RESEARCH ARTICLE

Regulatory Sequences of Arabidopsis Drive Reporter Gene Expression in Nematode Feeding Structures

Nathalie Barthels,a Frederique M. van der Lee,b Joke Klap,b Oscar J. M. Goddijn,b Mansour Karimi,a Piotr Puzio,c Florian M. W. Grundler,c Stephan A. Ohl,b Keith Lindsey,d Lee Robertson,e Walter M. Robertson,a Marc Van Montagu,a,b Godelieve Gheysen,a,b and Peter C. Sijmons,b,2

aLaboratorium voor Genetica, Department of Genetics, Flanders Interuniversity Institute for Biotechnology (VIB), Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium
bMOGEN International nv, Einsteinweg 97, NL-2333 CB Leiden, The Netherlands
cInstitut für Phytopathologie, Christian-Albrechts-Universität Kiel, Hermann-Rodewald-Strasse 9, D-24118 Kiel, Germany
dDepartment of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, United Kingdom
eScottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, United Kingdom

In the quest for plant regulatory sequences capable of driving nematode-triggered effector gene expression in feeding structures, we show that promoter tagging is a valuable tool. A large collection of transgenic Arabidopsis plants was generated. They were transformed with a β-glucuronidase gene functioning as a promoter tag. Three T-DNA constructs, pGV1047, pAgusBin19, and pMOG553, were used. Early responses to nematode invasion were of primary interest. Six lines exhibiting β-glucuronidase activity in syncytia induced by the beet cyst nematode were studied. Reporter gene activation was also identified in galls induced by root knot and ectoparasitic nematodes. Time-course studies revealed that all six tags were differentially activated during the development of the feeding structure. T-DNA-flanking regions responsible for the observed responses after nematode infection were isolated and characterized for promoter activity.

INTRODUCTION

Among the numerous species of nematodes, the relatively small group of economically important root parasites has recently excited interest among molecular biologists. In general, plant root parasitic nematodes belong to two orders, Tylenchida and Dorylaimida. The tylenchids show a broad diversity in parasitic adaptations and comprise both migratory and sedentary ectoparasites and endoparasites. Sedentary endoparasitic nematode–plant interactions, involving species such as root knot and cyst nematodes, are intriguing to study because of the highly specialized feeding structures (giant cells and syncytia, respectively) that become established in the root during the prolonged and unique relationship between the nematode and host. Feeding structures maintenance and nematode survival are preserved only when the mutual interaction remains established (Bird, 1962). These interactions often cause extensive crop damage in infested fields and hence severe economic losses. The order Dorylaimida comprises migratory ectoparasites. Some of these parasites can feed at a particular site for long periods of time (e.g., Xiphinema spp) rather than browsing along the roots. Similarly, these ectoparasitic species represent an economically important threat as vectors of soil-borne viruses (Taylor and Brown, 1981) and as a cause of direct damage to the roots.

Infective second-stage (J2) juveniles of cyst and root knot nematodes migrate intracellularly or intercellularly, respectively, toward the vascular cylinder, where they select an initial feeding cell. Secretions are injected via the stylet, eliciting a series of cellular responses that result in the production of metabolically active, multinucleate feeding cells with elaborate cell wall ingrowths characteristic of transfer cells (Jones and Northcote, 1972). Once feeding begins, the infective juveniles become sedentary, after which they mature by ingesting
food from the feeding cell. This process is an absolute re-

quirement for the nematode to complete its life cycle.

In contrast, migratory dorylaimid ectoparasitic nematodes feed at multiple sites, although mainly at root tips, which then cease to grow and develop into terminal galls. With their long needlelike styles, these nematodes pierce a column of subepidermal cells, injecting secretions to predigest the cytoplasm of the recipient cell. A path of collapsed necrotic cells is left behind. They are surrounded by multinucleate and expanding meristematic cells that are responsible for gall formation (Wyss et al., 1988).

For many years, the ultrastructure of such nematode–plant interactions has been comprehensively investigated (reviewed in Endo, 1971, 1991; Jones, 1981). Anatomical and cytological observations using advanced microscopic techniques have broadened our knowledge of the nematode feeding apparatus and the secreted glandular granules (Rumpenhorst, 1984; Endo, 1987; Hussey, 1989; Hussey and Mims, 1990; Jones and Robertson, 1997) as well as of plant tissue responses to nematode attack (Melillo et al., 1990; Grundler et al., 1994a; Golinowski et al., 1996, 1997; Robertson, 1996; Gheyesen et al., 1997; Sobczak et al., 1997). High-resolution video-enhanced contrast microscopy has also allowed the direct observation of feeding nematodes parasitizing living plant cells (Wyss, 1987; Wyss et al., 1992).

Underlying mechanisms are now being explored by using molecular genetic techniques to elucidate further the re-
differentiation processes involved and to develop genetic engi-
ingering strategies for effective nematode control in crops (Opperman and Conkling, 1994; Bird, 1996; Gheyesen et al., 1996). To pursue the identification of nematode-responsive plant promoters, we took advantage of a T-DNA system based on a randomly integrated promoter tag containing a promoterless dominant screenable marker. In previous re-
search, this strategy has proven to be successful in the identifica-
tion of molecular markers in plant development (Kertbundit et al., 1991; Lindsey et al., 1993; Topping et al., 1994). Further exploitation of its use was achieved in the iso-
lation of environmental and hormonal stress-responsive reg-
ulatory sequences or genes (Lindsey et al., 1993; Mandal et al., 1995). Encouraged by these results, we generated large collections of transgenic Arabidopsis lines harboring a pro-
moterless β-glucuronidase (gus; uidA gene from Escherichia
coli) gene, and subsequently conducted large-scale screening for activation of the reporter gene in the nematode feeding structures (NFSs).

The strength of this promoter-tagging strategy, compared with other molecular approaches (reviewed in Niebel et al., 1994a; Topping and Lindsey, 1995), rests on the immediate visual detection of tagged promoter activity and the assess-
ment of its spatial specificity. Here, we report on several ex-
pression patterns resulting from the induction of the gus reporter gene by a number of tagged nematode-responsive Arabidopsis regulatory sequences, and we discuss the use of this strategy as a tool in furthering our understanding of the interaction between plants and parasitic nematodes.

RESULTS

Tagging Novel Promoter Activities in NFSs

We generated Arabidopsis promoter tag lines by using bi-
nary T-DNA vectors pGV1047, pAgusBin19, and pMOG553. Figure 1 depicts the T-DNA constructs. One thousand pMOG553 and several hundred pAgusBin19 lines are now publicly available through the Arabidopsis seed stock center (Nottingham, UK). Transformation efficiencies, T-DNA integ-

Figure 1. T-DNA Structures in the Binary Vectors pGV1047, pAgusBin19, and pMOG553.

gus, gus gene encoding the GUS reporter; gus intron, intron-containing gus gene; hpt, hygromycin phosphotransferase gene; lacZ3', β-galactosidase 3' sequence; lacZ5', β-galactosidase 5' sequence; 3'nos, nopaline synthase 3' sequence; nos, nopaline synthase gene; nptII, neomycin phosphotransferase gene; 3'ocs, octopine synthase 3' sequence; P35S, cauliflower mosaic virus 35S promoter; Pnos, nopaline synthase promoter; supF, suppressor gene; 3'35S, cauliflower mosaic virus 35S 3' sequence.
Table 1. Overview of the GUS Patterns from Six Promoter Tag Lines

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<th>0025</th>
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*aHs, H. schachtii; MI, M. incognita; Xd, X. diversicaudatum. The asterisk indicates that expression patterns from in vitro-grown plants as described here occasionally differ slightly from those of soil-grown plants (see text for details). bF&P, flowers and pods; Hid, hydathodes; Lr, lateral root initiation; Lvt, leaf vascular tissue; Rvt, root vascular tissue; Sti, stipules; Vs, vegetative shoot. c(++++) indicates very strong reporter gene expression; (+ + +), strong expression; (+ +), moderate expression; (+), weak expression. The (+/-) indicates weak staining for GUS activity; the (-) indicates that no GUS activity could be seen; ND, not determined.

below) are summarized in Table 1 and Figures 2 and 3. The lines studied contain either the pΔgusBin19 T-DNA (Att0001, Att0728, and Att1712) or the pMOG553 T-DNA (Att0025, Att0651, and Att1164); no line transformed with pGV1047 Att0728, and Att1712) or the pMOG553 T-DNA (Att0025, Att0651, and Att1164) was found to show GUS activity in the NFSs, or additive and/or synergistic effects between the individual tagged sequences could account for the expression pattern. Because the inverse polymerase chain reaction (IPCR) using Att0025 plant DNA resulted in the recovery of only one of the four tagged sequences, we constructed a genomic library of this line. DNA gel blot analysis using Att0025 revealed four pMOG553 T-DNA copies (data not shown). Any of these tagged sequences could by itself be responsible for the observed GUS phenotype in the NFSs, or additive and/or synergistic effects between the individual tagged sequences could account for the expression pattern. Because the inverse polymerase chain reaction (IPCR) using Att0025 plant DNA resulted in the recovery of only one of the four tagged sequences, we constructed a genomic library of this line. Screening resulted in the isolation of all four T-DNA insertions, designated Att0025-1 through Att0025-4. For each right border (RB; possibly containing a promoter) and left border (LB), except for the Att0025-4 LB, a flanking 200-bp fragment was sequenced. None of the tagged sequences revealed an open reading frame (ORF) at the LB. Each RB tag was reintroduced into Arabidopsis (see Methods) to identify and confirm the regulatory capacity of the tag responsible for NFS-directed expression of the gus gene. The resulting lines were designated Att0025-R/1 through Att0025-R/4. Att0025-R/4 transgenic plants were found to express gus in root tips and at the sites of lateral root initiation. gus expression in syncytia was found only with Att0025-R/1 (Figure 2D); of 29 independent lines tested, 21 showed GUS staining in NFSs. Confirmation that reporter gene expression was restricted to the syncytium inside the NFS was provided by examination of histological sections (Figures 2F and 2G). The Att0025-R/1 lines exhibited considerable variation in gus expression levels, which may be explained by the different chromosomal contexts in which the reintroduced constructs reside. Compared with that of the original Att0025 line, the overall background expression in the Att0025-R/1 lines was greatly reduced (Table 1) and mainly restricted to the hydathodes, stipules, and vascular tissue of lateral roots (Figure 2C). The entire 3.4-kb RB flanking region from the Att0025 tag was cloned and sequenced (EMBL accession number Y12497).

From the same class of transgenic plants, Att1164 was found to show GUS activity in the syncytia (Figure 3) and at some other sites, as indicated in Table 1. In this line, gus expression levels in cells outside of the NFSs was much weaker than in the line Att0025. Genomic DNA gel blot analysis revealed the presence of a single T-DNA copy (data not shown). A fragment of 1480 bp of the RB-tagged sequence was isolated by using PCR and cloned in front of gus (see Methods). Plants transformed with this construct (Att1164-R/1 plants) were analyzed after infection with cyst nematodes; the gus expression pattern corresponded to that in the original Att1164, demonstrating that the correct regulatory
Figure 2. Reporter Gene Activation in Syncytia of Att0025 and Its Derivative Att0025-R/1 at 6 Days Postinoculation with H. schachtii.

(A) GUS activity in Att0025. The vegetative shoot and the hypocotyl vascular tissue are clearly GUS positive. Also, some regions of the root vascular tissue beyond the NFSs are GUS stained.

(B) High GUS activity in Att0025 syncytium.

(C) Att0025-R/1. Compared with the original Att0025, the background disappeared in the vegetative shoot, hypocotyl, and root vascular tissue beyond the NFSs. Strong GUS staining was retained in the stipules.

(D) GUS activity in Att0025-R/1 syncytium.

(E) Soil inoculation of Att0025. The resulting GUS activity pattern is similar to or the same as that obtained in vitro; the nematode is stained with acid fuchsin.

(F) Cross-section through Att0025-R/1 syncytium using dark-field microscopy.

(G) Cross-section through Att0025-R/1 syncytium using bright-field microscopy.

Under dark-field microscopy, GUS is visualized as a red precipitate and is more clearly observed compared with the blue color seen with bright-field microscopy. HVT, hypocotyl vascular tissue; N, nematode; RVT, root vascular tissue; S, syncytium; Sti, stipule; VS, vegetative shoot. Bars in (A) to (G) = 50 μm.

sequence had been cloned. A corresponding 4-kb genomic clone was isolated from a wild-type C24 genomic library by using the PCR fragment as a probe. No large ORFs or significant homologies to sequences in the database were found over a region of ~2 kb upstream and downstream from the insertion point (EMBL accession number Y12496).

In line Att0651 (pMOG553 T-DNA), the gus gene was predominantly expressed in NFSs (Figure 3G), although weak activity was also detectable in the vegetative shoot. DNA gel blot analysis revealed the insertion of two independent T-DNA copies in this line. In contrast to the other lines described here, Att0651-infected roots exhibited GUS-stained syncytia at a much lower frequency (only 50% or less of the induced syncytia showed GUS activity), suggesting a more strict regulation by nematode and/or plant developmental factors.

Att0728 (pAgusBin19 T-DNA) responded very early after infection with cyst nematodes. GUS histochemical staining could be observed within 6 hr after inoculation. Mechanical wounding experiments, however, did not result in the induction of reporter gene expression (data not shown), indicating that the rapid activation of expression was independent of a wound response. Under in vitro conditions, GUS activity...
Figure 3. Reporter Gene Activation in Syncytia after Cyst Nematode Infection of Arabidopsis Promoter Tag Lines.

(A) Att1712 syncytium 4 DPI.
(B) Cross-section through an Att1712 syncytium 4 DPI. Cell wall dissolution at this stage is still in progress.
(C) GUS-stained lateral root initiation site in Att1712.
(D) Att0001 syncytium 4 DPI.
(E) Cross-section through an Att0001 syncytium 4 DPI.
(F) GUS-stained lateral root initiation site in Att0001.
(G) Att0651 syncytium 7 DPI. The nematode is stained with acid fuchsin.
(H) Longitudinal section through an Att0728 syncytium 3 DPI.
(I) Att1164 syncytium 11 DPI.

(B) and (H) were taken with dark-field optics. N, nematode; S, syncytium. Bars in (A) to (I) = 50 μm.
was found to be located primarily in the developing NFSs (Figure 3H), but some activity was also detectable at sites of lateral root initiation. Soil-grown plants showed stronger activation in initiating lateral roots, together with some staining of the root vascular tissue. DNA gel blot hybridization analysis demonstrated the presence of a single T-DNA insertion.

Att0001 and Att1712 had similar gus expression patterns after nematode inoculation. Strong GUS staining was observed in the syncytia 4 days postinoculation (DPI) (Figures 3A, 3B, 3D, and 3E). Sites of lateral root initiation were also clearly stained in both lines (Figures 3C and 3F). Nevertheless, these tags were located at different chromosomal loci, as confirmed by DNA gel blotting (data not shown). Some Att0001 plants showed additional GUS activity at other sites, as indicated in Table 1 (in both soil- and in vitro-grown plants). This pattern of staining was never observed in Att1712 under in vitro conditions; however, soil-grown Att1712 plants occasionally displayed GUS activity in the leaf vascular tissue.

DNA gel blot analysis of S1 segregants (originating from seeds of the primary transformant) from Att0001 revealed the presence of three T-DNAs. Two of these T-DNAs were arranged in an inverted repeat over the RB (data not shown). After segregation, the locus containing the inverted T-DNA arrangement appeared to be responsible for NFS-directed expression and was analyzed further. Att1712 contained two T-DNAs in the S1 generation, but analysis of S2 progeny showed only one insert for progenitor Att1712a, indicating independent segregation of the two T-DNAs in the original line. This segregant showed a GUS activity pattern identical to that of the original Att1712.

On genomic Att0001 plant DNA, we performed IPCR to isolate the plant DNA regions flanking both T-DNA LB. Both clones, designated ARM1a and ARM1b, were reintroduced into Arabidopsis (see Methods). ARM1a was demonstrated to be the tagged sequence responsible for the observed nematode-induced gus expression. Sequence analysis indicated that the T-DNAs were inserted into a short putative ORF of 159 bp (EMBL accession number Y12834). However, no homology in the database was found. A GUS histochemical assay at 4 DPI on root knot nematode–infected Att0001-Rf1 S1 plants (Table 1) confirmed the regulatory character and revealed a gus expression pattern in the NFSs with a timing similar to that of the original Att0001 line.

### gus Expression Patterns Are Temporally Regulated

Feeding cell development by nematodes is a dynamic process and involves gene sets regulated by developmental stage–specific factors produced by nematode and host. Because such a temporal gene regulation might be reflected in the six selected lines, we monitored gus expression at different time points during syncytium development. Ten-day-old plants were inoculated, and the number of GUS-positive syncytia was determined at various intervals. The results are presented in Figure 4. Maximum GUS levels in syncytia were found at different times after inoculation of each of the six lines. The time points ranged from 2 DPI (Att0728) to 7 DPI (Att0651). Lines Att1712 and Att0001 showed maximum activities at 4 DPI, whereas Att0025 and Att1164 showed maximum GUS activities over a longer period during nematode infection rather than exhibiting transient patterns of activity. Att0025-Rf1, Att1164-Rf1, and Att0001-Rf1 retained the timing of maximum response from the original lines. Att0001-Rf1 and Att0025-Rf1 plants, however, displayed blue-stained syncytia with a lower frequency than did Att0001 and Att0025 plants, respectively (data not shown).

It should be emphasized that the data presented in Figure 4 correlate with the number of stained syncytia irrespective of the GUS level. Therefore, the time point corresponding to the maximum GUS activity in a given syncytium does not necessarily coincide with the time point corresponding to the maximum number of GUS-positive syncytia during the infection period. The fact that not all syncytia are stained at a given time point might be the consequence of the transient expression and of the fact that infections are not completely synchronous. When GUS activities were determined 7 DPI at different developmental stages of the host plant (10, 20, 30, 40, and 50 days), a lower percentage of stained syncytia was observed with increasing plant age for all six lines as well as for the reintroduced ones (data not shown).

![Figure 4. Monitoring Reporter Gene Expression in Syncytia over Time.](image)

The occurrence of reporter gene activity in the function of the developmental stage of the nematode (H. schachtii) or syncytia is presented. Per time point, the average percentage of stained syncytia is set in the diagram. GUS-positive (+) and GUS-negative syncytia were counted on 20 plants. Each plant was inoculated with 30 nematodes, from which ~50% induced a feeding structure.
Infections with Gall-Forming Nematodes

All lines with induced GUS activity in galls after root knot and ectoparasitic nematode infections are depicted in Figure 5. GUS staining levels in galls induced by root knot nematodes were similar to those observed in syncytia for lines Att1164 and Att0728 (Figures 5L and 5M). Despite repeated inoculation experiments, GUS was never observed after root knot nematode infection in Att0651, although galls were successfully formed (data not shown). Different levels of GUS staining in galls and syncytia appeared in lines Att1712 and Att0025, suggesting lower promoter activity in galls (Att0025) or syncytia (Att1712) (Figures 2B, 3A, 5G, and 5J). The presence of GUS in Att0025 giant cells is shown in Figure 5K.

Although Att0001 and Att1712 have a similar expression pattern (Figures 5A to 5D), as previously mentioned for cyst nematode–infected lines, the different nature of the tag in these two lines became clearly apparent by infection with the root knot nematode: an average of 70% of the established Att1712 galls showed an unstained zone at 4 DPI (Figure 5G). Subsequent cross-sectioning of these galls revealed strongly GUS-stained parenchymatous cells surrounding an unstained giant cell (Figure 5F). In contrast, Att0001 galls displayed very strong and uniform staining (Figures 5D and 5E) with GUS inside of the giant cells (similar to that shown for line Att0025 in Figure 5K).

Despite their ectoparasitic nature, Xiphinema nematodes feed for longer periods and can be regarded as being sedentary. Their ability to transform root tips into galls made this nematode species interesting to add to our study. As in the root knot nematode experiments, line Att0651 exhibited no induced GUS activity after inoculation with X. diversicaudatum. In addition, no galls were formed. It is not known whether this inability to induce a feeding structure implies a form of resistance associated with T-DNA integration.

Att0001, Att0025, Att0728, Att1164, and Att1712 exhibited GUS-positive galls when infected with X. diversicaudatum (Figure 5I). A cross-section through a X. diversicaudatum–induced Att1712 gall indicated that reporter gene expression in this line occurred in the multinucleate cells induced at the nematode penetration site (Figure 5H). The same pattern was observed in Att0001 galls (data not shown).

Screening for GUS Activity in Callus Tissue, Flowers, and Pods

The reproduction capacity of transgenic plants, engineered with a nematode-inducible promoter–cytotoxin construct, should be ensured when one seeks to develop nematode resistance in plants. This implies the absence of tagged promoter activity in the reproductive organs.

From the six selected lines (infected and noninfected), flowers were carefully dissected to screen for GUS activity. Pollen was GUS negative for all six lines. Att0025 plants showed weak staining in pistil, receptacle, and seeds, whereas Att1164 displayed weak GUS activity in pistil and siliques (data not shown). No GUS staining was observed in either flowers or seeds of any other lines studied.

As demonstrated in Figure 6, monitoring GUS activity after 6 days of incubation on callus-inducing medium (see Methods) revealed a variety of responses among the six uninfected lines. Att0001 and Att1712 both expressed gus in the root vascular tissue regions abutting the protruding calluses (Figure 6A). In the case of Att1712, this GUS staining occasionally extended into the vascular cylinder of developing lateral roots. Att1712 also showed clear reporter gene activation in cells at the cut surfaces of the explants (data not shown). It is intriguing that unlike Att1712, Att0001 displayed GUS staining in the tips of main and lateral roots, a so-called three-zone pattern (Figure 6B), which was also seen in auxin-treated Arabidopsis plants containing a cell cycle regulator (cdc2) promoter–gus construct (Hemerly et al., 1993). A very specific pattern was observed in callus tissue of Att0025 and its derivative Att0025-R/1. High GUS levels were present in the center of all calluses (Figure 6C). Explants of Att0651, Att0728, Att1164, and Att1164-R/1 showed no significant effect when an external hormone was applied. Extension of the incubation period for 5 more days did not alter the observed patterns.

A peculiar response was seen in the cauliflower mosaic virus 35S–gus control root explants. Except for root tips and calluses, GUS activity in the remaining root parts declined after incubation for 6 days on callus-inducing medium (Figure 6E). Newly formed lateral roots did not show any GUS staining except in the root tips (Figure 6D). Extension of the incubation time up to 12 days led to a 35S promoter activity confined solely to root tips and callus tissue (Figure 6F).

The influence of Agrobacterium infection was determined (see Methods) by mimicking the initial steps of the root explant transformation process. In general, Agrobacterium infection did not significantly alter the patterns seen after callus induction alone, except for the cut surfaces that showed weak GUS staining for all tags. By adding an Agrobacterium cocultivation step, GUS activity in Att0001 explants was no longer confined to vascular regions juxtaposed to the developing calluses, as described earlier, but extended throughout the entire vascular cylinder. Also Att0025-R/1 explants showed GUS staining in the vascular region upon Agrobacterium cocultivation. Att0728 and Att0651 displayed weak GUS activity in a few calluses, which was not observed after hormone treatment alone.

DISCUSSION

Reporters Gene Expression in Nematode Feeding Structures

The successful application of Arabidopsis in plant–nematode interaction studies (Sijmons et al., 1991; Wyss and
Figure 5. Reporter Gene Activation in NFSs Induced by Gall-Forming Nematodes.

Inoculations were performed with root knot nematodes, except when stated otherwise.

(A) Att1712 gall 4 DPI.
(B) Total Att1712 plant 4 DPI.
(C) Total Att0001 plant 4 DPI.
(D) Close-up of Att0001 gall (4 DPI) boxed in (C).
(E) Att0001 gall 4 DPI. The plant was inoculated in the soil.
Nematode-Induced Reporter Gene Expression

Grundler, 1992; Grundler et al., 1994b; Niebel et al., 1994b), the advantage of easy transformation (Valvekens et al., 1988; Márton and Browse, 1991; Clarke et al., 1992), and the thin roots allowing easy in vivo determination of gus reporter gene activity make this species a valuable model host plant in this promoter-tagging strategy.

To date, we have screened 1472 promoter tag lines for gus expression in developing syncytia. Because nematodes exploit the plant's gene regulation system to meet their needs, it was not unexpected that tagged regulatory sequences expressing the reporter gene in NFSs would also direct expression in other cell types. Nevertheless, antifeeding cell strategies in the development of resistance to nematodes call for promoters with specific spatial and temporal expression patterns. Confinement of the tagged promoter activity to the feeding structures might be possible by cloning promoter fragments containing only the nematode-responsive element, as was achieved for the TobRB7 promoter (Opperman et al., 1994).

Among the 103 transgenic plants displaying a distinct response to nematode infection, 39 lines also exhibited activity at sites of lateral root initiation, occasionally persisting during lateral root branching. In Arabidopsis, syncytia originate from a procambial cell or from derivatives of the pericycle (Golinowski et al., 1996). As its development progresses, the initial syncytial cell undergoes cytoplasmic changes, including increased density and streaming. Lateral root formation is initiated in pericycle cells that acquire dense cytoplasm while dividing periclinally (Esau, 1977). The results presented are consistent with a view that the processes of syncytium formation and lateral root development, which share common anatomical features, also share common patterns of gene expression.

Here, we describe six tags with the capacity for inducing transgene expression in NFSs. Nematode-triggered gus expression thereby reflects differences in timing of expression and differences in regulation by other nematodes. Moreover, only a fraction of the syncytia express the gus fusion at any particular stage of development. The hypothesis that small differences in the developmental stage of the nematode and/or host might generate significantly different transcript levels could explain a diversified induction of expression.

Figure 6. GUS Patterns in Roots of Transgenic Arabidopsis Lines after Incubation on Callus-Inducing Medium.

(A) Att1712 displays strong GUS staining in the root vascular tissue at the base of protruding calluses.
(B) Att0001 root tips show a typical three-zone pattern 6 DPI on callus-inducing medium.
(C) High GUS levels were observed inside callus tissue of Att0025-R/1.
(D) Shown are roots of P35S-gus—transformed plants after 6 days of growth on callus-inducing medium. Except for the root tips, newly formed lateral roots are not stained for GUS.
(E) Shown are P35S-gus roots after growth for 6 days on callus-inducing medium. Strong promoter activity in callus is evident. GUS activity starts to diminish (arrowheads) in root parts juxtaposed to the callus.
(F) Shown are roots of P35S-gus—transformed plants after 11 days of incubation on callus-inducing medium. GUS staining is confined solely to root tips and callus tissue. C, callus; LR, lateral root; RT, root tip; RVT, root vascular tissue. Bars in (A) to (F) = 50 μm.

Figure 5. (continued).

(F) Cross-section through an Att1712 gall 4 DPI.
(G) Att1712 gall 4 DPI. A nonstained zone is frequently observed.
(H) Cross-section through an Att1712 X. diversicaudatum—induced gall 4 DPI.
(I) Att0001 X. diversicaudatum—induced gall 4 DPI.
(J) Att0025 gall 7 DPI.
(K) Cross-section through an Att0025 gall 7 DPI.
(L) Att1164-R/1 gall 11 DPI.
(M) Att0728 gall 2 DPI.
(F), (H), and (K) were taken with dark-field optics. G, gall; GC, giant cell; MC, multinucleate cells; N, nematode; Nps, nematode penetration site; VP, vascular parenchyma. Bars in (A) and (D) to (M) = 50 μm; bars in (B) and (C) = 1 mm.
Resistance constructs should not interfere with sound regeneration and reproduction of transgenic plants. When strategies are used in which plant cytotoxic proteins are produced to destroy the NFS, expression correspondingly has to be avoided in callus and reproductive tissues. Therefore, one should avoid the use of promoters induced as a consequence of the transformation process itself. The inducibility of all six tags was monitored upon external hormone application with or without an Agrobacterium cocultivation step. Because calluses share characteristics with root knot nematode—induced galls, GUS activity could be expected in this tissue. Secretions emanating from root knot nematodes cause hypertrophy and to some extent hyperplasia (Endo, 1971), and the role of growth substances in these reactions seems plausible. Att0025 and its reintroduced derivative Att0025-R1 showed high GUS activity in parenchymatous callus tissue. No significant alterations in GUS pattern were observed after cocultivation with Agrobacterium.

Promoter activities beyond the NFS can be dealt with by employing the two-component system proposed by Sijmons (1993). However, one has to be cautious with this built-in safety feature. Constitutive promoters should be chosen carefully to ensure a complete counterbalance to any undesired effector gene expression driven by the nematode—responsive promoter in a given system. Because none of the six examined tags is expressed in pollen, the 3SS promoter would be a suitable promoter to abolish nematode—responsive promoter activity in tissues other than those in the NFS. In addition, because the 3SS promoter has been shown to be highly active in callus material, effector gene expression in this tissue, as observed for Att0025 and Att0025-R1, would be neutralized to allow proper shoot development.

Several LB and/or RB T-DNA fragments (Att0001, Att0025, and Att1164) have been cloned. Sequences upstream of the gus coding region were expected to contain the regulatory sequence responsible for the observed expression pattern, and this was indeed confirmed for Att0001, Att0025, and Att1164. Sequences downstream were expected to be part of the gene interrupted by the tag. None of the sequenced regions, however, showed significant homology to known genes. A small putative ORF of 159 bp was identified in the case of Att0001. Identification of other ORFs may have been hampered by the presence of introns. The regulatory capacity of Att0651, Att0728, and Att1712 needs to be confirmed, and for all six tags, the functionality of the putative, corresponding genes must be determined.

Previous molecular nematode—host interaction studies, focusing on the detection of upregulated transcript levels in feeding structures, have led to several findings: the isolation of structural protein genes belonging to the extensin family (Niebel et al., 1993; Van der Eycken et al., 1996), a late-embryogenesis abundant (Lea)—like gene (Van der Eycken et al., 1996), and a gene called pPMR1 of unknown function (Gurr et al., 1991). Several genes were also identified from a pool of upregulated root knot nematode—elicited transcripts in giant cells (Bird and Wilson, 1994a, 1994b).

In addition, promoters from known genes were fused to gus, and transgenic plants were monitored for a nematode—induced expression profile. Intriguing results obtained by Goddijn et al. (1993) demonstrated downregulation in syncytia of several constitutive promoters [the cauliflower mosaic virus 3SS promoter, the napinlike synthase [nos] promoter, the hairy root genes of Agrobacterium rhizogenes [roIA, roIB, roIC, and roID], a tonoplast— intrinsic protein [γ-TIP] promoter, and a cytokinin gene [T—cyt]]. High GUS levels in galls were obtained with the promoters of the hydroxymethylglutaral dehydrogenase (hmgD) (Cramer, 1992; Fenoll et al., 1997), TobRB7 (Opperman et al., 1994), and the Parasponia andersonii hemoglobin (Ehsanpour and Jones, 1996) genes. Niebel et al. (1996) also demonstrated that the cell cycle regulator genes cdc2a and cyc1At are expressed in the initial stages of syncytium and giant cell formation. Root meristems of cdc2a—gus plants showed a zone devoid of GUS staining amid two stained zones. In this study, Att0001 root tips showed a similar three—zone pattern when incubated on callus—inducing medium. Such a meristematic pattern also appeared in histone H4 promoter—gus (Atanassova et al., 1992) and auxin—treated cdc2a promoter—gus transgenic plants (Hemerly et al., 1993).

Although the isolation of nematode—responsive promoters is the primary goal of our research, the question remains whether the observed patterns of reporter gene activity accurately reflect the expression patterns of the tagged native genes or are merely caused by fortuitous promoter—like sequences that are not part of a functional gene (so—called pseudopromoters or cryptic promoters; Lindsey et al., 1993). Fobert et al. (1994) have described the activation of a promoter trap in tobacco by sequences that did not appear to be associated with a functional gene. In contrast to that description, all tagged loci of five analyzed transgenic Arabidopsis lines, containing pΔgusBin19, were demonstrated to correspond to an expressed gene (Topping et al., 1994; Muskett and Lindsey, 1995; Ferreira da Rocha et al., 1996; Wei et al., 1997). This correspondence was determined by RNA gel blot analysis or by the identification of a CDNA corresponding to the tagged locus, or both. In the two cases studied in greatest detail, the T—DNA was integrated into an intron of the respective tagged gene (Ferreira da Rocha et al., 1996; Wei et al., 1997). RNA gel blot analysis further demonstrated that the Proliferating gene (Springer et al., 1995) and the LRP1 gene (Smith and Fedoroff, 1995) were expressed in a pattern similar to that of the observed GUS activity in the tagged line.

To date, we do not know whether the tags reported here represent functional genes, because the available sequence data share no significant homologies with known genes. A lack of ORFs in neighboring genomic DNA, except in the case of Att0001, might represent promoter, intron, or intergenic sequences, and this remains to be determined.

We conclude that promoter tagging represents a powerful approach to identify plant sequences that can readily be cloned and used as tools to drive transgene expression in
NFSs. Potentially, promoter tagging will also facilitate the identification and cloning of genes that play a role in the development of the feeding structure and allow investigations into signaling mechanisms that activate gene expression in response to nematode infection in plants.

METHODS

Binary T-DNA Vectors and Agrobacterium tumefaciens Strains

Construction of pMOG553 was performed as described by Godijn et al. (1993), except that the β-glucuronidase (gus) gene was followed by a cauliflower mosaic virus 35S terminator and not by a nopalone synthase (nos) terminator (as erroneously stated in the article by Godijn et al., 1993). Construction of pGV1047 has been reported by Kertbundit et al. (1991). pGusBin19 comprises the uidA coding region at the left T-DNA border and has been described by Topping et al. (1991). Mobilization of pMOG553 and pGV1047 from Escherichia coli into Agrobacterium has been described by Godijn et al. (1993) and Kertbundit et al. (1991), respectively. pAgusBin19 was transferred from E. coli MC1022 into Agrobacterium strain C58C1RifR (Holsters et al., 1980) harboring either the octopine vir plasmid pGV2260 (Deblaere et al., 1985) or the nopaline vir plasmid pMP9O (Koncz and Schell, 1986).

Plant Transformation

Att0025, Att0651, and Att1164 were generated as described by Godijn et al. (1993). Att0001, Att0728, and Att1712 were recovered according to the transformation procedure described by Clarke et al. (1992) with some modifications (Barthes et al., 1994; Karimi et al., 1994).

Callus Induction

Roots from 2-week-old plants were incubated on callus induction medium (CIM) (Valvekens et al., 1991). Wild-type C24 and transgenic 35S-gus Arabidopsis thaliana plants were used for control purposes. GUS histochemical assays were performed after incubation for 6 and 11 days.

Agrobacterium Infections

Roots from 14-day-old promoter-tagged plants were incubated for 3 days on CIM (Valvekens et al., 1991). Subsequently, whole-root systems were cut into small explants and mixed with a C58C1RifR (pGV2260) Agrobacterium solution (OD600 of 0.1), after which the cocultivated material was further incubated on CIM for an additional 3 days. Root explants were washed several times to remove all overgrowing agrobacteria and were stained for GUS activity. Wild-type C24 and transgenic 35S-gus Arabidopsis plants were used for control purposes.

Nematode Cultures and Hatching Procedures

Root knot nematode (Meloidogyne incognita) cultures were maintained in vitro on tomato (Lycopersicon esculentum) hairy roots continuously subcultured on hormone-free Gamborg’s B5 medium (Flow Laboratories, Bioggio, Switzerland; pH 6.2) supplemented with 2% sucrose and 1.5% Bacto agar (Difco, Detroit, MI). Cyst nematodes (Heterodera schachtii) were grown in vitro on mustard (Sinapis alba) roots in Knop medium (Sijmons et al., 1991). Hatching was stimulated by putting cysts (H. schachtii) or galls (M. incognita) on 70-μm nylon sieves (Falcon 2350 Cell Strainer; Becton Dickinson, Bedford, MA) submerged in filter-sterile root exudate extracted from rapeseed (Brassica napus) and sterile deionized water, respectively. The migratory ectoparasitic nematodes (Xiphinema diversicaudatum) were cultured on raspberry (Rubus idaeus cv Glen Moy) in soil. Its size of 6 mm allowed us to isolate nematodes quite easily from the sand by using a sieve.

Nematode Inoculation of Tagged Arabidopsis Lines

For the X. diversicaudatum inoculations, seven to 10 Arabidopsis seeds were sown in a 1:1 sand/compost mixture in 30-mL plastic pots. These pots were then arranged in small propagation trays with a clear plastic cover. The trays were placed in a greenhouse at 18°C with 16 hr of light. The Arabidopsis plants were grown for 14 days before infection. Pots were inoculated twice (1 week apart) with five to 10 nematodes. After 1 more week, the roots were washed and stained for GUS activity. Plants were scored immediately using reflected light so that the GUS activity signal could be detected easily in the optically dense galls.

For cyst and root knot nematode soil inoculations, 2-week-old Arabidopsis seedlings were transferred to a 1:2 mixture of cutting soil (M. Snebbou s.a., Kaprijke, Belgium) and potting soil (M. Snebbou s.a.) in open translucent plastic tubes. By placing these tubes on a slant in rectangular flower boxes, the roots were forced to grow along one side of the tube, allowing more controlled inoculations and reproducible infections. Inoculations were performed after 2 more weeks of growth at 22°C and 16 hr of light by injecting a suspension containing 250 second-stage juveniles (5 to 7 days after hatching) of beet cyst or root knot nematodes in 1.5 mL of H2O per root system. One to 2 weeks later, three to five plants were washed carefully and stained for GUS.

In Vitro Inoculations with Cyst and Root Knot Nematodes

S2 seeds can be sown directly on selective Knop medium (Sijmons et al., 1991). On the other hand, S2 plants frequently showed abnormal growth when cultured for 2 weeks or longer on Knop medium, impeding sound analysis of inoculation and staining results after this time.

Surface-sterilized seeds (2 min in 70% ethanol and 15 min in 5% sodium hypochlorite) were germinated on germination medium (Valvekens et al., 1988) supplemented with either 50 mg L−1 kanamycin monosulfate (Sigma) or 20 mg L−1 hygromycin B (Calbiochem, La Jolla, CA). Two-week-old seedlings were subsequently transferred to and lined up on a thin layer of Knop medium. Petri dishes were placed slightly tilted to promote unidirectional root growth. After growth for 2 more days at 22°C (16-hr-light/8-hr-dark cycle), roots were incubated with 5- to 7-day-old hatched beet cyst or root knot nematode second-stage juveniles at an average density of 20 juveniles per root system. The plants were then incubated again under the same tissue culture conditions. Five to 10 plants were examined for the presence of GUS activity 4 to 6 days postinoculation (DPI).
GUS Histochemical Assay

Histochemical localization of GUS activity was performed with the substrate 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc; Europa Research Products, Ely, UK), according to Jefferson (1987) with minor modifications: 50 μL of X-gluc (20 mg in 1 mL of N,N-dimethylformamide) was diluted to a final concentration of 2 mM in 1 mL of 0.1 M NaPO₄, pH 7.2. Oxidative dimerization of the produced indoxyl derivative was enhanced by adding the oxidation catalyst K₃ ferrocyanide/ferrocyanide to a final concentration of 0.5 mM. Incubation of whole plantlets in phosphate buffer was preceded by a short treatment (15 to 30 min) with 90% ice-cold acetone followed by several washes with 0.1 M sodium phosphate, pH 7.2. The GUS reaction was incubated overnight at 37°C. Stained tissues were subsequently fixed for a few hours to overnight in 2.5% glutaraldehyde (Agar Scientific Ltd., Stansted, UK) at 4°C to prevent diffusion of the GUS product during the subsequent incubation in chloroalginatoxydine (2:1:1 mixture of chloral hydrate, lactic acid, and phenol) (Beeckman and Engler, 1994). Incubation of the material in chloroalginatoxydine removes all pigments and brown phenolics, producing transparent tissues that were further monitored for GUS activity by using a dissecting light microscope (Jenalumar; Zeiss, Oberkochen, Germany).

Sectioning and Microscopic Analysis

Blue-stained syncyta and galls were fixed in 4% paraformaldehyde/1% glutaraldehyde; samples were vacuum infiltrated for 20 min and further incubated overnight at 4°C in fresh fixing solution. After several dehydration steps, material was embedded in LR white medium-grade resin (London Resin Co., Basingstoke, UK) or butyl-methyl acrylate resin (Merck-Schuchardt, Hohenbrunn bei München, Germany, and BDH Laboratory Supplies, Poole, UK). Sections (2 to 2.5 μm thick) were examined by using dark-field optics (Diaplan; Leitz, Wetzlar, Germany). Sectioned material was sometimes stained for examination by using bright-field optics (Diaplan, Leitz); after removing the butyl methyl acrylate resin with acetone (15 min incubation), sections were immersed in a 0.1% ruthenium red (Sigma) solution for 7 to 20 min.

Nematode Staining

Nematodes inside root tissues can be visualized according to the McBryde method (Daykin and Hussey, 1985). After GUS histochemistry, fixation in 2.5% glutaraldehyde, and clearing in chloroalginatoxydine, root material was left in acid fuchsine dye for 16 hr and subsequently destained for 3 hr in a saturated chloral hydrate solution.

Inoculation Time-Course Experiments

All time-course experiments were performed in vitro (Knop medium containing 1% sucrose) with cyst nematodes. For each line, several 9-cm Petri dishes were prepared with 20 seeds lined up in two rows. The seeds had been vernalized at 4°C for 3 days to break dormancy. Ten days after germination, each plant was inoculated with 30 second-stage juveniles. Plants were monitored for GUS activity at 2, 4, 7, 12, and 30 DPI. The GUS assays with the infected plants were performed immediately. In a Petri dish, 4 mL of X-gluc solution, including Fe-cyanide, was poured on top of the agar, and the plates were incubated at 37°C for 24 hr. Subsequently, plants were examined for GUS activity in the nematode feeding structures (NFSs).

DNA Extraction

For lines Att0001, Att0728, and Att1712, 0.2 to 2 g of plant material was used for the preparation of DNA, as described by Dellaporta et al. (1983), with some modifications. The DNA pellets were dissolved in 400 μL of Tris-EDTA to which 20 μg of RNase was added. After an incubation period of 20 min at 37°C, 400 μL of 0.2 M Tris-HCl, pH 7.5, 2 M NaCl, 0.05 M EDTA, and 2% (w/v) cetyltrimethylammonium bromide was added; the mixtures were incubated for another 15 min at 65°C. The samples were extracted with 800 μL of chloroform–isoamylalcohol (24:1) and precipitated. For the Att0025, Att0651, and Att1164 lines, plant DNA was prepared as described by Mettler (1987).

T-DNA Number Determination by DNA Gel Blot Analysis

Att0001, Att0728, and Att1712 plant DNA was digested with HindIII and/or EcoRI in a double or single digest. Separation of the digested samples on a 1% agarose gel was followed by an overnight blotting to a Hybond N membrane (Amersham, Aylesbury, UK). The DNA on the membrane was fixed through UV cross-linking (GS Gene Linker; Bio-Rad, Hercules, CA). The 1.7-kb Nri gus coding region of pGUS1 (Peleman et al., 1988) was used as a probe. Radioactive labeling was performed with the Ready-To-Go DNA labeling kit (–dCTP) (Pharmacia, Uppsala, Sweden), according to the manufacturer's instructions. The nylon membrane was incubated in a hybridization buffer (450 mM NaCl and 45 mM sodium citrate, pH 7.0), 0.1% SDS, 0.25% milk powder (Gioria, Vevey, Switzerland), and 20 μg mL⁻¹ herring sperm DNA (Promega, Madison, WI) for 3 hr at 65°C. Hybridization was performed overnight in fresh hybridization buffer to which the corresponding α-32P-dCTP-labeled probe was added.

Inverse Polymerase Chain Reaction

Target DNAs with the corresponding primer annealing sites are shown in Figure 7. Att0001 DNA was digested with Spl and EcoRI and circularized under conditions favoring self-ligation (Sambrook et al., 1989). Inverse polymerase chain reaction (IPCR) was performed with primer sets 1 and 2, and 3, respectively (Figure 7A). The PCR mix consisted of 50 ng of self-ligated DNA, 200 ng of each primer, 1 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates, 2.5 μL of 10× Taq buffer, and 0.5 μL of Taq polymerase (5 units μL⁻¹) (Beckman, Fullerton, CA), in a total volume of 25 μL, and 25 μL of mineral oil. A total of 35 cycles was used. For both primer sets 1 and 2 and 1 and 3, the same temperature program was followed, except that the annealing temperatures were 64 and 60°C, respectively. The cycle order is as follows: cycle 1, 4 min at 95°C, 2 min at 64/60°C, 10 min at 72°C; cycles 2 to 35, 1 min at 95°C, 2 min at 64/60°C, 3 min at 72°C; and finally, for 10 min at 72°C. The following primers were used: primer 1, 5'-CCAGCGTGGACCGCTTGCTGGAAC-3'; primer 2, 5'-GTATTGCCAACGACCCGATACCCG-3'; and primer 3, 5'-CCCAGTCACGACGTTGTAAAAC-3'.
Figure 7. Schematic Presentation of the IPCRs Performed in This Study.

(A) pAgusBin19 inverted T-DNA repeat structure in Att0001. Primer sets 1 and 2 and 1 and 3 were used to isolate both LB flanking sequences.

(B) Restriction sites and primer combinations used for Att0025 and Att1164 genomic DNAs to isolate RB flanking regions. Att0025 genomic DNA was digested with PstI followed by IPCR using primers 5 and 8. Only one of the four insertions could be recovered. Att1164 genomic DNA was digested withMspI followed by IPCR using primer sets 5 and 7.

Abbreviations are as given in Figure 1. Numbered arrows indicate the different primer-annealing positions.

Att1164 genomic DNA was digested withMspI followed by IPCR (Does et al., 1991); primer sets 5 and 7 were used (Figure 7B). Standard conditions for Perkin Elmer Cetus (Branchburg, NJ) Taq polymerase were used; the total reaction volume was 50 μL. Cycle 1 was for 3 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C. Cycles 2 to 30 were for 1 min at 94°C, 1 min at 50°C, and 1.5 min at 72°C. Cycle 31 was for 1 min at 94°C, 1 min at 50°C, and 5 min at 72°C. Primer 5 (5'-CTTTCCCACCAACGCTGATC-3') and primer 7 (5'-GTAATGCTCTACACCACGCCG-3') were used.

Att0025 genomic DNA was digested withPstI. IPCR (Does et al., 1991) was performed with primers 5 and 8 (Figure 7B). Cycle order and primer 5 were as given for Att1164, with primer 8 being 5'-CGCATCGTCGGCTAC-3'.

Construction of Genomic Libraries

The Arabidopsis C24 library was constructed by partial digestion of total DNA withSau3A. After partial filling in of the overhang sites with nucleotides T and C, the fragments were ligated inXhoI-digestedλGEM11 arms in which the restriction sites had been partially filled with nucleotides A and G. The partial filling makes the Sau3A site compatible withXhoI and prevents the cloning of multiple inserts in the vector.

For the construction of the Att0025 genomic library, total DNA was isolated according to the method of Mettler (1987). The DNA was partially digested withSau3A and size fractionated on a sucrose gra-

dient to obtain a more uniform range of insert sizes. A block gradient of 40, 35, 30, 25, 20, 15, and 10% sucrose in Sephadex G-50 equilibrated in Tris-EDTA, pH 8.0, containing 0.1 M NaCl was layered stepwise in 5-mL ultracentrifuge tubes, and 80 μg of DNA per tube was centrifuged for 18 hr at 75,000g. Fractions of five droplets were collected and analyzed on a 0.3% Tris-borate agarose gel. DNA fractions of 9 to 20 kb were used for the genomic library construction inλGEM11 dephosphorylatedBamHI arms (Promega). We screened 250,000 recombinant phages (approximately fivefold the genome size) with a radiolabeledNcol-EcoRV5' gus fragment from pMOG18 (Sijmon et al., 1990). This process resulted in 63 positive signals. A selection of these phages was purified, and the inserts were analyzed.

Vector Constructions for Reintroduction

Vectors used for reintroduction into Arabidopsis of promoter-containing T-DNA-flanking regions are presented in Figure 8. The binary T-DNA vectorpTHW136 (Figure 8A) contains theP35S-gus-intron-3'358 cassette from P35SGUSINT (Vancanneyt et al., 1990). pTHW136 was kindly provided by Plant Genetic Systems nv (Gent, Belgium).

ConstructpMOG402 was derived from binary plasmidpMOG23 (Sijmons et al., 1990) by substitution of the wild-type neomycin phosphotransferase II(nptII) coding region for a mutantnptII coding region (Yenofsky et al., 1990). pMOG800 (Figure 8B) was made from pMOG402 through replacement of the EcoRI-BamHI linker to introduce the...
a new KpnI site in the polylinker. pMOG819 (Figure 8C) was constructed by cloning a BamHI-EcoRI fragment of pMOG553, harboring the gus-intron gene, into the binary vector pMOG800.

Reintroduction of Regulatory Regions

The reintroduction of the Att0025-1 right border (RB) was accomplished by transformation with pMOG821, resulting from the cloning of a 3.4-kb Smal genomic fragment containing the putative regulatory sequence in front of gus in the binary vector pMOG819. Because Smal cuts only 20 bp from the RB in the T-DNA, practically the entire 3.4-kb fragment is plant sequence. From tags Att0025-2, Att0025-3, and Att0025-4, BamHI fragments of 1.2, 1.6, and 2.7 kb, respectively, were cloned in front of gus in pMOG819, yielding pMOG820, pMOG847, and pMOG848, respectively.

The reintroduced 1480-bp PCR RB-flanking Att1164 sequence was obtained as follows. A 1.6-kb RB fragment was initially amplified by IPCR. Based on this sequence, PCR amplification was performed using Phu DNA polymerase with 3’ to 5’ proof-reading activity (Stratagene, La Jolla, CA) in combination with primers to introduce new cloning sites. The resulting PCR fragment of 1480-bp RB-tagged sequence was cloned upstream of the gus coding region in pMOG819 to make pMOG849.

From Att0001, both LB flanking regions were isolated by IPCR. Amplified fragments of ~0.6 and ~2.8 kb, designated ARM1a and ARM1b, respectively, were cloned in front of gus in pTHW136. After introduction into Arabidopsis, pthARM1-a600, corresponding to the cloned ~0.6-κb ARM1a fragment, revealed a nematode response similar to that of the original tag.

REFERENCES


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