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Glucuronidase Expression in Transgenic Tobacco Roots with a *Parasponia* Promoter on Infection with *Meloidogyne javanica*[1]

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Abstract: The expression of a *gus* reporter gene linked to a *Parasponia andersonii* hemoglobin promoter has been studied in transgenic tobacco plants after infection by *Meloidogyne javanica.* Transgenic roots were harvested at different times after nematode inoculation, and stained histochemically for expression of the *gus* gene. During the early stages of infection (0–2 weeks) there was little expression in giant cells, in contrast to other cells of the root. In later stages of infection (3–6 weeks) there was strong *gus* expression in giant cells, with virtually no expression in other cells of the root. The *Parasponia* hemoglobin promoter therefore appears to direct down-regulation of linked genes on induction of giant cells, but up-regulation in mature giant cells. This reflects different metabolic activities in the giant cells depending on their stage of development. The *Parasponia* hemoglobin promoter may respond to oxygen tension in giant cells. This suggests that oxygen tension may be limited in the metabolically active giant cells that are associated with egg-laying females.

Key words: down-regulation, gene expression, glucuronidase, *Meloidogyne javanica,* nematode, *Nicotiana tabacum, Parasponia andersonii,* root-knot nematode, tobacco, up-regulation.

The expression of genes in cells of host plant roots is affected by infection with nematodes. This is clearly evident, for example, in the formation of giant cells in susceptible host roots that are induced by root-knot nematodes, and from which the nematodes feed (12–14). Knowledge of genes that are up- or down-regulated during nematode infection will be useful in devising molecular approaches to engineer host resistance (6,20,22). Various strategies can be employed to understand more about which genes are up-regulated or down-regulated as a result of nematode infection (6,22). These include cloning cDNAs of expressed genes in control non-infected and infected roots, subtracting those expressed in control roots and characterizing the selected clones to check the specificity of their expression (2,3,22,23), "tagging" up-regulated promoters with marker genes (9), and examining transgenic plants containing specific promoter-glucuronidase (*gus*) inserts (8,9,19,21).

Because most endoparasitic nematode-host plant interactions occur in roots, it is relevant to study the effect of nematode infection on expression of root-specific promoters. One such promoter, from the hemoglobin gene of *Parasponia andersonii* Planch. (a nitrogen-fixing non-legume) was reported by Landsmann et al. (16,17). The *Parasponia* hemoglobin gene is strongly expressed in the transfer cell zone of nodules formed with a nitrogen fixing symbiont (*Rhizobium*), and also in roots of non-nodulated plants (4). When the promoter was cloned and introduced into tobacco driving a *gus* reporter gene, a high level of expression was found in roots of transgenic plants (5). The proposed func-

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tion of root-expressed hemoglobins in non-legumes is that they may act either as sensors of oxygen or oxygen transporters in the root (1,5). In addition, nitrogen fixing nodules have some features in common with nematode-induced giant cells (e.g., presence of transfer cell wall ingrowths in some nodule cells, related to solute movement, and metabolically active cells). Therefore, we have studied the effect of nematode infection on reporter gene expression driven by the *Parasponia* hemoglobin promoter. In replicated experiments, transgenic tobacco plants containing the *Parasponia* hemoglobin promoter linked to the *gus* gene have been infected with juveniles of *Meloidogyne javanica* (Treub) Chitwood, and analyzed for the expression of the *gus* reporter gene in nematode-induced giant cells, and other root and control tissues, during the life cycle of the nematode-host association.

Materials and Methods

Nematode eggs and juveniles were prepared as described by Hussey and Barker (10). Roots of tomato plants (cv. Grosse Lisse) infected with *M. javanica* were washed, cut into small pieces (3–5 cm), and placed in 100 ml of 5% to 10% (v/v) sodium hypochlorite in 200-ml flasks, which were then shaken vigorously for 5 to 10 minutes. The supernatant was passed through 100-μm stainless steel sieves and centrifuged at 2,000 rpm in a bench-top centrifuge (Hettich Universal). The pellet, containing nematode eggs, was washed three times with distilled water. Seeds from transgenic tobacco plants, *Nicotiana tabacum* L., containing the *Parasponia* hemoglobin promoter fused to the β-glucuronidase (GUS) reporter gene were kindly supplied by D. Llewellyn, CSIRO Division of Plant Industry, Canberra, Australia. The construct is a transcriptional fusion of 1 kb of the hemoglobin promoter fused to the glucuronidase reporter gene with a nopaline synthase termination sequence (5). Seeds were surface-sterilized with 10% sodium hypochlorite for 15 minutes followed by three washes with sterile distilled water, and were germinated and grown on MS medium (18) with sucrose (30 g/liter) and solidified with agar (0.7 g/liter). Seedlings germinated in tissue culture vessels were transferred to 500-ml clean plastic pots containing steam-sterilized river sand and grown in a glasshouse (normal daylight at 25-26°C). After 2 weeks growth, each plant was inoculated with 2,000 or 6,700 eggs or juveniles of *M. javanica* (experiments one and two, respectively). Pots were fertilized with commercial fertilizer (Spring) every 2 weeks. Plants were examined for *gus* gene expression at various times after infection by histochemical staining with 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-gluc) (11). In experiment one, three plants each from inoculated plants and non-infected plants (control) were carefully harvested at 3, 4, 10, 18, and 27 days after inoculation, and roots were washed gently. Ten to 15 roots (5–10 cm long) from each individual plant were cut, put in 2.5-ml petri dishes, and 1 to 2 ml of fresh X-gluc solution (1 mM X-gluc, 100 mM sodium phosphate pH 7.0, 0.5% Triton X-100) was added to each dish. The dishes were sealed with Nescofilm and incubated at 37°C in the dark overnight. After staining, roots were washed with distilled water and then examined with an inverted light microscope or stereo microscope. The tissue location of blue staining and the number of roots in which the *gus* gene was expressed were recorded. In some cases nematodes inside the roots were stained with acid-fuchsin as described by Byrd et al. (7) after X-gluc staining. In experiment two, five infected and five noninfected transgenic plants were analyzed for expression of *gus* at each time point, as described above, 10, 23, 31, and 46 days post-infection. The roots were handled carefully on harvesting and during subsequent handling to minimize damage.

Results

The roots were carefully harvested at the different time points and, based on the

presence or absence of blue staining, the expression of the glucuronidase reporter gene was scored in the different root tissues (root tip, vascular tissue, cortex, giant cells, galls). As is normally the case when transgenic plant tissues are stained with X-gluc for expression of the *gus* gene, there was occasional patchiness of staining. However, the large number of roots examined provided a clear picture of the overall *gus* expression in the experiments. In experiment one, a total of 148 infected and 148 uninfected transgenic roots were examined and scored; in experiment two, 216 infected and 216 uninfected roots were scored. Tissues were scored as expressing the *gus* gene if blue staining was present in some, but not necessarily all, of its constituent cells. The results were converted to percentage of each tissue, which showed cells with *gus* expression, and these are summarized and presented graphically in Fig. 1A,B and as color illustrations in Fig. 2. During these experiments, dramatic differences of expression between early and later stages of infection were observed (Figs. 1,2).

At 4 days post infection, in both control (noninoculated) and nematode-inoculated transgenic roots, blue staining indicating *gus* expression was observed in cells of the cortex and vascular tissues throughout the length of young roots (Figs. 1A; 2D), except that there was much less staining in meristematic regions of root tips. (Occasionally root tips also stained blue, but this was the exception rather than the rule.) As indicated in Fig. 2E, with careful examination it was possible to identify developing giant cells and associated nematodes 3 to 4 days post infection. *Gus* expression was not observed in such giant cell initials at this stage. By 10 days after infection there was a reduction in *gus* expression in vascular tissues and cortical cells of inoculated transgenic plants (72% to 75% expression), whereas *gus* expression was observed in 10% to 15% of the giant cell complexes associated with individual nematodes (Fig. 2F). With increasing time after infection (18–27 days), *gus* expression gradually increased in giant cell complexes (55% to 82% expression) (Fig. 2G) and decreased in other tissues of the root. At this stage, a few blue staining cells were observed in galls or in the cortex of the roots. From 31

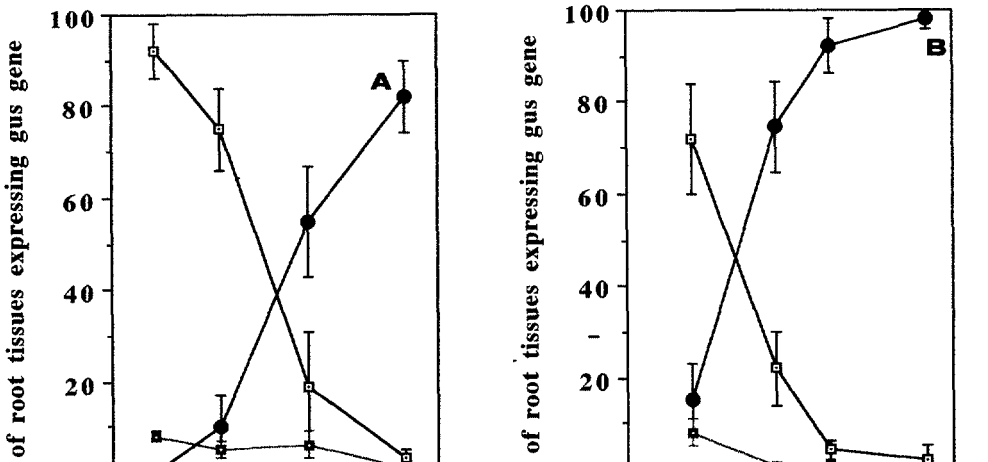

FIG. 1. Percentage of root tissues expressing the *Parasponia andersonii-gus* gene product in *Nicotiana tabacum* roots infected with *Meloidogyne javanica*. Error bars indicate standard error of the mean for each time point. A) Experiment 1; B) Experiment 2. *Gus* expression in cortical and vascular cells (□). *Gus* expression in giant cell complexes (excluding gall tissue) (●) *Gus* expression in root tips (■).

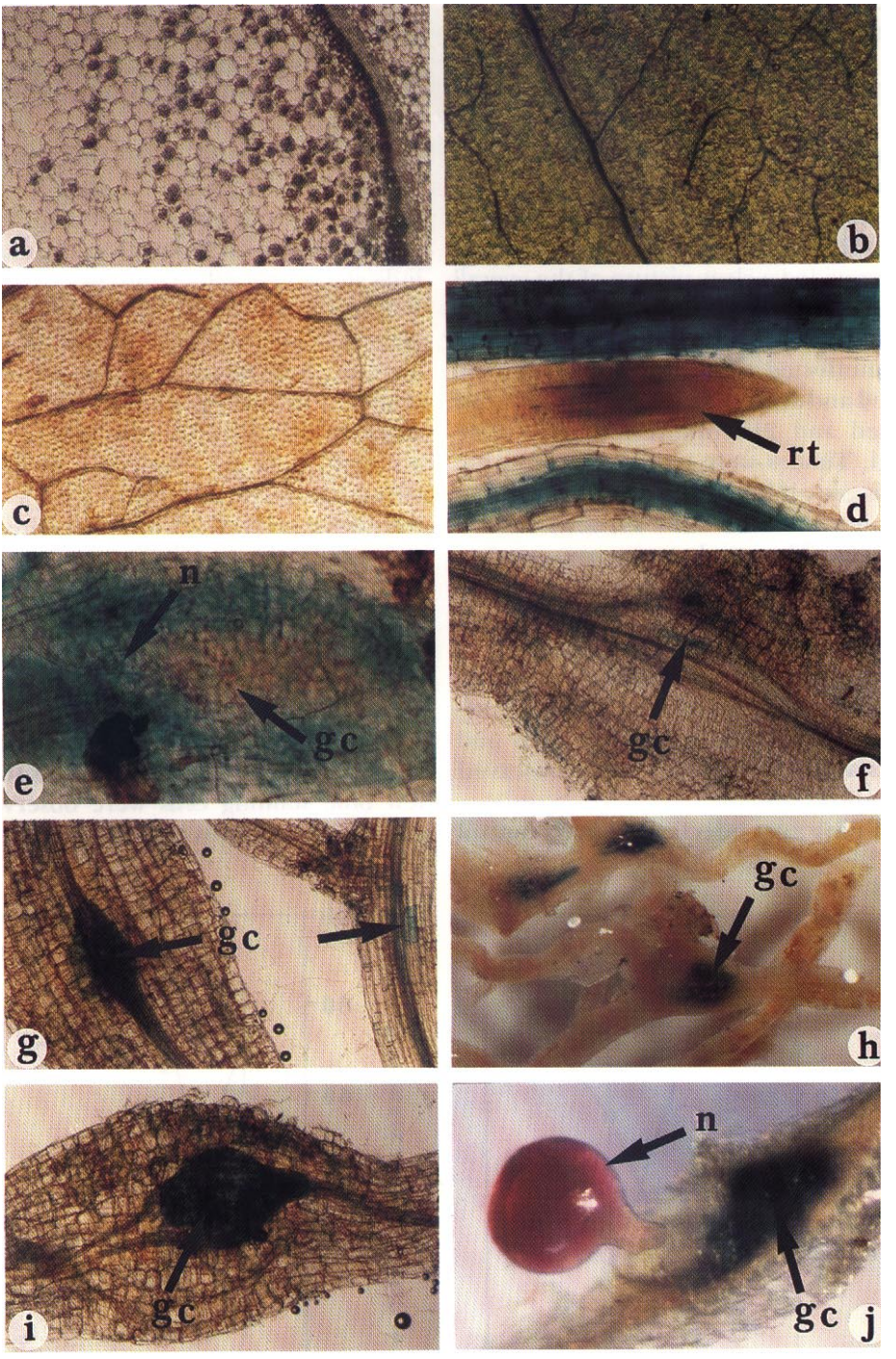

FIG. 2. Color micrographs of control (A–D) and *M. javanica*-infected tissues (E–J) of transgenic *Parasponia-gus* plants after staining with X-gluc. A) Stem tissues (no blue staining) (×45); B) Leaf tissues (no blue staining) (×45); C) Petal tissues (no blue staining) (×45); D) Non-infected young root tissues, with blue staining cells in cortical and vascular tissues; the root tip is unstained (×30); E) Root 3 days after infection with *M. javanica*. A nematode juvenile (n) is clearly visible with its head by a group of giant cell initials (gc) (×80); F) Root 10 days after infection; slight blue staining in giant cells (×55); G) Root 23 days after infection; the blue staining is mostly confined to giant cells, with occasional individual cortical cells also blue (unlabelled arrow) (×55); H) Galled roots 5 weeks after infection (low magnification) (×15); I, as H, individual gall and giant cell complex; note intense blue stain only in giant cells (×55); J) Root 6 weeks after infection; partially dissected to show a female counter-stained with acid fuchsin (pink); note the intense blue stain only in giant cells (×62.5). *gc* = giant cells, n = nematode, rt = root tip.

to 46 days post infection, however, *gus* expression was observed exclusively in giant cell complexes (92% to 98% of those examined stained blue) (Fig. 2H–J), with no expression elsewhere in roots of similar age.

Other organs of transgenic plants also were stained for *gus* expression by histochemistry, including stem, leaf, petals (Fig. 2A–C), and flower parts; with one exception, no expression was observed. The exception was when whole 10-day-old seedlings were stained with X-gluc. In this case, blue staining was observed continuing from the vascular tissue in the root to fade-out in the vascular tissue of the midrib of attached cotyledons.

Discussion

Because of the relatively small size of cells fed from by phytoparasitic nematodes, there is little direct information on metabolic changes in the cells. So far, evidence from various methods has indicated up-regulation of expression of some genes in giant cells or syncytia (e.g., hydroxymethyl CoA reductase, mitotic cycle genes cdc 2a and cyclin, extensin and the tobacco RB7 water channel protein gene) (3,8,19, 20) and down-regulation of other genes (e.g., driven by promoters such as *nos, rol,* phenylalamine ammonia lyase, and T-*cyt* genes) (9). The report that the CaMV 35S promoter down-regulates expression of linked genes in giant cells (9) appears equivocal; in fact, it appears to up-regulate expression of linked genes (unpubl. obs.). The work presented here is based on analysis of a well-characterized line of transgenic tobacco containing the *Parasponia* hemoglobin promoter (1,5,16,17). In this work we have demonstrated that on infection of tobacco plants containing the *Parasponia* hemoglobin promoter and *gus* reporter gene, *gus* staining is not detected during the early stages of giant cell formation but is strongly expressed in mature giant cells. This suggests that the hemoglobin promoter may be down-regulated in young giant cells but highly active in giant cells as they mature. In contrast, non-giant cell root tissues express the reporter gene in younger tissues, but the gene is switched off completely in mature root tissues. Although levels of *gus* mRNA were not analyzed in giant cells and the *Parasponia* promoter used is functioning in a heterologous system (i.e. tobacco), other studies using this promoter suggest that the observations reported are valid (1,5,16,17; D. Llewellyn, pers. comm.).

The observations on gene expression driven by the *Parasponia* promoter indicate that it is important to carry out a time course to study gene expression in giant cells. It is not sufficient to examine gene expression at one time point because it is clearly not constant throughout the life cycle of the giant cells and their associated nematodes. For example, during early stages of giant cell development, the cells are characterized by large vacuoles and accompanied by cell expansion and nuclear divisions without cytokinesis. With time, the cells become highly cytoplasmic, with enlarged, lobed nuclei; transfer cell wall ingrowths; numerous mitochondria; and small vacuoles (15). This point should be borne in mind when considering published reports on gene expression in giant cells.

The fact that genes may be down-regulated early in giant cell development and up-regulated later on indicates that giant cell metabolism is not constant. The initial phase probably involves establishment of the giant cells at the feeding site, with low nutrient demand. As the nematode grows and matures, the nutrient demand must increase dramatically and the metabolism may gradually switch to a solute transport and maintenance role with up-regulation of genes required for energy production, transport, and maintenance of cell homeostasis in response to that nutrient demand. This is consistent with the view that giant cells act as large transfer cells (14). The implications of these observations for molecular approaches to host resistance are that control may be achieved either by inhibition of es-

tablishment of giant cells early in the association with a juvenile or by disruption of the transfer cell function later in the association.

The two proposed roles for root expression of non-legume hemoglobin genes are as oxygen tension sensors or oxygen transporters to metabolically active cells. Bogusz et al. (5) favor the latter role, in which the hemoglobin functions to facilitate diffusion of oxygen to rapidly respiring cells. The pattern of expression of the *gus* gene, driven by the hemoglobin promoter, is consistent with this function in nematode-induced giant cells. During development of giant cell complexes, radial diffusion of oxygen is sufficient to meet their respiratory needs. However, at later stages, when the associated nematode is fully mature and producing eggs and the giant cells are surrounded by gall cells, oxygen tension in the metabolically active giant cells may be limiting and may lead to up-regulation of the hemoglobin promoter as observed.

In considering the question of whether the up-regulation of *gus* fused to the *Parasponia* hemoglobin promoter reflects a basic aspect of giant cell function, or is only an incidental aspect of maintenance or feeding, it is relevant that hemoglobin genes have now been found in a range of non-legume plants (e.g., *Arabidopsis* and barley, E. Dennis, pers. comm.). The *Arabidopsis* hemoglobin promoter is known to activate gene expression at pO_2 of <5% and is thought to act as a facilitator of oxygen diffusion. These observations suggest that hemoglobin genes, if present in all plants, will be switched on if pO_2 is limiting. The up-regulation of such a gene in giant cells is therefore unlikely to be a primary event involved in giant cell induction or controlled in some way by the nematode. Rather, it would be a secondary event which, nevertheless, may be important in the efficient functioning of giant cell metabolism. If the pO_2 becomes limiting, metabolic and transport functions of giant cells will slow; thus, the giant cells may not be capable of supplying as many nutrients to the associated nematode. This, in turn, would lead to a reduced reproductive capacity of the nematode.

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