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Activation of a Pollenin Promoter upon Nematode Infection

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Abstract: Three glycine-rich protein genes of *Arabidopsis thaliana* (*Atgrp-6, Atgrp-7,* and *Atgrp-8*) that correspond to putative genes coding for pollenins (*AtolnB;2, AtolnB;3,* and *AtolnB;4,* respectively) are expressed predominantly in the anthers and, more specifically, in the tapetum layer. Tapetal cells are responsible for nutrition of developing pollen grains and show some functional similarities to nematode feeding sites (NFS) induced in plant roots by sedentary parasitic nematodes. The aim of this study was to analyze promoter activity of the *Atgrp* genes in NFS. Transformed *Arabidopsis* plants containing a promoter-β-glucuronidase (*gus*) fusion of the *Atgrp-7* gene were inoculated with the root-knot nematode *Meloidogyne incognita* and the cyst nematode *Heterodera schachtii.* GUS assays were performed at different time points after infection. Histochemical analysis revealed an up-regulation of *Atgrp-7—gus* expression 3 days after inoculation in the feeding sites of both nematodes. Maximal *Atgrp-7—gus* staining levels in NFS were observed 1 week after nematode infection.

Key words: Arabidopsis thaliana, Atgrp, glycine-rich proteins, *Heterodera schachtii, Meloidogyne incognita,* oleosin, pollenin, tapetum.

Nematodes that are sedentary endoparasites infect roots of host plants and induce the formation of complex feeding sites. Morphological and physiological changes in nematode feeding sites (NFS) are correlated with alterations of host gene expression. Feeding sites function as transfer cells and provide nourishment for nematode growth, development and reproduction (Hussey, 1985). Molecular techniques, such as differential screening of cDNA libraries (Bird and Wilson, 1994; Van der Eycken et al., 1996), promoter tagging (Barthels et al., 1997; Favery et al., 1998), and differential display (Hermsmeier et al., 1998; Vercauteren et al., 2001) have been used to identify and analyze plant genes that are up- or down-regulated by nematode infection. Furthermore, the study of known genes that could be involved in feeding site formation or function increases our knowledge about the plant-nematode interaction. For instance, activation of cell cycle genes correlates with increased size and number of nuclei in the early feeding cell stages and points to the involvement of these events in NFS development (Gheysen et al., 1997).

Tapetal cells are responsible for nutrition of developing pollen grains and therefore show similarities with NFS (Mascarenhas, 1975). In both systems, cells are multinucleate (D'Amato, 1984; Jones and Northcote, 1972), have cell wall ingrowths, and act as transfer cells (Pate and Gunning, 1972).

Oleosins are a group of proteins associated with lipid bodies (Huang, 1992). The oil bodies in pollen of many species are used as food reserves for the growth of the pollen tube (Mascarenhas, 1975). Oleosins have been suggested to extract lipids in the tapetum to form lipid particles and facilitate their storage in pollen exine (Wang et al., 1997). The aim of this study was to analyze the expression of oleosin genes in NFS, which have previously been reported to be tapetum specific (Alves Ferreira et al., 1997) and have been renamed pollenins (Murphy et al., 2001). The tandemly arrayed genes *Atgrp-6, Atgrp-7,* and *Atgrp-8* have been isolated from *Arabidopsis thaliana* by differential screening of a genomic library and encode three different glycine-rich proteins (GRPs) (de Oliveira et al., 1990, 1993). These genes have a high sequence homology in the hydrophobic domain with the oleosin protein family (Alves Ferreira et al., 1997) and are expressed predominantly in anthers, specifically in the tapetum layer (Alves Ferreira et al., 1997). The expression pattern of a fusion between the *Atgrp-7* promoter and β-glucuronidase (*gus*) in transgenic *Arabidopsis* lines was determined during a time course after inoculation with the root-knot nematode *Meloidogyne incognita* or the cyst nematode *Heterodera schachtii.*

Materials and Methods

Plant material: *Arabidopsis thaliana* (L.) Heynh. C24 was transformed by the pTC025 construct (Fig. 1) containing a genomic fragment corresponding to the promoter of the glycine-rich protein gene *Atgrp-7* fused to the β-glucuronidase (*gus*) reporter gene (Franco, De Oliveira Manes, Sachetto-Martins, de Oliveira, unpubl. data). A complex *grp-gus* fusion was used that contains the genomic region with the *Atgrp-6* promoter (P*Atgrp-6*) and coding sequence and the *Atgrp-7* promoter (P*Atgrp-7*) (Fig. 1). Deletion experiments have shown that sequence elements upstream of the *Atgrp-7* promoter (including those in the *Atgrp-6* gene region) are required for correct expression of the *Atgrp-7* gene (Franco, De Oliveira Manes, Sachetto-Martins, de Oliveira, unpubl. data). Seeds were kindly provided by D. E. de Oliveira (Laboratório de Genética Molecular Vegetal, Rio de Janeiro, Brazil).

In vitro culture of M. incognita *and* H. schachtii: Hairy

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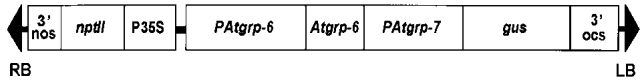

FIG. 1. The pTC025 construct used to assess pollenin gene expression in *Arabidopsis thaliana* (*Atgrp*). The neomycin phosphotransferase II (*nptII*) gene and *Atgrp-gus* fusion (see Materials and Methods) are located at the right border (RB) and left border (LB), respectively. *gus*, β-glucuronidase gene; P35S, promoter of the cauliflower mosaic virus 35S; 3′nos, 3′ region of the nopaline synthase gene; 3′ocs, 3′ region of the octopine synthase gene; P*Atgrp-6* and P*Atgrp-7*, promoters of *Atgrp-6* and *Atgrp-7*, respectively.

roots of tomato (*Lycopersicon esculentum* L.) were used to maintain the *Meloidogyne* culture (Verdejo et al., 1988). Egg masses of root-knot nematodes were hatched in sterile distilled water. Cyst nematodes (*H. schachtii*) were cultured on monoxenically grown mustard (*Sinapis alba*) roots (Sijmons et al., 1991). Hatching was initiated by submerging cysts in 5 mM filter-sterilized $ZnCl_2$.

Inoculation of transformants: Seeds of transformants were surface-sterilized (Valvekens et al., 1988) and germinated on Murashige and Skoog (1962) medium containing 1% sucrose and 50 mg/liter kanamycin (pH 5.7) in solidified 0.8% (w/v) Select agar® (Invitrogen, Carlsbad, CA). Seedlings were incubated in a growth room at 22 °C (16 hours light/8 hours dark photoperiod). Two-week-old kanamycin-resistant plants were transferred to Knop medium (Sijmons et al., 1991). Two days later, the root tips of these plants were inoculated with hatched second-stage juveniles (J2) of *M. incognita* or *H. schachtii* (10 nematodes/root tip). The inoculated plants were incubated in the growth room at 20 °C with a light/dark regime of 16 hours/8 hours.

GUS histochemical assay: At different time points after inoculation with nematodes, *A. thaliana* plants were subjected to a GUS histochemical assay (Jefferson et al., 1987). Prior to the GUS assay, plants were incubated for 30 minutes in 90% cold acetone followed by several washes with 100 mM sodium phosphate (pH 7.2). After the GUS assay, plants were bleached with 70% (v/v) ethanol and examined under the light microscope.

RESULTS

For expression analysis of the P*Atgrp-gus* fusion, GUS histochemical assays were performed on the seedlings transformed with the pTC025 construct (Fig. 1) during a time course after inoculation with *M. incognita* and *H. schachtii*. Three days after inoculation, GUS activity could be detected in galls (*M. incognita*) and syncytia (*H. schachtii*) but was absent in control roots (Fig. 2). GUS staining was limited to the nematode infection site, and no staining was observed in the non-infected parts of infected roots, except for the vascular cylinder close to the feeding site. GUS staining in NSF was maximal 1 week after inoculation with cyst and root-knot nematodes. Two weeks after inoculation, GUS staining in NSF was less strong as compared to the first week after inoculation. One month after inoculation, GUS was very weak. Although the *gus* expression pattern over time was similar in the NFS of both nematodes, the intensity of GUS staining in *M. incognita*-infected roots was reproducibly stronger at all time points than in those infected by *H. schachtii*. In non-infected plants, *gus* expression was predominantly visible in the anthers (data not shown).

DISCUSSION

One way to understand the molecular mechanisms underlying plant-nematode interactions is to study known plant genes with a possible function in the development of NFS. This strategy has been used to study the expression of several genes in NFS, such as those encoding the cell wall protein extensin (Niebel et al., 1993), a water channel protein *TobRB7* (Opperman et al., 1994), *Parasponia* hemoglobin (Ehsanpour and Jones, 1996), a copper diamine oxidase *atao1* (Møller et al., 1998), and cell cycle genes in NFS (de Almeida Engler et al., 1999; Niebel et al., 1996). The similarity between tapetal cells and NFS has been described by several authors; both cell types contain a dense and granular cytoplasm, have a high DNA content (Hussey and Grundler, 1998; Jones, 1981), and function as transfer cells (Jones and Northcote, 1972; Pate and Gunning, 1972). Nematode feeding sites provide food to the developing nematode (Huang, 1985) and tapetal cells for the pollen grain (Roberts et al., 1995). These functional analogies predict at least some similarities in gene expression. The *Arabidopsis thaliana* pollenin genes (*Atgrp*) that are expressed mainly in the tapetum (Alves Ferreira et al., 1997; de Oliveira et al., 1993) were selected to address this hypothesis.

Oleosins are glycine-rich proteins that were found on lipid bodies in desiccation-tolerant seeds. Certain anther-specific proteins, now called pollenins, contain an oleosin-like domain and are localized initially on lipid bodies in the tapetum. However, after the oleosin domain has been cleaved off, the mature proteins are major components of the pollen coat where they may be involved in pollen rehydration by making water channels (Mayfield and Preuss, 2000; Murphy et al., 2001). We have shown that the P*Atgrp-gus* fusion is strongly expressed in the NFS of *M. incognita* and *H. schachtii*, with a peak 1 week after inoculation. Therefore, transcriptional activity of *Atgrp-7* in NFS must culminate around this time point, although more time points and RNA quantification methods would be needed to determine exactly this maximum. Regardless of the plant species and nematode, most morphological changes occurring in the NFS are visible within 1 week after infection. Whereas the nutrient demand of nematodes still increases after the first week of infection, the promoter

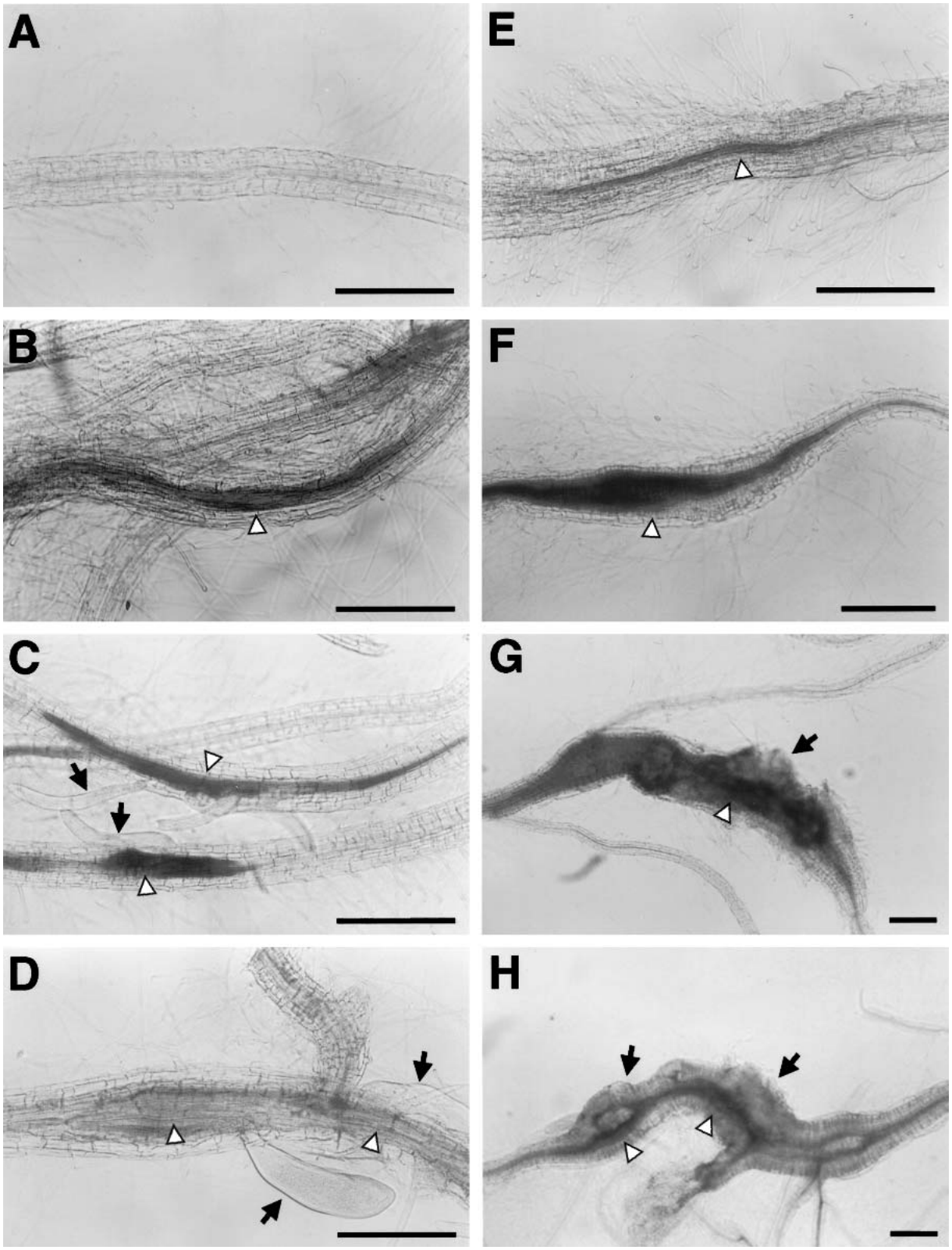

FIG. 2. *GUS* expression patterns upon nematode infection in *Arabidopsis* plants harboring the P*Atgrp-gus* fusion. A) Non-infected control root without GUS staining. B) Strong GUS staining 3 days after infection by *Heterodera schachtii*. C) Very strong GUS staining 1 week after infection by *H. schachtii*. D) Weak GUS staining 2 weeks after infection by *H. schachtii*. E) Strong GUS staining 3 days after infection by *Meloidogyne incognita*. F) Very strong GUS staining 1 week after infection by *M. incognita*. G) Very strong GUS staining 2 weeks after infection by *M. incognita*. H) Strong GUS staining 1 month after infection by *M. incognita*. Arrows and arrowheads indicate nematodes and feeding sites, respectively. Scale bars equal 300 μm.

activity of the *Atgrp-7* gene was not as strong during this time period.

Several pollenin genes have been identified, but a precise role for the *Atgrp* genes in the NFS is difficult to propose because the function of the GRP proteins is not fully understood (Frandsen et al., 2001; Sachetto-Martins et al., 2000). Nevertheless, based on the two possible functions of pollenins, different hypotheses can be formulated. Oleosin promoters have been shown to be activated in young root tips; this observation has been correlated with the presence of lipid bodies (Murphy et al., 2001; Næsted et al., 2000). GUS staining was also detected in some root tips of *Arabidopsis* plants transformed with P*grp-gus* (data not shown). There is evidence that numerous lipid bodies are present in mature syncytia (Sobczak, pers. comm.); therefore, pollenins might play a role in lipid stabilization of these NFS (Sachetto-Martins et al., 1995). Alternatively, the possible water-channel function of pollenins may be involved in cell water regulation of NFS, as proposed for aquaporins that have been demonstrated to be up-regulated in giant cells (Opperman and Conkling, 1994).

In summary, we have demonstrated that the promoter activity of the *Atgrp-7* genomic region is activated strongly in feeding sites induced by root-knot and cyst nematodes. Further experiments should be done to specify the sequences of the *Atgrp-7* region that are responsible for up-regulation in the NFS.

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