Induction of the *Hahsp17.7G4* Promoter by Root-Knot Nematodes: Involvement of Heat-Shock Elements in Promoter Activity in Giant Cells

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Root-knot nematodes feed from specialized giant cells induced in the plants that they parasitize. We found that the promoter of the *Hahsp17.7G4* gene, which encodes a small heat-shock protein involved in embryogenesis and stress responses, directed GUS expression in tobacco galls induced by the root-knot nematode *Meloidogyne incognita*. In roots containing a GUS reporter fusion to the *Hahsp17.7G4* promoter, 10% of the galls stained for GUS expression 1 to 3 days after infection and the fraction stained increased to 60 to 80% 17 to 20 days after infection. A DNA fragment from –83 to +163, which contains heat-shock element (HSE) core sequences, is sufficient to support a promoter activity largely restricted to giant cells within the galls. Two-point mutations in HSE cores, previously reported to abolish the heat-shock response and to strongly reduce the embryogenesis response of the same promoter, did not reduce expression in giant cells. This suggests a distinct regulation of the promoter by nematodes. However, additional point mutations located at positions crucial for binding of heat-shock transcription factors (HSFs) caused a severe decrease in the nematode response. These results demonstrate that HSEs are involved in the promoter activation in giant cells and suggest that HSFs may mediate this response.

Root-knot nematodes use poorly understood mechanisms to maintain the specialized cells (giant cells) that constitute their feeding sites. The dramatic changes leading to the differentiation of a giant cell are known to involve changes in gene expression (Fenoll et al. 1997; Gheysen and Fenoll 2002). Several studies have reported differential promoter activity in nematode feeding sites (NFSs) (Barthels et al. 1997; Bertoli et al. 1999; Escobar et al. 1999; Favery et al. 1998; Gheysen and Fenoll 2002; Goddijn et al. 1993; Möller et al. 1998; Niebel et al. 1996; Opperman et al. 1994; Puzio et al. 1999; Urwin et al. 1997), but identification and analysis of minimal regulatory sequences from these nematode-activated promoters is scarce. Nematode induction of the promoters of *TobRB7*, *AtPYK10*, *AtPYK20*, and *CaMV35S*, among others, have been studied by deletion analysis (Bertoli et al. 1999; Nitz et al. 2001; Opperman

et al. 1994; Puzio et al. 1999). Furthermore, putative *cis* elements associated with NFS expression have been proposed based only on in vitro analysis evidence (Escobar et al. 1999, 2001). Similarly, a few genes encoding putative transcription factors have been reported as expressed in NFSs. Among them are *PYK20*, which codes for a protein with a glutamine-rich domain (Puzio et al. 1999); *WRKY1*, which belongs to a large family of genes with different roles in plant defense and development (Gheysen and Fenoll 2002); *ABI3*, whose promoter is induced in syncytia (Gheysen and Fenoll 2002), and *KNOX* and *PHAN*, which encode transcription factors required for meristem establishment and whose transcripts accumulate in giant cells (Koltai et al. 2001). However, no functional analyses have been reported so far to demonstrate a role of such putative transcription factor genes in NFSs. Thus, a detailed analysis of regulatory sequences in nematode-inducible promoters and of their interacting transcription factors will contribute to the understanding of the regulatory signal transduction pathways involved in the dramatic changes in gene expression that occur in NFSs.

Several genes induced by nematode infection also are activated during embryogenesis, such as the embryogenesis-specific *ABI3* (Gheysen and Fenoll 2002) and genes encoding LEA-like proteins (Gheysen et al. 1996; Vaghchhipawala et al. 2001; Van der Eycken et al. 1996), which also are induced during desiccation processes. One group of genes that is induced during the late desiccation stages of embryo development encodes small heat-shock proteins (sHSPs) (Almoguera et al. 1998; Coca et al. 1996; Prändl and Schöffl 1996; Wehmeyer et al. 1996). Several studies indicate the participation of heat-shock factors (HSFs) as well as additional transcription factors in the regulation of sHSP expression during development and in response to environmental cues (Almoguera et al. 1998; Carranco et al. 1999; Dong and Dunstan 1996; Medina-Escobar et al. 1998; Rojas et al. 1999; Wehmeyer and Vierling 2000; Zimmerman et al. 1989). There also are reports of differential regulation of sHSPs by pathogen attack, such as during fungal haustorium formation (Tada et al. 2000), and of *HSP70* during viral infections (Aranda et al. 1999). Moreover, cyst nematodes induce the accumulation of a heat-shock protein (*HSP70*) transcript in soybean (Vaghchhipawala et al. 2001).

In this study, we demonstrate that the promoter of *Hahsp17.7G4*, a gene that encodes a sunflower sHSP, is activated in giant cells formed after *Meloidogyne incognita* infection in transgenic tobacco plants. A translational *GUS* fusion carrying a promoter region of only 83 bp contains the regu-

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latory sequences sufficient for its nematode-dependent activation. Furthermore, point mutations in heat-shock element (HSE) cores crucial for HSF binding caused a severe decrease in the nematode response. This fact suggests an involvement of HSEs and, possibly, of HSFs in promoter activation in giant cells.

RESULTS

The *Hahsp17.7G4* promoter is active in giant cells.

To analyze the inducibility of the *Hahsp17.7G4* promoter by root-knot nematodes, we used transgenic tobacco plants that carried a fusion of the *Hahsp17.7G4* promoter (up to position -1,132) linked to the reporter gene *uidA* (*GUS*) (Coca et al. 1996). Several independent transgenic lines (see Material and Methods) were infected with *M. incognita*, and GUS activity was determined at two time points after infection. Under the conditions used in the assays, roots showed no detectable GUS activity, while intense GUS staining was detected in galls (Fig. 1A). Thin sections showed a strong localized signal in the giant cells (Fig. 1B). On some occasions, a weak staining was observed in the cell layers adjacent to the giant cells (Fig. 1B). We attribute this signal to some diffusion of GUS reaction product or to some expression in the surrounding cells, because this appeared sporadically. At early infection stages (1 to 7 days post infection [dpi]), the percentage of stained galls was low ($\approx 10\%$). However, there was an increase during the infection period, reaching values of 60 to 80% at 13 to 20 dpi in most of the plant lines (Fig. 2A and B).

The proximal region of the *Hahsp17.7G4* promoter is sufficient to confer expression in galls.

Transgenic plants carrying 5' truncations of the *Hahsp17.7G4* promoter to positions -395 and -83 bp showed induction of GUS in galls very similar to that of the control (-1,132::GUS) lines (Fig. 1C and E). Plants with the two deleted promoter constructs showed higher frequencies of stained galls compared with the control, both at early and later infection stages (Fig. 2B). However, statistical analysis indicates that no significant differences were observed between the control and each of the deleted transformants in pairwise comparisons by the U-Mann-Whitney test (-1,132::GUS/-395::GUS, $P = 0.375$; -1,132::GUS/-83::GUS, $P = 0.306$). Tissue localization in the tobacco lines with deleted promoters showed that the signal also was concentrated strongly in giant cells (Fig. 1D and F). Both constructs exhibited higher frequency of stained galls at later infection stages, as did the transformants with the longest promoter version (Fig. 2B). A more detailed time course of stained galls in the two deleted lines showed a clear increase in the frequency of blue galls along the infection period, starting at values of $\approx 10\%$ at 1 to 3 dpi, and reaching a value close to 80% at the latest infection stages (17 to 20 dpi) tested (Fig. 2A). These results indicate that a region of 83 bp in the *Hahsp17.7G4* promoter is sufficient to maintain the induction pattern observed in galls with the longest (-1,132 bp) promoter version tested, both temporally and spatially (Figs. 1 and 2).

Four point mutations in the HSEs of the *Hahsp17.7G4* promoter abolish its nematode inducibility.

The minimal promoter region sufficient for nematode-inducibility contains several HSE core sequences (Almoguera et al., 1998; Coca et al. 1996) (Fig. 3). To determine the involvement of these HSE cores in nematode inducibility, plants containing a mutated version of the -1,132::GUS gene (mutant A) (Almoguera et al. 1998) (Fig. 3) that affected two different HSE core sequences (Fig. 3, first and third HSE cores of box I) were infected with *M. incognita* and stained for detection of GUS activity at different time points after infection. GUS

staining was localized in the center of the gall, with a strong signal in giant cells (Fig. 4A and B). The two nucleotide substitutions in box I of mutant A (Fig. 3) had no obvious qualitative effect on GUS expression, because the staining pattern was comparable to that of the -1,132-bp control line (compare Figs. 1 and 4, panels A and B). Moreover, there were no significant differences in the percentage of blue galls in pairwise comparisons by the U-Mann-Whitney analysis between mutant A and the rest of the transformants (-1,132::GUS/mutant A::GUS, $P = 0.074$; -395::GUS/mutant A::GUS, $P = 0.157$; and -83::GUS/mutant A::GUS, $P = 0.157$). Therefore, the mutant A promoter behaved similarly to the wild-type promoter (-1,132::GUS) and to the two deletion versions tested (-395::GUS and -83::GUS).

Plants containing additional mutations in box I and box II also were tested. We used mutant E (Fig. 3) because it carries two mutations in box I which are essential for HSF binding and which render the promoter nonresponsive to embryogenesis and heat shock (Almoguera et al. 1998). Thus, we could establish similarities or differences in the promoter regulation between these processes and the nematode response. Compared with mutant A, mutant E carries two extra point mutations in box II (second and fourth HSE cores) and two extra point mutations in box I (third and sixth HSE cores). In mutant E plants, nematode inducibility was almost completely abolished at all infection times tested (Figs. 4C and D, and 2B). Differences between mutant E and each of the constructs (control, deletions, and mutant A) were significant (mutant E::GUS/-1,132::GUS, $P = 0.04$; -395::GUS, $P = 0.027$; -83::GUS, $P = 0.014$; and /mutant A::GUS, $P = 0.025$, respectively). Thus, the four extra point mutations present in mutant E compared with mutant A impaired nematode-mediated promoter activation.

DISCUSSION

A short fragment of the *Hahsp17.7G4* gene drives giant cell expression in transgenic tobacco plants.

Our results demonstrate that the *Hahsp17.7G4* promoter is active in tobacco galls induced by *M. incognita*. The promoter is activated from early stages of infection (1 to 5 days), similarly to other nematode-inducible promoters related to embryogenesis or cell water deficit, such as *LEMMI9* or *LEA14A* (Escobar et al. 1999; Gheysen et al. 1996). However, *Hahsp17.7G4* promoter activity appears to be restricted to giant cells and, to a lesser extent, some immediately adjacent cells, whereas *LEMMI9* also is expressed in vascular tissues surrounding the gall and *LEA14A* is not expressed in giant cells, but in the rest of the gall. Maximum *Hahsp17.7::GUS* activity was detected 13 to 20 dpi (Fig. 2), indicating that the promoter is active throughout early to middle infection stages. This suggests that the promoter activity may be more related to tissue (giant cells) differentiation than to an immediate response to infection.

The deletion analysis showed that the tobacco lines carrying the construct that included only 83 bp upstream from the transcription start point was as active in galls as the longest promoter version tested (Figs. 1 and 2). This indicates that the region from -1,132 to -83 bp is dispensable for the activation of *Hahsp17.7G4* promoter in tobacco giant cells induced by *M. incognita*. Therefore, a minimal nematode-inducible promoter region that comprises the 83 bp upstream from the transcription start point has been identified (Fig. 3).

Involvement of HSEs in giant cell expression.

The effect of specific mutations in the HSE regions of *Hahsp17.7G4* on its nematode inducibility suggests that a minimal HSE, including five HSE cores, is crucial for the

Fig. 1. Activity of various *Hahsp17.7G4* promoter versions analyzed by GUS staining in tobacco plants infected with *Meloidogyne incognita*. Whole mounts (left panels) show GUS activity in galls of representative transformant lines containing wild type and deleted promoter versions (**A**, -1,132::GUS; **C**, -395::GUS; **E**, -83::GUS). Thin sections (10 μ m) of galls 10 to 15 days after *M. incognita* infection (right panels) localize GUS activity mainly in giant cells (**B**, -1,132::GUS; **D**, -395::GUS; **F**, -83::GUS). GC, giant cells and N, nematode. Scale bars: **A** and **C**, 420 μ m; **E**, 525 μ m; and **B**, **D**, and **F**, 25 μ m. GUS staining is observed as blue color in whole mounts and as a red precipitate in the dark field micrographs of the sections.

nematode inducibility (Fig. 3). Mutant A plants and plants with the wild-type version of the promoter were similarly induced in giant cells, both in terms of cell specificity and temporal regulation. In contrast, the two HSE point mutations in mutant A caused a severe depletion of heat shock and late embryogenesis

responses (Almoguera et al. 1998). Mutant E, which has four extra point mutations at positions crucial for HSF binding (Almoguera et al. 1998) (Fig. 3), showed no detectable induction in galls (Figs. 2 and 4). Both box II and the first HSE core from box I (Fig. 3) seem not to be crucial for giant cell activa-

Fig. 2. Frequency of GUS activity in galls of tobacco plants transformed with different *Hahsp17.7G4* promoter versions. **A**, Time course of the deleted promoter lines -83::GUS (light gray) and -395::GUS (dark gray). **B**, Wild-type, deleted, and mutated promoter lines: -1,132::GUS, -83::GUS, -395::GUS, mutant A::GUS, and mutant E::GUS. The different time intervals analyzed are indicated. Data are means of four independent plant lines for the deletions and the wild-type promoter versions, three lines for mutant A, and six lines for mutant E. Data from four independent experiments for each line were pooled. Bars represent the standard errors as deviations of the values of the different transgenic lines from the mean for each construct and time point.

tion, because the –83-bp deletion does not contain these sequences, but it is fully active in galls. However, we cannot exclude a contribution of these sequences, in the context of the longest promoter version, to the regulation of the *Hahsp17.7 G4* in galls. In contrast, the two extra mutations in the third and sixth HSE cores of box I in mutant E (Fig. 3) show a severe reduction in promoter activation in giant cells. Therefore, these

core nucleotides in box I (Fig. 3) could be considered crucial for the nematode inducibility of *Hahsp17.7 G4*, and mutant E behavior in this response should reflect mostly the effect of the mutations in the third and sixth cores of box I.

HSEs mediate transcription of heat-shock-responsive promoters through their interaction with HSFs (Schöffl et al. 1998). Thus, it is probable that giant cell activity of the

Fig. 3. Proximal *Hahsp17.7G4* gene promoter region. The transcription start point (arrow) and the ATG for translation initiation are indicated. The sequences from position –83 bp up to position +163 are included in all the constructs used in this work. The positions of the two boxes containing heat-shock element (HSE) cores are shown (box I and box II) and their sequences are displayed. The core repeats crucial for heat-shock transcription factor binding in each box are indicated in bold and underlined. The core sequences changed in mutant A and mutant E are shown below the wild-type sequence. Nucleotide substitutions in the HSE cores potentially crucial for nematode inducibility are indicated in lower case and the cores identified by gray boxes. The dotted bar at the top represents the minimal region of the gene required for giant cell expression as delimited in this work, and the dotted bar at the bottom represents the minimal nematode inducible HSE identified in the –83::GUS construct.

Fig. 4. Activity of mutated versions of the *Hahsp17.7G4* promoter analyzed by GUS staining in representative transformed tobacco lines infected with *Meloidogyne incognita*. Left panels show whole mounts of infected roots (A, mutant A::GUS; C, mutant E::GUS). Right panels show thin sections (10 μ M) of galls 10 to 15 days after infection (B, mutant A::GUS; D, mutant E::GUS). GC, giant cells. Scale bars: A and C, 328 μ m; B and D, 25 μ m; and B, 50 μ m. GUS staining is observed as blue color in whole mounts, and as a red precipitate in the dark field micrographs of the sections.

Hahsp17.7 promoter involves HSFs, because mutations described to impair HSF binding (Almoguera et al. 1998), such as those present in mutant E, abolish nematode induction.

How is giant cell activation of the *Hahsp17.7* promoter related to its activation during heat shock and embryogenesis? The -83-bp promoter version essentially retained the regulation in the three processes, indicating that sufficient elements required for all of them are present in this sequence. The HSE positions mutated in mutant A are dispensable only for nematode induction. Therefore, at least part of the HSE (and hence HSF)-mediated responses during heat shock and embryogenesis do not seem to be crucial for giant cell expression. However, the additional mutations in mutant E abolished nematode inducibility as well as the late-embryogenesis and heat-shock responses (Almoguera et al. 1998). This suggests that common HSE cores in box I are essential for the three responses. As with *Hahsp17.7*, *HSP70* expression during viral infection seems to be regulated through pathways different from those operating in the heat-shock response, because the HSF needed for *HSP70* transcription in the heat-shock response is not present during viral infection (Aranda et al. 1999).

Sequence elements other than HSE present in the -83-bp promoter version also may contribute to the nematode inducibility of the *Hahsp17.7G4* promoter. All promoter constructs analyzed were translational fusions and included, besides the promoter region, an additional 163 bp downstream from the transcription initiation site. A search for putative *cis* elements in the 246 bp that constitute the minimal nematode-inducible sequence using three different data bases, TRANSFAC (Wingender et al. 2000), PLACE (Higo et al. 1999), and PLANTCARE (Rombauts et al. 1999), identified a number of homologies to known regulatory sequences from other plant genes (data not shown). The most remarkable putative *cis* elements overlapping HSEs are the CCAAT boxes, which are known to interact with HSEs enhancing the expression of chimeric heat-shock genes in transgenic tobacco (Rieping and Schöffl 1992) and which might play a quantitative role on *Hahsp17.7G4* expression in galls. However, the participation of any of these *cis* elements on nematode inducibility of this promoter remains purely speculative until functional analyses are performed.

The present study is based on the analysis of the plant response to nematodes in a heterologous system. Preliminary results from our laboratory indicate that the longest promoter construct, -1,132 bp, also is active in galls formed in *Arabidopsis* spp. (C. Escobar, M. Portillo, M. Barcala, and C. Fenoll, unpublished data). This suggests that common regulatory components in both heterologous plant systems (tobacco and *Arabidopsis* spp.) can mediate the induction of the sunflower *Hahsp17.7G4*, and reinforces the idea of a general mechanism involved in regulation of expression of this specific gene in root-knot nematode giant cells.

Our data pose the question of why HSP genes are activated in giant cells. The fact that the *Hahsp17.7* promoter is induced in giant cells does not necessarily imply that the gene product accumulates in such cells or that, if it does, it plays a role in giant cell ontogeny or function. However, *HSP70* transcripts also have been shown to accumulate in roots infected by cyst nematodes (Vaghchhipawala et al. 2001), and viral infection also triggers *HSP70* transcript accumulation (Aranda et al. 1999). Work is in progress to determine whether the *Hahsp17.7G4* transcript and protein are present in giant cells as a first step to assess the involvement of sHSPs in giant cell functioning.

The specific differences between promoter regulation during embryogenesis, in response to heat stress, and in giant cells that we have identified could be exploited to engineer giant cell-specific chimeric promoters that will knock out the heat

shock and late embryogenesis responses, while allowing giant cell expression.

MATERIALS AND METHODS

Nematode populations and infections.

An *M. incognita* MIK population (obtained from F. Grundler, Vienna) was maintained in vitro on cucumber roots. Egg masses were collected at maturity and used to inoculate transgenic tobacco plants grown in Gamborg medium (Gamborg et al. 1968) supplemented with sucrose at 15 g/liter, at 27 to 28°C, a 16-h photoperiod, and 70% humidity. Galls were monitored visually every 24 h after inoculation for at least 7 days, and newly formed galls were individually labeled. Therefore, gall age could be determined with an error of ± 1 day after infection. A minimum of 30 galls was scored in each independent experiment.

Plant material.

Transgenic tobacco plants containing different versions of the *Hahsp17.7G4* promoter were described by Coca and associates (1996). We used four independent transgenic lines for the nematode infection experiments for both the full-length (-1,132::GUS) and each of the two *Hahsp17.7G4* shorter promoter versions (-395::GUS and -83::GUS) (Coca et al. 1996), and three and six lines, respectively, for the mutated promoter constructs (mutant A and mutant E) (Almoguera et al. 1998). Seed from T₀ plants were selected in MS medium (Murashige and Skoog 1962) supplemented with kanamycin at 50 μ g/ μ l. Resistant plants were transferred to soil and grown at 20 to 25°C and a 16-h photoperiod, and T₁ seed were collected for the experiments. Transformant lines were selected among 10 to 12 lines whose responses to heat shock or during embryogenesis, adjusted to the average data, measured as GUS activity in previous works (Almoguera et al. 1998; Coca et al. 1996).

Histochemical detection of GUS activity.

Plants were stained for GUS activity in sterile conditions and at 28°C to avoid heat shock, as described by Almoguera and associates (1998). Whole-mount specimens were observed and photographed under a Leica binocular microscope MZ6. For tissue localization of GUS expression, root samples were washed in 10 mM phosphate buffer, pH 7, immediately after collection. Material was fixed in 2.5% glutaraldehyde in 25 mM sodium phosphate buffer, pH 7, by vacuum infiltration for 10 min and incubated at 4°C for 150 min. After a wash in 10 mM phosphate buffer, pH 7, for 10 min, samples were dehydrated in an ethanol series, treated with 100% acetone, and subsequently embedded in 1/1 acetone/Araldite (CY212, Taab) (vol/vol). Finally, tissue was embedded in 100% Araldite and semithin sections (10 μ m) were obtained with an ultramicrotome (Reichert's ultracut S). Micrographs were taken with a stereoscopic microscope (OLYMPUS BX61).

Statistical analysis of data.

The value distributions for the percentages of GUS-stained galls were compared between plants carrying different *Hahsp17.7G4*::GUS fusions by the nonparametric test of Mann-Whitney, and using the program Statistical Package for the Social Sciences (SPSS, Inc., Chicago). Pairwise comparisons between all possible groups were performed to analyze the differences of the effect of each chimeric gene in GUS expression. The level of significance was set to $P \leq 0.05$.

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