The Role of Callose Deposition Along Plasmodesmata in Nematode Feeding Sites

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Infective second-stage juveniles of the obligate plant-parasitic root-knot and cyst nematodes invade plant roots to induce specialized feeding structures. Here, we present data on the distribution of plasmodesmata in cell walls of syncytia and giant cells induced by cyst and root-knot nematodes. An *Arabidopsis* and a tobacco line were used, containing viral movement proteins fused to green fluorescent protein as a localization marker for plasmodesmata. Plasmodesmata were detected in walls between giant cells but also in walls toward neighboring cells. In syncytia, plasmodesmata were mainly detected at later stages. In young syncytia, few plasmodesmata were observed and a specific temporal callose deposition along plasmodesmata indicated impaired symplasmic exchange. In order to study the relevance of callose deposition for successful cyst nematode development in *Arabidopsis*, two mutant lines inhibited in callose synthesis and degradation, respectively, were used in nematode infection assays. Histological analyses showed that syncytia were smaller when callose degradation was reduced, indicating a significant importance of this process to cyst nematode development.

Plant-parasitic cyst nematodes (*Heterodera* and *Globodera* spp.) and root-knot nematodes (*Meloidogyne* spp.) evolved highly sophisticated strategies to manipulate their hosts. Infective second-stage juveniles (J2) invade plant roots and move towards the central cylinder to induce the formation of nematode feeding structures (NFS) (Wyss et al. 1992; Golinowski et al. 1996). Juveniles of cyst nematodes induce syncytial cells as a result of cell wall dissolution along plasmodesmata (PD) of up to several hundred cells (Golinowski et al. 1996). Root-knot nematodes trigger the formation of a number of multinucleate feeding-giant cells by successive karyokinesis events without cytokinesis (Huang and Maggenti 1969). Both NFS are characterized by a dense cytoplasm, enlarged nuclei, a fragmented central vacuole, and proliferation of organelles (Jones 1981; Golinowski et al. 1996). The main function of the NFS is to provide plant solutes and assimilates to the parasitic nematodes.

Phloem-derived solutes may be imported into NFS either through PD (symplasmically) or by transport protein activity (apoplasmically). Although, up till now, giant cells were found

to be symplasmically isolated from neighboring cells (Jones and Dropkin 1976; Hoth et al. 2008), there is a controversial discussion on the connectivity status of syncytia (Jones and Payne 1977; Grundler et al. 1998; Hoth et al. 2005, 2008; Hofmann and Grundler 2006). Although there is consensus that syncytia are symplasmically connected to phloem elements at later stages of development, differing reports exist on the presence and functionality of PD at early stages.

PD are unique membrane channels in plant cell walls that provide cytoplasmic continuity, cell-to-cell transport, and an intercellular communication network. The status of PD may fluctuate between closed or opened, allowing the passage of small metabolites, and extended opened, thus allowing the passage of smaller or larger metabolites, respectively (Crawford and Zambryski 2000). Whereas passage of most molecules is primarily controlled by the size exclusion limit, macromolecules such as proteins and RNA rely on specific trafficking processes. Due to the significant importance of information and solute exchange to plant cell development and function, passage through PD has to be tightly regulated. PD frequently change their configuration between closed and opened, especially during developmental processes of specific tissues and organs such as embryos (Kim and Zambryski 2005), seed (Asthir et al. 2001; Wilson et al. 2006), meristems (Oparka et al. 1994; Rinne and van der Schoot 1998; Gisel et al. 1999), fibers (Ruan et al. 2001), and root nodules (Complainville et al. 2003), as well as during dormancy (Rinne et al. 2001). PD frequency and distribution have been studied by transgenic plants containing fusions of viral movement proteins (MP) and fluorescing proteins such as green fluorescent protein (GFP) (Roberts and Oparka 2003). Most viral MP attach to branched PD that are mainly secondary PD which are formed in existing cell walls. Primary PD, in contrary, often are not branched and are formed during cell wall formation after cell division.

Passage through PD may be controlled by a transient or reversible deposition of callose (β -1,3-glucan) in the cell wall surrounding PD at both ends of the channel. This leads to an inward compression of the plasma membrane, reducing the size exclusion limit of the PD or eventually cutting off solute passage (Wolf et al. 1991). An *Arabidopsis* mutant lacking a β -1,3-glucanase expression showed reduced callose degradation and a limited GFP diffusion between neighboring cells (Levy et al. 2007). Rinne and van der Schoot (2003) suggest that 1,3- β -D-glucan synthase and 1,3- β -D-glucanase are the key enzymes controlling alterations in symplasmic permeability. Biotic and abiotic stresses induce rapid callose deposition along PD in order to isolate the affected areas. Molecular mechanisms underlying callose deposition after pathogen attack (*Hyaloperono-*

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spora spp.) on *Arabidopsis* showed that five of 12 *Arabidopsis* callose synthase genes were upregulated (Dong et al. 2008). Silencing the putative callose synthase gene *GSL5* led to decreased callose formation after mechanical wounding and infection with powdery mildew (*Blumeria graminis*) on *Arabidopsis* (Jacobs et al. 2003). Fungal growth was significantly impaired on the mutant, thus indicating a critical role of callose for the infection process. In plant–nematode interactions, callose was detected around nematode stylets inserted into plant cells of *Cricodemella xenoplax* (Hussey et al. 1992) and *Heterodera schachtii* (Sobczak et al. 1999) and along PD of syncytia (Jones and Payne 1977; Grundler et al. 1998). The role of callose deposition and degradation in NFS is presently unknown but may be involved in regulating the functioning of PD.

This study aims to localize PD and callose deposition along PD in the cell walls of giant cells induced by root-knot nematodes and syncytia induced by cyst nematodes. Furthermore, two T-DNA insertion mutants impaired in callose synthesis or callose degradation, respectively, were employed to elucidate the potential regulatory role of callose deposition for nematode development.

Our experiments revealed PD in cell walls of giant cells and in syncytia of advanced stages but specific temporal callose deposition along PD of advanced-stage syncytia (in *Arabidopsis* and tobacco) and giant cells (in tobacco), indicating impaired symplasmic exchange. The absence of callose deposition in giant cells of *Arabidopsis* suggests unrestricted functionality of the occurring PD. The importance of callose degeneration for syncytium development became apparent in a mutant analysis of *Arabidopsis*.

RESULTS

PD localization in cell walls of NFS.

In order to localize branched PD in the cell walls of galls and syncytia induced by root-knot and cyst nematodes, respectively, a tobacco line was used containing the MP of the *Tobacco mosaic virus* MP30^{TMV}-GFP fusion (Kragler et al. 2003). The analysis was complemented by using an *Arabidopsis* line containing an MP of the *Potato leaf roll polerovirus* (MP17^{PLRV}) fused to GFP (Hofius et al. 2001). This line has been previously used to study nematode-induced syncytia (5 and 14 days after inoculation [dai]) and root galls at late stages of development (18 dai) (Hoth et al. 2008). The tobacco GFP line was inoculated with two cyst nematode species, *Globodera tabacum* and *H. schachtii*, and one root-knot nematode species, *Meloidogyne incognita*. Although tobacco is not a natural host for *H. schachtii*, nematodes were able to complete their life cycle and produce infective offspring (Supplementary Fig. S1). Fresh slices (100 to 200 µm) of syncytia and giant cells induced in tobacco and *Arabidopsis* associated with nematodes at J2 to J4 stages were analyzed in vivo under the confocal microscope. A clear punctuated fluorescent signal throughout NFS indicates the presence of PD in both transgenic plant species (Fig. 1A, B, C, D, and F). In the tobacco line, a number of green fluorescing PD were localized in syncytia induced by *G. tabacum* (15 dai) and *H. schachtii* (10 dai) along cell walls toward neighboring cells (Fig. 1A and B). Many PD were visible between the giant cells induced by *M. incognita* (10 dai) and significantly fewer PD were observed between giant and neighboring cells (Fig. 1C).

In the *Arabidopsis* line, root galls induced by *M. incognita* (13 dai) showed numerous PD along the cell walls between giant cells and in cell walls toward neighboring cells (Fig. 1D), indicating symplasmic connection between these cells. Due to the current discussion about symplasmic connectivity of nematode induced syncytia (Hoth et al. 2005; Hofmann and

Grundler 2006), experiments were performed in a way that allowed us to determine the exact time point of nematode infection and syncytium induction. No PD were observed in syncytia induced by *H. schachtii* during the first 2 days after infection, in contrast with numerous PD observed in uninfected root primordia (Supplementary Fig. S2). Although PD were still rare in syncytia at 4 days after infection (Fig. 1E), a higher number of PD were detected mainly in the expanding fringe area at 7 days after infection (Fig. 1F).

Callose deposition at cell walls of NFS.

Callose deposition at cell walls along PD has been described to reduce their size exclusion limit and, therefore, alters solute transfer (Roberts and Oparka 2003). In the present work, immunolocalization was performed to visualize callose deposition in NFS of tobacco and *Arabidopsis* (Fig. 2). In tobacco roots infected with *G. tabacum* (15 dai), callose was deposited along the outer cell walls of the syncytia (Fig. 2A). Sections through syncytia in tobacco induced by *H. schachtii* (10 dai) showed a similar pattern, additionally revealing callose deposition along the outer walls of recently incorporated cells (Fig. 2B). In NFS induced by *M. incognita*, no callose deposition was observed along cell walls between giant cells but frequently along cell walls toward neighboring cells (Fig. 2C).

In *Arabidopsis*, syncytia induced by *H. schachtii* showed callose deposition at cell walls of margin syncytial cells, decreasing between 4 and 7 dai (Fig. 2E and F). Giant cells in *Arabidopsis* roots showed no callose deposition; callose was only detected in some neighboring cells (Fig. 2D). Thus, there is no indication that PD in the outer walls of giant cells are impaired by callose deposition; therefore, they should provide unrestricted symplasmic exchange between giant cells and neighboring cells.

Sampling may induce callose formation; therefore, in these experiments, specific attention was paid to controls. In noninfected *Arabidopsis* and tobacco roots, positive signals were almost absent and callose was deposited solely along the cell walls of a few phloem elements that are sensitive to sample treatments (Fig. 2G and H). Control experiments omitting the primary antibody resulted in no fluorescence, as shown for giant cells in tobacco roots (10 dai) (Supplementary Fig. S3).

Callose localization along PD.

In order to show that callose is deposited along PD, immunolocalization was performed on root sections of infected *H. schachtii* tissue of the MP17^{PLRV}-GFP *Arabidopsis* line. In treated tissue sections, GFP fluorescence has been preserved as confirmed by spectral analysis at the confocal microscope. The same sections were then used for immunolocalization of callose, resulting in a red fluorescent signal (Alexa 594) discernible from GFP. Potential co-localization is depicted as a yellow fluorescence (Fig. 3A). A closer look and detailed observations clearly showed deposition of callose on a number of PD while other PD were free of callose (Fig. 3B).

The relevance of callose synthesis and hydrolysis for nematode development.

The relevance of callose synthesis and hydrolysis for nematode development was studied in two *Arabidopsis* mutant lines used for nematode infection assays. Because callose was not found to be deposited along giant cells in *Arabidopsis*, this study was focused on syncytia induced by *H. schachtii*. The first line has a T-DNA insertion in the putative callose synthase gene *GLUCAN SYNTHASE-LIKE5* (*AtGSL5*), showing a strong reduction in wound callose and papillary callose formation after mechanical wounding and infection with *Sphaerotheca fusca*, respectively (Jacobs et al. 2003). The second line has the T-DNA

Fig. 1. A to C, Localization of plasmodesmata in the tobacco cell walls of nematode feeding sites expressing the MP^{TMV}-GFP fusion protein. A, *Globodera tabacum* (15 days after inoculation [dai]); B, *Heterodera schachtii* (10 dai); and C, *Meloidogyne incognita* (10 dai). Localization of plasmodesmata in the cell wall of nematode feeding cells of *Arabidopsis* plants expressing the movement protein of the *Potato leaf roll polerovirus* fused to green fluorescent protein (GFP) inoculated with D, *M. incognita* at 13 dai, E, *H. schachtii* 4 days after infection (dainf), and F, 7 dai. Cell walls representing the border of the nematode feeding structures are indicated in white. S = syncytium, * = giant cell, arrows point to GFP fluorescence. Bar = 20 μ m (A through D) and 50 μ m (E and F).

insertion in the exon of a β -1,3-glucanase (*AtBG_ppap*), an enzyme that degrades callose deposited along PD (Levy et al. 2007). The mutation revealed 45% higher callose accumulation and a significantly reduced diffusion of GFP compared with the wild type (Levy et al. 2007).

Nematode infection assays revealed that a lack of *AtGSL5* expression had no effect on nematode development (Fig. 4). These data show that reduced callose synthesis and, thus, callose deposition in cell walls of syncytia had no effect on nematode development. The impaired callose deposition in syncytia in the *Atgsl5* mutant was shown by immunolocalization (Supplementary Fig. S4). On the other hand, reduced callose degradation led to a significant shift of the female/male ratio in the *AtBG_ppap* mutant line (Fig. 4).

Decreased callose degradation in the *AtBG_ppap* mutant line does not influence connectivity to the phloem.

Callose deposition along cell walls of syncytia toward the phloem may affect PD opening during syncytium differentiation by narrowing solute import or retarding PD opening. Such changes could affect female nematode development that is highly sensitive to altered nutrient availability (Betka et al. 1991; Grundler et al. 1991). Thus, loading experiments with 5(6)-carboxyfluorescein diacetate (CFDA) were performed at 5, 7, 10, and 15 dai with the *AtBG_ppap* mutant line compared with the wild type (Supplementary Fig. S5). In the wild type, an increasing number of syncytia took up the dye, as shown previously (Hofmann et al. 2007). However, there was no difference in the number of CFDA positive syncytia in the wild type compared with the mutant at the studied time points. Therefore, we conclude that the mutation in the *AtBG_ppap* line does not influence the connection of syncytia to the phloem.

Callose degradation plays a pivotal role in syncytium expansion.

Apart from gating of PD toward phloem elements, callose degradation may play a significant role for syncytium enlargement (Grunder et al. 1998). In the *AtBG_ppap* line, the degradation of callose deposited along PD in young syncytia may be delayed compared with the wild type, reducing the expanding capacity of the syncytium by the incorporation of neighboring cells. Therefore, the size of syncytia was measured during the

Fig. 4. Nematode infection test using *Atgsl5* and *AtBG_ppap* T-DNA insertion lines. Plants inoculated with *Heterodera schachtii* showed a significant shift in female:male ratio in the *AtBG_ppap* line but not in the *Atgsl5* compared with the wild type (WT). Asterisks indicate significant differences (least significant difference test, $P < 0.05$); values are means $\pm$ standard error; $n = 15$.

Fig. 2. Localization of callose in cell walls of nematode feeding sites with an anticalllose antibody. Nematode feeding cells in tobacco wild-type roots inoculated with **A**, *Globodera tabacum* (15 days after inoculation [dai]); **B**, *Heterodera schachtii* (10 dai); and **C**, *Meloidogyne incognita* (10 dai). Nematode feeding cells in *Arabidopsis* wild-type roots of **D**, *M. incognita* at 7 dai and *H. schachtii* at **E**, 4 dai and **F**, 7 dai. **G**, *Arabidopsis* wild-type noninfected root and **H**, tobacco wild-type noninfected root. S = syncytium, X = xylem, * = giant cell, N = nematode, arrows point to callose depositions. Bar = 50 μ m (A, B, D, G, and H), 20 μ m (C and F), and 10 μ m (E).

Fig. 3. **A**, Double localization of callose and green fluorescent protein (GFP) in cell walls of syncytia of *Heterodera schachtii* (10 dai) in the movement protein (MP) of the *Potato leaf roll polerovirus* fused to GFP *Arabidopsis* line. **B**, Detail of **A**, as indicated by marked area. S = syncytium, arrow points to MP-GFP signals, arrow heads indicate MP-GFP-callose co-localization. Bar = 20 μ m (A) and 10 μ m (B).

first days of infection. Plants were grown on thin glass plates and nematode infection in wild-type and *AtBG_ppap* plants was marked after 1 day. The area of these syncytia was measured after 1, 2, 4, and 7 days. For the analysis, only syncytia were chosen that were associated by one single nematode and were sufficiently translucent so that their size could easily be measured with the AxioVision A.C. 4.1 software (Zeiss, Hallerbergmoos, Germany) (Supplementary Fig. S6). At 1 day after infection, syncytia developing in the roots of *AtBG_ppap* were already clearly smaller than those in the wild type (Fig. 5). In the following days, this difference in size increased, resulting in twofold smaller syncytia in the mutant line at 7 days after infection compared with the wild type (Fig. 5). These results indicate a clear effect of callose degradation on syncytium expansion.

DISCUSSION

The occurrence and functionality of PD in the cell walls of nematode-induced feeding sites has been under investigation and discussion during the last years (Jones and Northcote 1972; Jones and Dropkin 1976; Jones and Payne 1977; Dorhout et al. 1993; Böckenhoff and Grundler 1994; Böckenhoff et al. 1996; Grundler et al. 1998; Hoth et al. 2005, 2008; Hofmann and Grundler 2006; Hofmann et al. 2007). Both the presence and functionality of PD may affect structure, development, maintenance, and morphogenesis of nematode feeding sites. Therefore, the results presented provide substantial information to the cellular communication and solute exchange of the studied interactions. Unrestricted PD passage, on one hand, facilitates intracellular communication and enables enhanced solute import; however, on the other hand, may lead to nutrient loss from the feeding cells to adjacent cells of the vasculature. Furthermore, PD are important start points for cell wall degradation, an essential step for syncytial formation and expansion.

To date, giant cells induced by the root-knot nematode *M. incognita* were believed to be symplasmically isolated from the vascular tissue and neighboring cells but tightly connected by PD among each other (Jones and Dropkin 1976; Hoth et al. 2008). The present study confirmed the existence of a high number of PD between the giant cells in tobacco roots. In contrast to earlier studies, PD were also found in the cell wall of giant cells toward neighboring cells. However, deposition of callose along these PD indicates impaired functionality as previously suggested (Wolf et al. 1991). In *Arabidopsis*, the situation was clearly different. PD were distributed along the entire

cell wall of giant cells and no callose deposition was detected. Thus, evidence is provided for the first time that giant cells are symplasmically connected to neighboring cells through functional PD. Mechanisms involved in NFS development may vary between different plant hosts. In the present work, giant cells from tobacco formed less secondary PD compared with *Arabidopsis* whereas callose deposition was detected in giant cells in tobacco roots but not in *Arabidopsis* (Fig. 2C and D). The high number of branched PD indicates a de novo formation of PD which may be the response to an increased metabolic sink triggered by nematode feeding. In contrast to secondary PD, the number of primary PD is not expected to increase in both NFS. Primary PD emerge during de novo cell wall synthesis after cell division, a process that does not occur during NFS formation. Up till now, there was no information on how phloem-derived solutes are unloaded in giant cells. Here, we show the presence of a large number of PD in the walls between giant cells and neighboring cells, indicating a symplasmic solute transfer toward giant cells. Apoplastic solute supply by transport proteins (Hammes et al. 2005) will mainly be relevant for specific reactions such as sugar transporters coupled with cell-wall-bound invertases or cellulases (Koch 2004). The presented results differ from previous studies using the same *Arabidopsis* MP17^{PLRV}-GFP line, where PD were observed in walls of neighboring cells but not within the cell walls flanked by giant cells (Hoth et al. 2008). Because we observed a clear and distinct GFP signal, the difference may be due to the use of different protocols: in fact, Hoth and associates (2008) inoculated 4-week-old seedlings with *M. incognita* juveniles and performed microscopic observations at 18 dai. *Arabidopsis thaliana* plants grown in petri dishes undergo an accelerated life cycle and, thus, are in an advanced developmental stage after 6 weeks. The advanced age of the inoculated plants may affect GFP gene expression (Kronberg et al. 2007) and, therefore, could have impeded microscopic detection of the MP-GFP signal. During our studies, younger seedlings (12 days old) were inoculated and observed at 13 dai. Further, the occurrence of PD in giant cells may depend on the age of the host plant, so that no secondary PD were established in the cell walls of giant cells in these older plants studied by Hoth and associates (2008). This option, however, is not probable because the absence of PD may affect nematode development that was not reported in this study.

In order to study PD distribution in syncytia induced by cyst nematodes, the infection sites of two nematode species (*H. schachtii* and *G. tabacum*) in tobacco and *Arabidopsis* were compared. All pathosystems presented PD in cell walls of syncytia toward neighboring cells and the phloem. PD were rarely detected in young syncytia of *Arabidopsis*, confirming our previous phloem-loading experiments, which indicated the isolation of syncytia during the first days of feeding site development (Hofmann and Grundler 2006; Hofmann et al. 2007). In addition, the observed callose deposition along PD may affect solute transfer by reducing the size exclusion limit (Wolf et al. 1991; Roberts and Oparka 2003; Levy et al. 2007). In this work, we successfully co-localized MP17^{PLRV}-GFP and callose using the previously published immunocytochemical detection method in feeding sites (de Almeida Engler et al. 2004). The observed preservation of the GFP signal after the different steps of fixation for immunolocalization has been previously described (Thompson and Wolniak 2008). Immunolocalization showed callose deposition mainly at the edges of syncytia, the area of syncytium expansion. Callose deposition during early stages of syncytium differentiation confirms previous observations (Grunder et al. 1998). The frequency of callose plugs decreased during syncytium and nematode development (4 to 7 dai). The increasing number of PD and de-

Fig. 5. Size of syncytia induced by *Heterodera schachtii* in *Arabidopsis* roots of wild-type (white) and *AtBG_ppap* (gray) lines. Syncytia developing in the mutant show a reduced ability to expand at 1, 2, 4, and 7 days after infection. Asterisks indicate significant difference (least significant difference test); ** and *** indicate $P < 0.01$ and 0.001 , respectively; values are means $\pm$ standard error; $n = 18$.

creased callose deposition along PD in cell walls of syncytia during nematode development is also in agreement with our previous and present fluorochrome transport studies. Phloem-loading experiments showed an increasing number of syncytia taking up the dye during nematode development (Hofmann and Grundler 2006; Hofmann et al. 2007).

It is a common phenomenon that plant tissues and organs switch between symplasmic isolation causing impaired solute passage and symplasmic connectivity during development. Initial cells of the *Arabidopsis* embryo consist of one single symplasmic domain forming subdomains as development proceeds (Kim and Zambryski 2005). Mutant lines maintaining PD with large size-exclusion limits were not able to form embryos, indicating that symplasmic isolation is essential during embryogenesis (Kim et al. 2002). During cotton fiber elongation, a 5-day period of transient symplasmic isolation accompanied by an increased expression of plasma membrane transporters has been described (Ruan et al. 2001). The reopening of the PD was observed at the end of cotton fiber elongation and was suggested to be coupled with turgor pressure release. During fiber elongation, callose was detected at the base of the fibers when PD were closed but never at opened PD, suggesting that callose was involved in PD gating (Ruan et al. 2004). In *Arabidopsis*, the root meristem forms one symplasmic unit and, during plant development, specific root cells such as the differentiated maturing phloem become symplasmically isolated from their surroundings (Oparka et al. 1994). Furthermore, PD are sensitive to biotic stress, which often leads to an immediate closure by rapid accumulation of callose in order to prevent spreading, as described for viruses (Rinne et al. 2005).

During nematode invasion and feeding site induction, callose may be deposited as a general defense mechanism of plants. *M. incognita* penetrates intercellularly, inducing little root cell damage during migration. Furthermore, we show that these root-knot nematodes induce no callose deposition along PD in cell walls of their giant-feeding cells; therefore, callose mutants may not affect nematode migration and development. In contrast, the two studied cyst nematodes move intracellularly, leaving a track of damaged cells. Infective juveniles of both types of pathogenic nematodes then select initial feeding cells by piercing various cells with their stylets (Wyss 1992; Wyss et al. 1992). Repeated retractions and reinsertions of the stylet during feeding events may provoke plant cell defense responses such as callose deposition (Wyss 1992; Wyss et al. 1992). However, the *Atgsl5* mutant line with reduced callose deposition affecting fungal infections (Jacobs et al. 2003) had no effect on nematode development or nematode infection, as shown in our study. Because callose is a plant defense reaction that separates the plant cell from the root symplast, reduced callose deposition may facilitate cell fusion along PD during syncytium formation. This may cause even slightly favorable conditions for female nematode development. On the other hand, we observed that reduced callose degradation in the *AtBG_ppap* mutant line significantly affected cyst nematode development. Prior to the incorporation of additional cells into the syncytia by cell wall dissolution, the deposited callose must be degraded. In our study, decreased callose degradation led to smaller syncytia. This may result in a number of effects, such as a diminished surface available for nutrient uptake and a reduced metabolic activity. Our results support earlier observations that reduced nutrient supply significantly affect nematode sexual differentiation, resulting in a higher proportion of male nematodes compared with the control (Betka et al. 1991; Grundler et al. 1991).

The present work gives insights into the role of PD frequency and distribution and callose deposition along cell walls of NFS. Numerous PD could be found in the cell walls of giant cells induced by the root-knot nematode in *Arabidopsis*, indi-

cating massive symplasmic solute transfer. Syncytia induced by cyst nematodes seem to benefit from both symplasmic isolation and connectivity according to their developmental needs. We further demonstrated that callose degradation is important for syncytium expansion and cyst nematode development.

MATERIALS AND METHODS

Plant and nematode culture.

Sterile *A. thaliana* and *Nicotiana tabacum* seed were sown on 0.2% Knop medium (Sijmons et al. 1991) and grown under conditions of 16 h of light, 8 h of darkness, and 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at 21°C. After 12 to 14 days, plants were inoculated with freshly hatched J2s of *M. incognita*, *G. tabacum*, or *H. schachtii* sterilized as previously described (Sijmons et al. 1991; Griesser and Grundler 2008).

Immunostaining and microscopy.

For immunostaining, root sections were fixed and treated as previously described by de Almeida Engler and associates (2004). Immunolocalization of callose was performed on tissue sections from infected roots using a 200 $\times$ diluted primary monoclonal (mouse immunoglobulin G [IgG]) anti-callose antibody (Biosupplies Australia, Parkville, Australia). As secondary antibody, Alexa Fluor 488 goat anti-mouse (Invitrogen, Carlsbad, CA, U.S.A.) was used, 300 $\times$ diluted in blocking solution (2% bovine serum albumin in 50 mM PIPES, pH 6.9). As control, the primary antibody was omitted. Sections were observed with an epifluorescence microscope (Axioskop; Zeiss) equipped with a standard filter set. Images were taken using the Zeiss AxioCam HRC digital camera.

Confocal laser scanning microscopy.

Tobacco and *Arabidopsis* plant containing the MP-GFP fusion genes (Hofius et al. 2001; Kragler et al. 2003) were grown and inoculated as described above. NFS (10 or 15 days old) were cut out from the roots and sectioned to 100 to 200 μm with a vibratome (Microm HM650V). Sections were imaged with a confocal laser scanning microscope (LSM510 META; Zeiss). GFP was excited with the 488-nm Argon laserline and imaged with a 505- to 530-nm BP emission filter. To have a complete picture of the presence of PD in nematode feeding sites, serial images were collected at different focal planes. In order to discriminate autofluorescence resulting from the cell walls from GFP fluorescence, images were taken with the spectral mode. For the co-localization of the MP17^{PLRV}-GFP and the callose signal, we have used the secondary antibody Alexa Fluor 594 goat-anti mouse IgG (Invitrogen), 300 $\times$ diluted. Images were generated in the multitrack mode to avoid signal overlap using the excitation lines 488 and 543 nm, respectively, from the Argon and the HeNe laser.

Phloem-loading.

Phloem-loading experiments with CFDA (Molecular Probes, Eugene, OR, U.S.A.) were performed on wild-type and *AtBG_ppap Arabidopsis* lines that were inoculated with *H. schachtii* as described previously (Wright and Oparka 1997; Hofmann et al. 2007). Phloem loading was performed at 5, 7, 10, and 15 dai in three independent experiments. NFS were screened for fluorescent signals with an inverted microscope (Axiovert 200M; Zeiss) using UV light excitation and a fluorescein isothiocyanate filter.

Nematode infection test.

Arabidopsis AtBG_ppap and *Atgsl5* mutant lines provided by Jacobs and associates (2003) and Levy and associates (2007) were tested for nematode infection compared with the

wild type. For the infection test, 15 petri dishes with 10 plants each were grown in three separate repetitions. Plants were grown as described above and root length was estimated according to Jürgensen (2001) before nematode inoculation. At 15 dai, roots were screened for developing female and male nematodes. Statistical differences were calculated by applying the analysis of variance least significant difference test.

Syncytium measurements.

Seed were sown on Knop medium poured on glass plates (0.15-mm thickness, 80-mm diameter). The glass plates were then placed into sterile petri dishes (90 mm in diameter). After 12 days, plants were inoculated as described above. One day after inoculation, glass plates were removed from the petri dishes and syncytia were marked. These syncytia were photographed consecutively at 1, 2, 4, and 7 days after infection by putting the glass plates under the inverted microscope (Axiovert 200M; Zeiss). For syncytium size analysis, syncytia were chosen that only fed one nematode, and that were translucent, so that their size could easily be measured with the Zeiss software (AxioVision A.C. 4.1; Zeiss) analog to Hewezi and associates (2008).

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