-3, 5), which subsequently gave rise to irregular-shaped ER-vesicles by budding (Figs. 3, 4). It was found that SER cisternae could be directly oriented to the cell wall (Fig. 5), possibly preparing to release their enzyme content into the cell wall, as shown in Figs. 7-9 via exocytosis. A few deposits of reaction product could be seen in the cell wall (Fig. 10). We failed to observe enzyme activity in Golgi bodies and Golgi vesicles (Figs. 2, 3, 6).

At zygotene stage, ER and ER-vesicles (ERVs) with pectinase reaction products were still rich in their cytoplasm. The prominent feature of pectinase distribution at this stage was that strong activity existed in the wall of the pollen mother cells, especially along the middle lamellae and in the vicinity of plasmodesmata. RPs were also observed in the middle lamellae between two adjacent simple plasmodesmata (Figs. 12, 13), and within simple (Fig. 14) and Y-shaped
plasmodesmata undergoing formation (Fig. 16). Again as expected, such localization patterns of pectinase activity were absent in the controls (Figs. 11, 15).

2.3 Dynamic distribution of pectinase in anther wall cells

Pectinase reaction products could be observed in the wall between tapetal cells. Of particular relevance, they were often present between two very neighboring intercellular connections (Fig. 18), or in the middle lamellae at the forming sites of CC (Figs. 17, 18). The amount of reaction deposits was greatly reduced in the walls of both tapetal cells (at pachytyene Fig. 19) and PMCs (at synizesis) after cytoplasmic channels reached their full sizes, which were usually filled with migrating cytoplasm or chromatin substances across cell walls (Figs. 19–22).

In the cells of other layers (epidermis, endothecium and middle layer) of anther wall, a similar distribution pattern of pectinase reaction products (RPs) to that in leptotene PMCs was observed (Table 2): pectinase activity was often observed in some distended SER cisternae and their derived vesicles, from which the enzyme seemed to be released into the wall by exocytosis, where the RPs were found to be present in a line along the middle lamellae (Figs. 23–28). Like PMCs and tapetal cells, CCs were also often observed between those anther wall cells, although their frequency is much lower than that observed between PMCs.

3 Discussion

3.1 Pectinase reaction products indicate the presence of pectinase activity

Ultrastructural localization of pectinase activity was first developed by Allen and Nessler (1984) through modification of the method reported by Bal (1974) for the localization of cellulase activity. Its principle is similar to that underlying the cellulase localization (Wang et al., 1998) and based on the fact that reducing sugars (galacturonic acid) liberated from the enzymatic hydrolysis of exogenous pectin can react with Benedict’s reagent, reducing cupric salts to cuprous oxide precipitates that appear as small electron-dense crystalline deposits in linear form under electron microscope. Since in our present experiment such deposits are only produced in the samples incubated in complete reaction medium at normal reaction conditions, but not in the negative controls, their presence must indicate pectinase activity. The same conclusion was also obtained by Li et al. (2004).

3.2 Delivery of pectinase to its action sites

Like cellulose, enzymatic hydrolysis of pectin is also considered to be a critical step in sPD formation (Ding and Lucas, 1996). However, the exact cellular mechanisms responsible for this process remain elusive. Ding and Lucas (1996) speculated that the localized degradation of the middle lamella and adjacent cell wall material could be achieved by several possible mechanisms. First, active enzymes may be directly transported, via the primary PD, into the middle lamella. Secondly, inactive form of enzymes may be secreted into the wall via vesicle fusion with the plasma membrane, and triggering molecules may be transported, via primary PD, into the middle lamella to activate the enzymes locally. Thirdly, the inactive enzymes may have been deposited in the walls by fusing Golgi vesicles during cell plate formation, and the triggering molecules may enter the middle lamella through PDs to activate the enzymes when the modification process begins. The presence of active wall-degrading enzymes (pectinase in this study and cellulase in our previous report (Wang et al., 1998)) within ER cisternae and their derived vesicles, and their subsequent movement toward the wall to secrete these enzymes in active form by exocytosis, does not favor the last two of the above speculated mechanisms; rather, our results suggest a concept of de novo synthesis and transport of enzymes in active form during or just before the process of sPD formation. Although studied in two separate experiments, the same picture found for the two enzymes for their transport to the action sites in the wall suggests that these two major wall-degrading enzymes may be co-delivered by the same cellular apparatus (SER and its derived vesicles), which made it possible for the cell to coordinate their actions in wall degradation, i.e. at exactly the same place and time, which is necessary for sPD and CC formation correctly. Double labeling of the two enzymes by immuno-cytochemical method should be used to directly check this possibility. It remains to be seen if this is also the case for other wall degrading enzymes such as hemicellulase. The absence of pectinase and cellulase activity in Golgi bodies and their vesicles indicated that this organelle played no role in this process. In addition, as shown in Figs. 14, 16, the enzymes might also be transported, via the forming plasmodesmata, into the middle lamella. So we proposed two alternative pathways for delivering pectinase to the middle lamellae during the development of sPD: the active enzymes may be either directly transported into the middle lamella through forming plasmodesma, or diffused into middle lamella after being released into the cell wall via ER-vesicles/plasma membrane fusion.

3.3 Possible roles of pectinase in secondary plasmodesmata and cytoplasmic channel formation

Parallel changes between the occurrence of pectinase
and the process of sPD and CC formation, as has been reported previously for cellulase (Wang et al., 1998), indicate that the enzyme plays an important role in this process. At leptotene stage, most pectinase were localized in the SER and ER-vesicles but scarce in the cell wall. At zygote stage, when large number of sPD and CC being formed, strong pectinase activity could be observed in the vicinity of or within PDs, as well as in the middle lamellae at the sites of CC being formed, which was subsequently reduced greatly at synizesis (PMCs) and pachytyene (tapetal cells) stages when CCs were completely formed to mediate cytomixis.

In the model proposed by Kollmann and Glocmann (1991) to explain the de novo formation of sPDs between heterografts of *Vicia faba* on *Helianthus annuus*, they guessed that cell wall degrading enzymes might be only involved in the initial stages of wall loosening/thinning and removal at the site of the graft union. Subsequent entrapment of ER cisternae during wall rebuilding by Golgi vesicles activity resulted in sPD formation, which could satisfactorily explain the complex branched morphology of the sPD in their case. In PMCs, however, we failed to observe such Golgi-mediated wall rebuilding process, indicating that it did not participate in the formation of sPDs and CC. Rather, both our present and previous studies (Wang et al., 1998) favor a more direct process of enzymatic perforation across the wall for sPD and CC formation, as proposed by Jone (1976). Based on cytochemical localization of cellulase activity, Wang et al. (1998) suggested that, if the ER cisternae providing the enzymes entered the perforations being formed during local wall digestion and were finally “trapped” in them by the surrounding wall, the formed connections would be sPD with appressed ER. If not, the simple PD without ER would be formed; if the released enzymes were very strong or remained active for a long time, the perforations formed will be large, leading to the formation of CC (Wang et al., 1998). Our present results indicated that pectinase could be co-released with cellulase from the same ER-vesicle (ERV) into the wall, where, in a closely cooperative manner with cellulase activity, it selectively degraded pectin in the local middle lamellae at the forming sites of sPDs and CCs. Its localization within the “Y”- and “H”-shaped sPDs (Figs.13, 16) clearly showed that in PMCs such complex sPDs could be formed by rather directly simple way of enzymatic perforations that did not require the process of wall rebuilding. Moreover, removal of pectin in middle lamellae by pectinase resulted in lateral fusion of two neighboring PDs, producing “H”-shaped PD as shown in Figs.12 and 13.

### 3.4 Cytoplasm channel formation is associated with cytomixis

CCs are distinct from PDs (Bisalpufra and Stein, 1966; Robards, 1975) in their sizes, structures and functions. They are non-branched pores lined by plasmalemma, and generally 200–600 nm in diameter, and can reach up to 3500 nm at its maximum, which are much larger than those of PDs (30–50 nm) (Weiling, 1965; Bisalpufra and Stein, 1966; Heslop-Harrison, 1966; Baquar and Husain, 1969, Wang et al., 2002). In both PMCs (Figs.21, 22) and tapetal cells (Figs.19, 20), cytomixis could be observed once the cytoplasmic channels had been formed. Cytomixis between tapetal cells has also been observed in lily under light microscope (Cheng et al., 1964). In the present investigation, we found that cytomixis appeared to closely follow the fully formation of CC but not precede it. These results suggested that, as huge intercellular connections much larger than PDs, CCs were closely related to the phenomenon of cell-to-cell migration of cytoplasmic organelle (such as mitochondria, plastids and vesicles, etc.) and nuclear material (Bisalpufra and Stein, 1966; Heslop-Harrison, 1966; Baquar and Husain, 1969; Cheng et al., 1975; Wang et al., 2002).

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