The Maize Pathogenesis-Related PRms Protein Localizes to Plasmodesmata in Maize Radicles

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Pathogenesis-related (PR) proteins are plant proteins induced in response to infection by pathogens. In this study, an antibody raised against the maize PRms protein was used to localize the protein in fungal-infected maize radicles. The PRms protein was found to be localized at the contact areas between parenchyma cells of the differentiating protoxylem elements. By using immunoelectron microscopy, we found that these immunoreactive regions correspond to plasmodesmal regions. This was also true for the parenchyma cells filling the central pith of the vascular cylinder, although PRms mRNA accumulation was not detected in these cells. These findings suggest that for one cell type, the parenchyma cells of the central pith, the protein is imported rather than synthesized. The localization of the PRms protein indicates the possible existence of mechanisms for sorting of plant proteins to plasmodesmata and suggests that this protein may have a specialized function in the plant defense response. These findings are discussed with respect to the structure and function of plasmodesmata in cell-to-cell communication processes in higher plants.

INTRODUCTION

In plants, when the individual cells are separated by an intervening wall, intercellular communication is established by way of intercellular junctional structures called plasmodesmata, which are cytoplasmic channels that traverse plant cell walls linking protoplasts into a symplastic continuum (for reviews, see Lucas and Wolf, 1993; Lucas et al., 1993; Epel, 1994; Overall and Blackman, 1996). Therefore, a basic knowledge of plasmodesmal functions is fundamental to the understanding of cell-to-cell communication processes in both the physiological and developmental aspects of plants.

Structurally, plasmodesmata are plasma membrane-lined pores that contain a strand of endoplasmic reticulum (ER) in the center of the pore that is continuous with the ER of adjacent cells. The presence of structural proteins in both the ER and plasma membranes causes the cytoplasmic bridge to divide into microchannels that establish a basal size exclusion limit of 800 to 1000 D for cell-to-cell diffusion of molecules. Initially, plasmodesmata were thought to be simple structures that functioned mainly as passive channels for diffusion of small molecules. However, a large body of evidence substantiates that plasmodesmata are both structurally and functionally complex (Russin et al., 1996). In many plants infected by viruses, cell-to-cell movement of infectious particles occurs via plasmodesmata (Lucas and Gilbertson, 1994; Gilbertson and Lucas, 1996). This function is secured by specific virus-encoded proteins—the movement proteins (MPs)—that allow the virus to move out of the infected cells into neighboring healthy cells.

One of the most comprehensively studied viral MPs is the MP from the tobacco mosaic virus (TMV), for which two functions have been described: binding to viral single-stranded nucleic acids and increasing the plasmodesmal size exclusion limit (Deom et al., 1987; Wolf et al., 1989; Citovsky et al., 1990). Microinjection experiments with fluorescently labeled viral MPs and dextrans revealed dextran movement as well as MP movement to adjacent cells (Fujisawa et al., 1993; Noueiry et al., 1994; Waigman et al., 1994). Most importantly, what these microinjection experiments revealed was that trafficking of the fluorescently labeled molecules occurs almost instantaneously. Consequently, it has been proposed that viruses may have evolved the ability to use a pathway for macromolecular trafficking via plasmodesmata. This pathway is already present in plant cells for their own cell-to-cell movement. The challenge is now to determine how, where, and when these mechanisms operate during biological processes in plants.

A recent study has shown that a maize transcription factor, KNO1TED1, which is thought to be involved in maintenance of the indeterminate state of the vegetative shoot apical meristem, has the capacity to move from cell to cell and can also mediate the selective transport of its own mRNA.

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(Lucas et al., 1995). Thus, experiments in which the fluorescently labeled KNOTTED1 protein was microinjected into the cytoplasm of mesophyll cells revealed that KNOTTED1 moves and facilitates the movement of dextrans, just as viral MPs do. Although not yet proven, it has been proposed that KNOTTED1 might be able to interact with plasmodesmata and potentiate its own cell-to-cell movement. Unlike TMV MP, which has been immunolocalized in plasmodesmata (Tomenius et al., 1987; Atkins et al., 1991; Ding et al., 1992), definitive cytochemical evidence proving that KNOTTED1 interacts with plasmodesmata has not yet been obtained. To date, endogenous protein transport through plasmodesmata has been proposed for one other plant system, the companion cell–sieve element complex of the phloem system (Lucas and Wolf, 1993).

Together, these findings have led to the concept that plasmodesmata must have an endogenous functional capacity for increasing their transport capabilities to allow the cell-to-cell transport of macromolecules. If plasmodesmal protein trafficking occurs in intercellular communication processes in plants, then we need to determine how endogenous proteins are targeted to plasmodesmata as well as how the structural and functional properties of plasmodesmata are regulated. New insights into these aspects again came from studies with viral MPs. Such studies have demonstrated that the TMV MP interacts with the plant cytoskeleton and in particular with the network of microtubules (Heinlein et al., 1995; McLean et al., 1995). Whether this system also operates for the cell-to-cell trafficking of endogenous macromolecules remains an open question. A model in which the cytoskeletal system and additional unknown host factors are involved in the cell-to-cell transport of macromolecules has been proposed recently (Gilbertson and Lucas, 1995). A complete understanding of the role of plasmodesmata in cell-to-cell communication processes first requires the identification of specific plant proteins that are targeted to plasmodesmata and either deposited or transported to the neighboring cell.

The PRms protein is a pathogenesis-related maize seed protein whose expression is induced in response to fungal infection in maize seedlings (Casacuberta et al., 1991, 1992; Raventós et al., 1995). The distribution of the maize PRms mRNA and PRms protein in fungal-infected maize radicles was investigated. Initially, the PRms protein was expressed in Escherichia coli and purified. Polyclonal antibodies that specifically recognized the PRms protein were then obtained and used for cellular and subcellular immunolocalization studies. PRms was found to be associated with the plasmodesmata between parenchyma cells of the vascular cylinder. The observed subcellular localization of PRms provides evidence for the existence of intracellular mechanisms for the transport and sorting of a plant protein to plasmodesmata and suggests a specialized function for this protein in the plant defense response against pathogens. Furthermore, results from the in situ hybridization experiments in conjunction with the immunolocalization data allow us to suggest that PRms, like KNOTTED1, is transported from cell to cell in fungal-infected maize radicles.

RESULTS

PRms Accumulation in Tissues of Fungal-Infected Maize Seedlings

To obtain antibodies against the PRms protein, the coding sequence of the PRms gene was cloned into the pMAL vector and expressed in E. coli. The maltose binding protein–PRms fusion protein was purified by affinity chromatography on amylose resin and cleaved with factor Xa to generate the PRms polypeptide. Polyclonal antibodies were then raised against the pure PRms protein. The specificity of the anti-PRms antiserum was assayed with total protein extracts from maize tissues. In protein extracts prepared from Fusarium moniliforme–infected and F. moniliforme elicitor-treated germinating maize embryos (henceforth, embryo tissues refer to embryonic axis plus scutellum), the antiserum reacted with a single polypeptide species with the expected mobility for this protein (Figure 1A, lanes a and b). No immunological reactions occurred with the same total protein extracts when the preimmune serum was used (Figure 1A, lanes d to f). These results correlate well with those previously reported on the accumulation of PRms mRNA in response to fungal infection or treatment with fungal elicitors (Casacuberta et al., 1991, 1992). As a control, we transcribed and translated mRNA in vitro from a plasmid carrying the PRms gene under the control of the T7 promoter. The antiserum reacted strongly and specifically with the PRms protein (results not shown).

The identity of the protein recognized by the anti-PRms antibody was further confirmed by immunoblot analysis of protein extracts that were obtained from fungal-infected embryos and were subjected to two-dimensional gel electrophoresis (Figure 1B). The anti-PRms antibody reacted with a single polypeptide with the expected mobility for the PRms protein (apparent molecular weight of 18 kD and isoelectric point of 8.5). Together, these results confirmed that the antiserum reacted specifically with the PRms protein present in total protein extracts from fungal-infected maize tissues. Finally, the anti-PRms serum was used to study PRms protein accumulation in the fungal-infected maize tissues (Figure 1C). We previously reported that during germination of maize embryos, there is a low level of PRms mRNA accumulation, and that this level is notably increased in F. moniliforme–infected tissues when compared with sterile tissues at all stages of germination (Cordero et al., 1994). Protein gel blot analysis of embryo tissues revealed that PRms protein accumulation could be detected as early as 26 hr after germination (which corresponds to 6 hr after inoculation with fungal spores—the shortest period of time used in this study) (Figure 1C, lane b). In agreement with results for PRms mRNA accumulation,
PRms accumulation was notably increased in extracts from fungal-infected embryos (Figure 1C, lanes d, f, and h). Unexpected results were found when protein extracts were prepared from radicles that had been dissected from the fungal-infected germinated embryos and probed separately with the anti-PRms antiserum. As shown in Figure 1C (lane i), the PRms protein accumulated at significant quantities in protein extracts prepared from infected radicles. However, RNA gel analysis of these tissues did not reveal PRms mRNA, at least at levels comparable to those observed for the fungal-infected embryo tissues (result not shown; however, the same result was previously reported in Casacuberta et al. [1992]). This discrepancy prompted us to conduct a more detailed study of the expression of the PRms gene in fungal-infected radicles by using in situ mRNA hybridization and immunocytochemistry.

**PRms mRNA Accumulates in Protoxylem Cells of the Vascular System**

In situ mRNA hybridization analyses with digoxigenin-labeled riboprobes were conducted using longitudinally cut serial and transverse sections prepared from *F. moniliforme*-infected maize radicles. Results are presented in Figure 2. Longitudinal sections revealed high levels of PRms mRNA accumulation at discrete areas localized along the pattern of the vascular system at the region of differentiation of the radicle. Analysis of longitudinally cut serial sections revealed that these regions are always associated with the protoxylem and are the first xylem elements that differentiate during radicle growth. As an illustration, two protoxylem elements can be seen in Figure 2A (arrows). Figures 2C and 2D present higher magnifications of two different protoxylem elements: a young immature protoxylem element (Figure 2C) and a protoxylem element in which lignification of tracheids has already started (Figure 2D). No PRms mRNA can be detected in association with fully differentiated protoxylem elements that are clearly identified by their characteristic reticulate secondary wall thickening (Figures 2C and 2D). Equally, PRms mRNA was not detected either in pith parenchyma cells or in phloem tissues of the vascular system.

In situ hybridization analyses were also conducted using sections obtained from lateral roots of infected maize seedlings. In lateral roots, the genesis and differentiation of tissues were identical to that of the primary root, but the locus of initiation of lateral roots is the pericycle, which is opposite the protoxylem strands. The types of cells are essentially similar, although some differences in the number and distribution of conducting elements and in the time of differentiation of these elements were found between the lateral roots and the primary root from which they originated. Thus, vascular differentiation occurs faster in the lateral root than in the primary root. As found in the primary root, in situ hybridization with the antisense probe, using a longitudinal section of a lateral root, revealed PRms mRNA accumulation at the xylem elements distributed along the vascular cylinder (indicated by arrows in Figure 2F). No labeling was observed in sections of the same lateral root when hybridized with the control riboprobe (results not shown).

To localize more precisely the type of cells in the protoxylem element in which PRms gene expression occurred, transverse sections obtained from the region of differentiation of the radicle were hybridized with PRms transcripts (Figures 2G to 2I and 2K). Examination of these sections...
Figure 2. Localization of PRms Transcripts in Fungal-Infected Maize Radicles.

In situ hybridization was with longitudinal and transverse sections from seedlings germinated for 3 days. Serial cross-sections were prepared, and adjacent sections were hybridized with digoxigenin-labeled PRms sense or antisense transcripts. In transverse sections (G to K), it was possible to localize PRms mRNA in the parenchyma cells of the protoxylem elements. (A) to (F) are longitudinal sections.

(A) Longitudinal section through the region of differentiation hybridized with the antisense probe. PRms mRNA was detected in protoxylem elements of the vascular cylinder (arrows). Bar = 40 μm.

(B) Section similar to the one shown in (A) hybridized with the sense probe. Bar = 40 μm.

(C) Magnification of an immature protoxylem element (arrow) showing strong PRms gene expression. Bar = 10 μm.

(D) Magnification of a protoxylem element (arrow) in an advanced state of maturation. Bar = 10 μm.

(E) Section similar to the one shown in (D) hybridized with the sense probe. Bar = 10 μm.

(F) Longitudinal section through a lateral root hybridized with the antisense PRms probe. The arrows indicate xylem elements. Bar = 40 μm.

(G) Transverse section through a young living metaxylem cell and its surrounding parenchyma cells of the central pith. The section was hybridized with the antisense PRms probe. Expression of the PRms gene was not observed. Bar = 4 μm.

(H) and (I) Transverse section of the region of differentiation hybridized with the antisense probe. The region of hybridization was localized in the protoxytem parenchyma cells. Asterisks denote mature trached with their lignified cell walls. Bars = 10 μm.

(J) Transverse section of the region of differentiation hybridized with the sense PRms probe. Bar = 10 μm.

(K) Transverse section from a lateral root hybridized with the antisense probe. Intense labeling of parenchyma cells at the xylem elements is visible. Bar = 10 μm.

(L) Section similar to the one shown in (K) hybridized with the sense PRms probe. Bar = 10 μm.

Co, cortex; end, endodermis; Ir, lateral root; Mx, metaxylem; Pe, pericycle; Ph, phloem; Px, protoxylem.
revealed that PRms mRNA accumulated in the parenchyma cells of the protoxylem strands (Figures 2H and 2I). Furthermore, no PRms mRNA was detected either in parenchyma cells associated with mature tracheid cells, which had already elaborated their lignified secondary cell walls, or in phloem strands. Furthermore, no PRms mRNA was observed either in parenchyma cells surrounding young, immature tracheary elements (Figure 2G) or in parenchyma cells of the central pith (Figures 2A, 2C, and 2D). Finally, in situ hybridization reactions using transverse sections of the lateral root also revealed strong labeling in parenchyma cells of xylem elements but not in the parenchymatous cells of the central pith (Figure 2K). The specificity of the in situ hybridization reactions was confirmed by the lack of appreciable reaction of the PRms sense strand probe with the paraffin-embedded sections (Figures 2B, 2E, 2J, and 2L).

From these results, we conclude that cell-type specific expression of the PRms gene occurs during growth of the fungal-infected maize radicle, with the PRms gene being highly expressed during the process of differentiation of the protoxylem elements, particularly in the protoxylem parenchyma cells. It is important to stress that during the course of this work, in situ hybridization analyses with both longitudinal and transverse sections from radicles that were at different developmental stages were conducted (from seedlings germinated 2 to 6 days) and that no PRms mRNA was ever detected in the parenchyma cells of the central pith. The significance of this result is discussed below.

**Immunolocalization of PRms**

Figure 3 shows the immunohistochemical detection of PRms in transverse sections prepared from the region of differentiation of the radicle. A pattern of immunoreactive material appeared at the vascular cylinder, where the protoxylem parenchyma cells were highly immunodecorated by the anti-PRms antibody (Figure 3A). The PRms-specific labeling was

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**Figure 3. Immunolocalization of PRms in Tissue Sections from Fungal-Infected Maize Radicles.**

Transverse sections were prepared from the region of differentiation of the radicle of seedlings germinated for 3 days.

(A) Transverse section of the vascular cylinder with its central pith incubated with the anti-PRms antiserum. PRms-specific labeling at the protoxylem parenchyma cells and cells of the central pith is visible. The labeling is always found at the regions of contact between contiguous cells (arrowheads).

(B) Magnification of an immature metaxylem cell from the section shown in (A) incubated with the anti-PRms antiserum. Arrows denote PRms-specific labeling at the regions of contact between contiguous parenchyma cells.

(C) and (D) Control sections incubated with preimmune serum.

End, endodermis; Mx, metaxylem; Pe, pericycle; Px, protoxylem. In (A) and (C), bars = 10 μm; in (B) and (D), bars = 2 μm.
always observed at discrete regions that were the contact regions between the parenchyma cells. Interestingly, a similar, highly localized deposition was also observed at the regions of contact between the parenchyma cells of the central pith (Figure 3A, arrows). A closer view of the parenchyma pith cells surrounding a young, immature metaxylem cell is presented in Figure 3B. Here, the characteristic immunolabeling at the contact regions between parenchyma cells is clearly observed (arrows). Control sections incubated with preimmune serum did not show any staining in cells of the vascular cylinder (Figures 3C and 3D). Whereas in situ mRNA hybridizations revealed the presence of PRms mRNA in protoxylem parenchyma cells (Figures 2H and 2I), PRms mRNA was not detected in pith parenchyma cells (Figure 2G).

Taken together, the in situ mRNA hybridization and immunohistochemical localization studies revealed that the accumulation of PRms mRNA did not parallel the pattern of PRms protein accumulation in tissues of the fungal-infected maize radicle. Thus, in situ mRNA hybridization experiments revealed the presence of PRms mRNA in specific cell types, namely, the parenchyma cells of the protoxylem system. In agreement with this result, the immunohistological observations showed the presence of PRms in these cells. In contrast, the PRms protein but not PRms mRNA was detected in parenchyma cells of the central pith. PRms always localized at the regions of contact between contiguous parenchyma cells.

### Plasmodesmata Are Sites of PRms Localization

A more rigorous analysis of the association of the PRms protein with the regions of contact between cells of the infected radicle was performed by immunoelectron microscopy. These studies revealed that those discrete regions showing PRms-specific labeling previously observed using light microscopy correspond to the wall regions between contiguous parenchyma cells in which plasmodesmata were present. The results are shown in Figure 4. Plasmodesmata either occurred packed in primary pit fields or were independently distributed along the contact regions between parenchyma cells (Figures 4A to 4E). No labeling was detected in wall areas of parenchyma cells in which plasmodesmata were not present (Figure 4H).

![Figure 4. Immunolocalization of PRms Protein in Plasmodesmata of Infected Maize Radicles.](image)
Different procedures for tissue fixation and immunocytochemical protocols were assayed for detection of PRms in maize radicle sections. Unfortunately, methods that ensure preservation of plasmodesmal ultrastructure do not permit preservation of antibody reactivity. The dense distribution of plasmodesmata observed in the pith fields did not allow us to determine whether PRms represents an integral component of the plasmodesmal channel or if this protein is deposited in the cell wall. However, the labeling observed for the individual plasmodesma (Figure 4E) suggests that PRms protein might be located either in the plasmodesmal channel or only in the cell wall adjacent to this junctional structure.

In addition, electron microscopy studies revealed that these radicle cells appear to be highly interconnected through plasmodesmata by a network of tubules in the ER. Thus, the presence of continuous strands of the ER connecting neighboring cells together with long segments of ER positioned close to the plasma membrane in the proximity of plasmodesma was frequently observed. In addition to its association with the plasmodesmata, the ER network of tubules also exhibited PRms-specific labeling (Figures 4D, 4F, and 4J). Controls incubated with the preimmune serum or omitting the anti-PRms antiserum did not show labeling either on plasmodesmata or in the ER strands (Figure 4G).

During the course of this work, an interesting observation was made. A polarity in plasmodesmal formation associated with the PRms-specific labeling was observed in fiber protoxylem cells. As an illustration, Figures 4I and 4J show a protoxylem fiber cell in which plasmodesmata and PRms-specific labeling occur only in the cell wall region that is contiguous with one of its neighboring parenchyma cells. In contrast, neither plasmodesmata nor gold particles were observed in the opposite cell wall.

**DISCUSSION**

One of the most important structural differences between animal and plant cells is that the latter are enclosed in a rigid cell wall. This structural feature determines that in plants, cell communication is largely dependent on the intercellular connections created by plasmodesmata. Because these junctional structures maintain cytoplasmic and ER endomembrane continuity between contiguous plant cells, a fundamental issue surrounding the role of plasmodesmata in intercellular communication in plants is whether they provide a continuous functional symplastic route for macromolecules. Traditionally, plasmodesmata were thought to serve as mere cytoplasmic bridges to facilitate symplastic movement of small molecules. However, functional studies have gradually pointed to the potential of plasmodesmata to be engaged in selective transport of macromolecules. Compelling evidence for macromolecular transport between plant cells via plasmodesmata comes from studies involving microinjection of fluorescent dyes and fluorescently labeled virally encoded MPs into plant cells (Fujiwara et al., 1993; Noueiry et al., 1994; Waigmann et al., 1994). Considering the fast response observed in the cell-to-cell movement of these fluorescent markers, it has been concluded that they move via plasmodesmata. To date, protein movement through plasmodesmata has been proposed for only one plant protein, the maize KNOTTED1 transcription factor, which displays functional properties similar to those of viral MPs (Lucas et al., 1995).

Taking into account the results of the protein localization data presented here, we come to the following conclusions. First, the PRms protein is localized at the contact regions between contiguous parenchyma cells in both protoxylem and pith tissues of the vascular cylinder, as determined by protein localization data obtained by light microscopy. Second, this protein is localized at or in plasmodesmata connecting these parenchyma cells, as determined by immunoelectron microscopy. The finding of a PR protein localized at the region of plasmodesmata suggests a specialized function of this protein in the plant defense response and the existence of mechanisms for sorting of endogenous proteins to this subcellular destination and supports a regulatory role of plasmodesmata in cell-to-cell communication processes for the control of physiological functions in plants. On the other hand, the in situ hybridization studies revealed that PRms mRNA accumulation could only be detected in a particular subset of cells, the parenchyma cells of the differentiating protoxylem elements. PRms, however, could be immunolocalized not only in these cell types but also in cells in which PRms mRNA was not detected. Thus, PRms but not PRms mRNA was detected in parenchyma cells of the central pith of the vascular cylinder. Together, these results suggest that PRms, like KNOTTED1, moves from cell to cell. In addition, the plasmodesmal localization of PRms suggests possible trafficking of this plant protein through these cell-to-cell junctional structures.

**Plasmodesmal Targeting of PRms**

The data suggest that PRms is targeted to plasmodesmata. Although the exact molecular mechanism by which PRms is targeted to this destination is still unknown, on the basis of our observations we currently favor the following mechanism for plasmodesmal targeting of PRms.

First, the PRms protein contains an N-terminal signal sequence for translocation into the ER lumen (Casacuberta et al., 1991). In vitro transcription and translation of the PRms gene result in a protein with an apparent molecular mass that is 2 kD higher than that of the tissue-extracted PRms protein (L. Murillo, L. Cavallarin, and B. San Segundo, unpublished results). This is consistent with the removal of the N-terminal signal peptide present on the PRms polypeptide during its translocation into the ER lumen.

Second, it is well known that the ER system of a plant cell forms a continuous network of tubules with the plasmodesmal...
ER and with the ER system of the adjacent cells. According to this feature, a possible scenario for PRms plasmodesmal targeting could be that once the PRms protein has been sequestered into the lumen of the ER, the network of ER tubules might channel the protein to plasmodesmata. That the ER provides a track for PRms to reach plasmodesmata is supported by our electron microscopic observations showing PRms labeling on the continuous strands of ER tubules connecting contiguous cells through plasmodesmata (Figure 4). Before concluding that the ER is engaged in plasmodesmal targeting, alternative explanations must be considered. Thus, a mechanism whereby an intracellular vesicular transport ER-Golgi-plasmodesma of the PRms protein occurs may also be considered. If so, one would expect to observe immunoreactive material concentrated inside of vesicles in cells in which the PRms gene is being expressed. During this immunocytochemical analysis, however, no labeling was observed within vesicles in those protoplasts of parenchyma cells that in fact showed labeling in their plasmodesmata, not even in cytoplasmic regions close to plasmodesmata. However, labeling in the ER tubules associated with plasmodesmata, or located close to them, was observed in these cells.

Clearly, nothing is known about the existence of plasmodesmal localization sequences that may direct and specify transport of proteins to plasmodesmata. In other studies, the virally encoded TMV and red clover necrotic mosaic virus MPs have been used as models to identify functional domains involved in the viral cell-to-cell movement. No homology has been found between the functional domains defined within these two proteins (Berna et al., 1991; Citovsky et al., 1992; Giesman-Cookmeyer and Lommel, 1993; Waigmann et al., 1994). Furthermore, experiments by Waigmann and Zambryski (1995) in trichomes of Nicotiana clevelandii suggest that the plasmodesmal targeting signal of the TMV MP resides within the MP sequence. A search for homology between the PRms and the viral MP amino acid sequences has been performed, and no homology could be found.

Recently, it was proposed that cytoskeletal elements could be involved in the intracellular transport and targeting to plasmodesmata of the TMV MP-viral RNA complexes in tobacco cells (Heinlein et al., 1995; McLean et al., 1995). In contrast to the PRms protein, synthesis of the TMV MP occurs in the host cell cytoplasm; therefore, this protein must move through the cytoplasm to the plasmodesmata. An intriguing question is whether the cytoskeleton is also associated with plasmodesmal targeting of endogenous cytoplasmic plant proteins, KNOTTED1 being an obvious candidate for this type of study (see the model proposed for cell-to-cell trafficking of macromolecules through plasmodesmata for both viral MP-nucleic acid complexes and endogenous plant proteins [Gilbertson and Lucas, 1996]). In any case, plasmodesmal targeting of virally encoded and endogenous plant proteins, either cytoplasmic proteins or proteins that enter into the secretory pathway, might or might not follow the same targeting pathway. It is possible that the cytoskeleton also plays a role in plasmodesmal targeting of secretory proteins by governing the distribution and disposition of the ER. Structural observations in plant cells have provided evidence for an association between the cortical network of ER and the cytoskeleton (Hepler et al., 1990).

The plasmodesmal location of PRms was found both in cells highly active in PRms gene expression (protoxylem parenchyma cells) and in cells in which the protein presumably has been imported (parenchyma pith cells). This finding implies that once the PRms protein has been synthesized and targeted to plasmodesmata, it can be either deposited or transported to the neighboring cell, where it is similarly targeted to plasmodesmata. Our observations suggest that the ER system would be engaged in both the intracellular plasmodesmal targeting and the cell-to-cell transport of the PRms. Molecular studies must now be performed to test whether the potential for plasmodesmal PRms sorting and targeting requires special amino acid signal sequences and/or particular post-translational modifications. In addition, the identification of additional endogenous proteins that enter into the secretory pathway and are targeted to these junctional structures is of paramount importance if we are to have a comprehensive understanding of the regulatory roles fulfilled by these unique plant intercellular organelles.

Function of PRms in the Plant Defense Response

The PRms protein belongs to a family of proteins collectively known as PR proteins. They have been defined as plant proteins that are induced in pathological or related situations (van Loon et al., 1994). A general property of PR proteins is that they are synthesized in a nonspecific manner with respect to the infectious agent that triggers their expression and accumulation (van Loon, 1985; Bowles, 1990; van Loon et al., 1994). Thus, expression of PR genes can be observed in response to different pathogens, such as viruses, viroids, fungi, and bacteria. The most extensively studied PR proteins are those that accumulate in tobacco plants reacting hypersensitively to infection by TMV. The maize PRms protein is homologous to the tobacco PR-1 group of proteins, which represents the most abundant of the PR proteins that accumulate in TMV-infected tobacco leaves. PRms and PR-1 proteins are serologically related. Nevertheless, their function in the plant defense response has not been elucidated. The plasmodesmal localization observed for the PRms protein provides the opportunity to investigate whether a similar subcellular localization for the homologous tobacco PR-1 proteins also occurs in plant-virus interactions.

A general defensive strategy to block the passage of the pathogen from one cell to another might be the location of proteins on plasmodesmata, as is the case for the PRms protein. If so, the plasmodesmal localization of PRms could play a role in preventing viral infections in maize plants. Systemic infection of a host plant by a virus has been considered to occur in two stages (Séron and Haenni, 1996). In the
first stage, the cell-to-cell movement involves transport through plasmodesmata. During the second stage, the entire plant becomes infected. For systemic infection, the viruses must enter into the vascular tissues, with the phloem system being the route by which most viruses move for long-distance transport. It is then possible that deposition of PRms in plasmodesmata of parenchyma xylem cells plays a defensive role by creating protective physiological domains. Thus, blocking the viral cell-to-cell movement through xylem parenchyma cells hinders the pathogen in reaching the xylem system. Clearly, this represents an effective strategy that might be critical for resistance to pathogens in plants.

It is worth recalling that the plasmodesmal localization of PRms in infected maize tissues implies that plasmodesmata between these cells could have altered or modified structures. There are possible explanations. First, plant cells have the ability to respond to signals that are triggered upon pathogen recognition by operating a general pathway for plasmodesmal gating in the infected plant tissues. Alternatively, the PRms protein itself is capable of interacting with plasmodesmata to alter the functional status of plasmodesmata. Macromolecular trafficking via plasmodesmata is not restricted to pathogen-specific processes, and mechanisms by which the normal plasmodesmal structure can be modified must operate in other physiological processes, such as in viral cell-to-cell movement, phloem loading, or maintenance of the cell differentiation state (KNOTTED1). Then, we predict that the general mechanism(s) for the regulation of plasmodesmal function(s) operates in the infected tissues. Once this mechanism is in operation, PRms would be targeted and deposited at modified plasmodesmata.

The results presented here suggest that plasmodesmal regulation could be involved in the plant defense response against pathogens and support the view that plasmodesmata can play a regulatory role in physiological processes in plants. Resolving the complexities of the cellular processes involved in controlling plasmodesmata in plant-pathogen interactions will also provide important information on resistance. In our opinion, special attention should be paid to the comparative analysis of plasmodesmal functioning in compatible and incompatible plant-pathogen interactions. This may allow an understanding of how hypersensitively reacting plants are able to limit pathogen spread throughout the plant. Indeed, the hypersensitive reaction consists of a controlled and localized necrosis of plant tissue around the infection site. This is a phenomenon similar to that of mammalian apoptosis, in which processes for cell-to-cell communication are known to be altered. In plants, trafficking of proteins through plasmodesmata could be a common event in cell-to-cell communication processes for the control and coordination of physiological processes in higher plants. Once additional endogenous plant proteins that are targeted and transported across plasmodesmata are identified, plant cell biologists will have powerful tools with which to explore the way plants coordinate their functions at a supracellular level.

METHODS

Plant Material

Maize (Zea mays W64A) seeds were used as the experimental material. The fungus Fusarium moniliforme was grown on potato dextrose agar plates (Difco Laboratories, Detroit, MI) at 25°C until the mycelium covered the surface of the plate. Conidial suspensions and fungal elicitors were prepared as previously described (Casacuberta et al., 1991, 1992). Embryos were germinated for 20 hr and then inoculated either with spores (50 µL; 10⁶ to 10⁷ spores/mL) or with elicitors (50 µL; 300 µg/mL) prepared from the fungus F. moniliforme (Casasuberta et al., 1992). Inoculated and control seeds were allowed to continue germination for the required period of time.

Preparation of Protein Extracts and Immunoblotting

Protein extracts were prepared from maize tissues by using 84 mM citric acid, 30 mM Na₂HPO₄, pH 2.8, 14 mM β-mercaptoethanol, and 6 mM ascorbic acid as the extraction buffer (Raventós et al., 1994). All protein concentrations were determined by the method of Bradford (1976), using the Bio-Rad dye reagent and BSA as a standard. SDS-PAGE was performed according to the method of Laemmli (1970). For two-dimensional gel electrophoresis, isoelectric focusing was run in the first dimension and electrophoresis in SDS in the second dimension (O’Farrell et al., 1979). Immunoblots were prepared essentially according to the procedure of Towbin et al. (1979). Blots were incubated for 60 min at room temperature with the anti-PRms antiserum (diluted 1:1000), followed by incubation with alkaline phosphatase-conjugated swine anti-rabbit IgG (Dakopatts, Copenhagen, Denmark). The color substrates nitro blue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate were used for detection of serological reactions.

Bacterial Expression, Purification of the PRms Protein, and Preparation of the Antiserum

To obtain the PRms coding sequence, the EcoRI fragment of the BBA2 plasmid (Casacuberta et al., 1991), containing the maize PRms cDNA lacking its N-terminal signal sequence as well as the 3′ untranslated region, was isolated by agarose gel electrophoresis. The DNA fragment was blunt ended with the Klenow fragment of DNA polymerase I and digested with XbaI to remove the poly(A) tail. The resulting DNA fragment was ligated into the StuI- and XbaI-digested pMAL vector (New England BioLabs, Beverly, MA), giving a fusion plasmid encoding a fusion protein that, after cleavage with factor Xa, yielded the PRms protein (starting at residue 2 of the mature PRms protein). The fusion plasmid was used to transform Escherichia coli (JM109 strain). Clones containing the pMAL-PRms plasmid were characterized by nucleotide sequencing by the dideoxynucleotide chain termination method (Sanger et al., 1977) on an automated laser fluorescence sequencing apparatus (Pharmacia Biotechnology). DNAs were sequenced directly from the pMAL vector, using the malE primer (New England BioLabs).

For purification of the fusion protein, cells from the isopropyl β-D-thiogalactoside-induced culture (1 liter) were lysed by incubation with lysozyme (1 mg/mL for 60 min on ice). The fusion protein was purified by affinity chromatography on amylose resin, concentrated to 1 mg/mL in Centricon-30 units (Amicon), and digested with...
factor Xa at a ratio of 1% of factor Xa fusion protein (w/w) in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, and 2 mM CaCl₂ at a fusion protein concentration of 1 mg/mL for 16 hr at room temperature. For purification of the PRms polypeptide from the fusion protein, products from factor Xa cleavage were subjected to preparative 15% SDS-PAGE. To localize the protein bands to be excised from the gel, a replica nitrocellulose filter of the electrophoresed proteins was obtained and subjected to silver staining (2% Na-citrate, 0.8% FeSO₄·7H₂O, and 0.2% AgNO₃ was used as the staining solution). Elution of proteins from the gel slices was performed by diffusion into 50 mM Tris-HCl, pH 6.8, 50 mM NaCl, and 0.1% SDS overnight at room temperature. The recovered protein was concentrated by filtration in Centricon-10 units (Amicon). The purified proteins from the preparative SDS-PAGE (~1 nmol of each) were subjected to N-terminal sequence analysis on an Applied Biosystems (Foster City, CA) automatic sequencer (model 477A) and used for immunization of rabbits.

Polyclonal antibodies were raised by multiple subcutaneous injections of purified PRms protein emulsified in complete Freund’s adjuvant (Sigma). Three triweekly injections (25 µg in each injection) were made, and the rabbits were bled 8 days after the final injection. Preimmune serum was collected from the rabbits 1 week before immunization.

**Light Microscopy**

Radicles from *F. moniliforme*-infected maize seedlings were harvested and immediately fixed with formaldehyde (ethanol/formaldehyde-acetic acid, 90:3.5:5 [v/v]) for 1 hr at room temperature. After one change, the tissue was kept in the fixation solution for 1 week at 4°C. Tissue was then washed twice with 70% ethanol and kept until used. The material was dehydrated through a series of ethanol solutions (30, 50, 70, 90, and 98%) for 60 min each and embedded in paraffin. Sections (8 µm thick) from paraffin-embedded material were mounted on glass slides coated with poly-L-lysine. Paraffin was removed from the tissue by incubating the slides in xylene twice for 10 min each and then once in 100% ethanol for 10 min. Rehydration of the tissue was accomplished by 5-min incubations of the slides in an ethanol/water series at room temperature just before antibody treatment. All washes and incubations with antibodies were performed at room temperature. Sections were blocked in PBST (PBST is PBS buffer and 0.05% Tween 20 [w/v] containing 3% skim milk powder) for 1 hr and then incubated with a 1:250 dilution of the anti-PRms antiserum in blocking buffer for 2 hr. The sections were washed three times for 5 min each with blocking buffer and incubated with an anti-rabbit IgG alkaline phosphatase conjugate (Dako-patts) diluted 1:200 in PBST for 60 min. After four washes of 5 min each with PBST, the slides were incubated in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM MgCl₂). Color reaction was in alkaline phosphatase reaction buffer containing 0.66 mg/mL nitro blue tetrazolium salt and 0.12 mg/mL 5-bromo-4-chloro-3-indolyl phosphate. The color reaction was allowed to proceed for 1 to 2 hr.

Several control tests were performed to assess the specificity of the labeling pattern obtained with the anti-PRms antiserum. Incubation of sections with preimmune rabbit serum instead of the anti-PRms antiserum resulted in the absence of labeling over the entire sections. To detect false positive signals, we included two additional controls. First, to detect endogenous alkaline phosphatase activity, sections not incubated with any antibody were used to detect activity. Second, incubation only with the secondary antibody, that is, omitting incubation with the anti-PRms antibody, was used to detect activity due to nonspecific binding of the secondary antibody to the tissues. No false signals were detected.

**Electron Microscopy**

For transmission electron microscopy, tissue samples were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in 100 mM sodium phosphate buffer (pH 7.2, at 4°C for 12 hr). After washing in PB (three washes in PB for 10 min each, two washes of 15 min each in PB and 0.15 M NaCl, and three washes of 10 min each in PB), tissues were gradually dehydrated with ethanol and embedded in London Resin white (Polysciences, Warrington, PA) at −20°C. Ultrathin sections were mounted on Formvar-coated nickel grids and incubated twice in phosphate/Gly buffer (100 mM phosphate and 20 mM glycine, pH 7.2) for 5 min each and once in phosphate/Gly buffer containing 2% BSA for 20 min to block nonspecific adsorption of antibodies. Sections were then incubated with the anti-PRms antiserum diluted with PB-Gly and 1% BSA for 2 hr at room temperature, washed with phosphate/Gly (three times for 5 min each), incubated for 1 hr at room temperature with the secondary antibody (15-nm gold–goat anti–rabbit) diluted with phosphate/Gly and 1% BSA, and washed with PB twice for 5 min each. Several dilutions of the anti-PRms antiserum were assayed, with the dilution 1:200 being used for the experiments described in this study. Controls were run with nonimmune rabbit serum to check for nonspecific adsorption of the primary antibody, with the PRms antiserum also being omitted. Sections were stained with unryl acetate and lead citrate and examined with an electron microscope (model H 600AB; Hitachi Ltd., Tokyo, Japan).

**mRNA in Situ Hybridizations**

To make the results comparable, similar sections were used for immunolocalization of PRms protein and hybridization with digoxigenin-labeled probes. Tissues were fixed and paraffin-embedded as described above. The slides were hybridized with digoxigenin-labeled antisense or sense strands of PRms RNA probes transcribed by T3 or T7 RNA polymerase from linearized pBluescript KS– harboring the PRms cDNA. Labeling with digoxigenin was performed following instructions from the manufacturer (Boehringer Mannheim). Hybridization of the probes to the slides was performed essentially as described by Langdale (1994), using 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.5), 3% SDS, 1.2 µg/µL tRNA, and 0.6 µg/µL poly(A) as the hybridization solution at 55°C overnight. Detection of hybridized probes was performed by using an anti-digoxigenin–alkaline phosphatase conjugate (Boehringer Mannheim) and the color substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

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