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Nematode-Responsive Activity of the Cauliflower Mosaic Virus 35S Promoter and Its Subdomains

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Root-knot and cyst nematodes are obligate plant parasites that induce complex biotrophic feeding structures in host roots. The mechanisms by which nematodes regulate host gene expression to produce feeding sites are unknown. The cauliflower mosaic virus (CaMV) 35S promoter has been reported to be repressed strongly in the feeding sites of both root-knot and cyst nematodes. In contrast, other work has indicated that this promoter is partially active in some feeding sites. Considering the importance of the 35S promoter in biotechnology, we have defined the nematode-responsive nature of this promoter in more detail. Transgenic tobacco harboring various 35S-*uid*A constructs was assayed for β-glucuronidase (GUS) activity after infection by root-knot nematodes (*Meloidogyne incognita)* and cyst nematodes (*Globodera tabacum* subsp. *tabacum*). The entire 35S promoter (–343 to +8) was active in giant cells induced by *M. incognita* and, to a lesser extent, the syncytia of *G. tabacum* subsp. *tabacum*. In the latter case, activity decreased as the feeding sites matured. Subdomains of the 35S promoter were also active in feeding sites, particularly B4 and B5 in giant cells. However, subdomain B3 was strongly down-regulated in gall tissue and syncytia. In total, 14 constructs were studied and nematode-responsive expression was always stronger and more consistent with the root-knot nematode than the cyst nematode.

Plant-parasitic nematodes are a diverse group of microscopic, semitransparent "worms" that feed on living plant cells. Many species are significant agricultural pests and cause large crop losses (Sasser and Freckman 1987). Two of the most economically damaging groups are the root-knot nematodes (*Meloidogyne* spp.) and the cyst nematodes (*Heterodera* and *Globodera* spp.); both groups are highly specialized parasites that induce and maintain complex feeding sites in host roots. These nematode feeding sites (NFS) have transfer cell-like properties and are the only source of nutrients for the nematodes. The feeding sites develop differently for the two nematode groups (Jones 1981; Sijmons et al. 1994a). Root-knot nematodes induce the formation of a variable number of discrete, multinucleate "giant cells" that develop by repeated mitosis without cytokinesis. The giant cell complexes are surrounded by gall tissue that is derived largely from the root cortex. In contrast, cyst nematodes induce a single, large, multinucleate syncytium that develops without mitosis by cell expansion and the incorporation of adjacent plant cells by cell wall degradation. There is no galling associated with syncytia. Despite these differences in development, both giant cells and syncytia are metabolically very active, and have dense granular cytoplasm, abundant mitochondria and endoplasmic reticulum, small vacuoles, and enlarged amoeboid nuclei (Jones 1981).

It has long been suspected that host genes play an active role in the development of NFS and that these structures might be unusual or unique in terms of their overall patterns of gene expression (Burrows 1992; Sijmons et al. 1994a). However, little is known about the spectrum of genes expressed in giant cells and syncytia and how nematodes manage to orchestrate the changes. Recently, research has focused on the identification and characterization of promoters that respond to feeding site initiation and development. Studies with transgenic plants containing various promoter-*uid*A (*gus*-A) fusions have shown that, in addition to promoters that up-regulate genes in response to nematode challenge (Cramer 1992; Goddijn et al. 1993; Opperman et al. 1994.), a number are also turned off or down-regulated in and around the NFS (Goddijn et al. 1993). This nematode-responsive repression is particularly striking because it extends beyond plant promoters to those of bacterial and viral origin, such as *rol* promoters from *Agrobacterium rhizogenes* and the cauliflower mosaic virus (CaMV) 35S gene promoter, which normally direct high levels of expression in roots and elsewhere.

The CaMV 35S promoter is one of the best-studied plant promoters. It was divided into six domains (the A domain and five B domains) by Benfey et al. (1990a, 1990b) and the activity of a number of derivatives of the promoter was studied. Binding sites for two transcription factors have been well characterized and at least two other factors probably interact with the promoter (Lam and Chua 1990). The 35S promoter seems to consist of multiple *cis* elements, some of which are synergistic and developmentally regulated (Benfey et al. 1990a, 1990b). The promoter is very active in most plant tis-

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sues and is, perhaps, the most widely used promoter for the expression of transgenes in plants. Despite the strong "constitutive" nature of the 35S promoter it has been reported that it is strongly silenced in a high proportion of nematode-induced giant cells and syncytial feeding sites. (Goddijn et al. 1993; Sijmons et al. 1994b). Due to the importance of this promoter in plant biotechnology and as part of a longer term study into the ability of sedentary nematodes to control host gene expression, we report here on the further characterization of the response of the 35S promoter to nematode infection. A total of 14 constructs, including the entire 35S promoter (–343 to +8) and constructs based on the five B subdomains, were examined. This is the most detailed analysis of a nematode-responsive promoter yet reported and has implications for our understanding of the changes in gene expression upon nematode infection and for those seeking to engineer nematode resistance by molecular genetic methods.

RESULTS

Expression of the *uid*A gene in tobacco driven by the various combinations of the 35S promoter A and B domains (Fig. 1) has

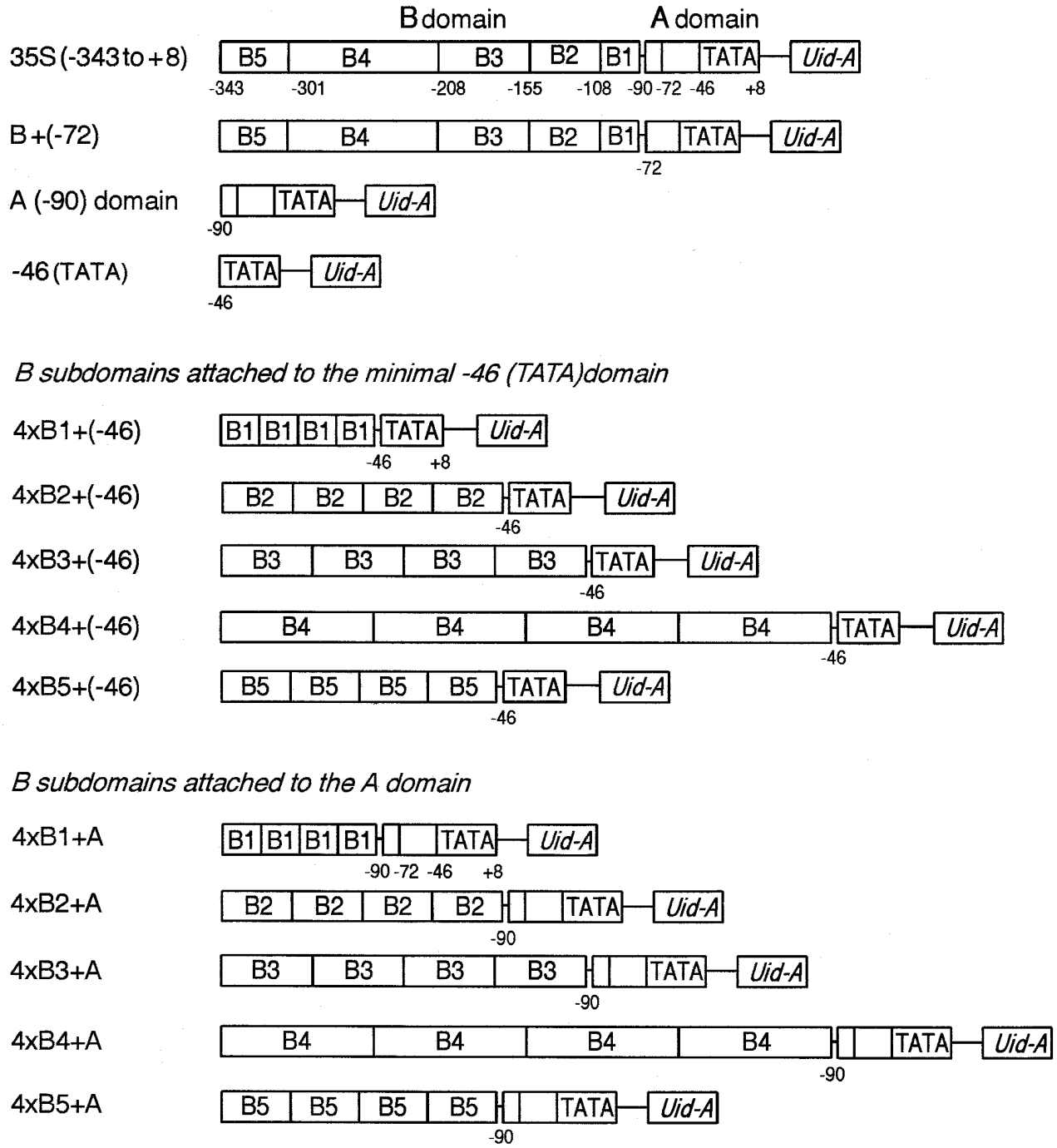


Fig. 1. 35S promoter and subdomain constructs analyzed for nematode-responsive expression.

already been described in detail (Benfey et al. 1989, 1990a, 1990b). In the current investigation, background expression patterns observed in the roots of uninfected (control) plants were essentially the same as described previously but there were a few minor differences. Nearly all of these were inconsistent and varied between independent transformants. However, the most consistent deviation from the described pattern was with the A (–90) domain. Benfey et al. (1990b) reported that the A domain alone gave β-glucuronidase (GUS) activity in the meristematic and cortex regions of the root tips, but in the four lines examined here there was no reproducible activity in roots with this construct. This could be explained by the different growth conditions employed, agar medium (Benfey) vs soil (this study) or the different relative ages of the plants at the time of observation. In general, the expression patterns observed in roots, whether infected by nematodes or not, were stronger in younger tissues and, for some constructs, e.g., 4xB1+A and 4xB4+A, expression in older tissues was weak or absent. Of the 14 constructs examined (Fig. 1), eight showed no GUS expression in either infected or uninfected roots (Table 1).

Nematode-responsive domains.

NFS in each root system were scored for presence or absence of GUS activity (Table 1). The scoring criterion used to assess activity of the constructs was frequency of GUS activity in NFS, i.e., the proportion of GUS-positive feeding sites in a single root system, rather than comparative intensity of GUS staining. This was taken to be a direct indication of the potential for the various 35S elements to respond to nematodes. The greater the frequency of blue feeding sites, the more nematode responsive the construct. No attempt was made to quantify GUS expression precisely but, by eye, most lines showed comparable intensity of GUS staining. The few cases in which lines showed obvious "strong" or "weak" expression relative to each other were noted.

35S (–343 to +8) promoter.

The entire 35S (–343 to +8) promoter gave strong *uid*A expression throughout the roots but it became weaker and more patchy in older regions. GUS staining could be seen clearly in a proportion of the galls and giant cell complexes of *Meloidogyne incognita* and the syncytial feeding sites induced by *G. tabacum* subsp. *tabacum* (Fig. 2A and B). In some cases, where the background expression in the root was weak, stronger expression was seen in the galls and giant cells (Fig. 2A). In these instances the 35S promoter appeared to be up-regulated by *M. incognita* relative to local background expression.

One of the most striking initial observations for this and other constructs was that only a proportion of the feeding sites in any one root system showed GUS activity. The relative proportion of feeding sites associated with up- or down-regulation of *uid*A differed greatly between the different constructs and the two nematode species used (Table 1). For example, with the entire 35S promoter construct 90% of *M. incognita* feeding sites examined showed GUS activity compared with only 27% for *G. tabacum* subsp. *tabacum*. In the case of the cyst nematode *(G. tabacum* subsp. *tabacum*) there was an obvious tendency toward greater silencing of *uid*A expression as the feeding sites became more mature (Fig. 2B).

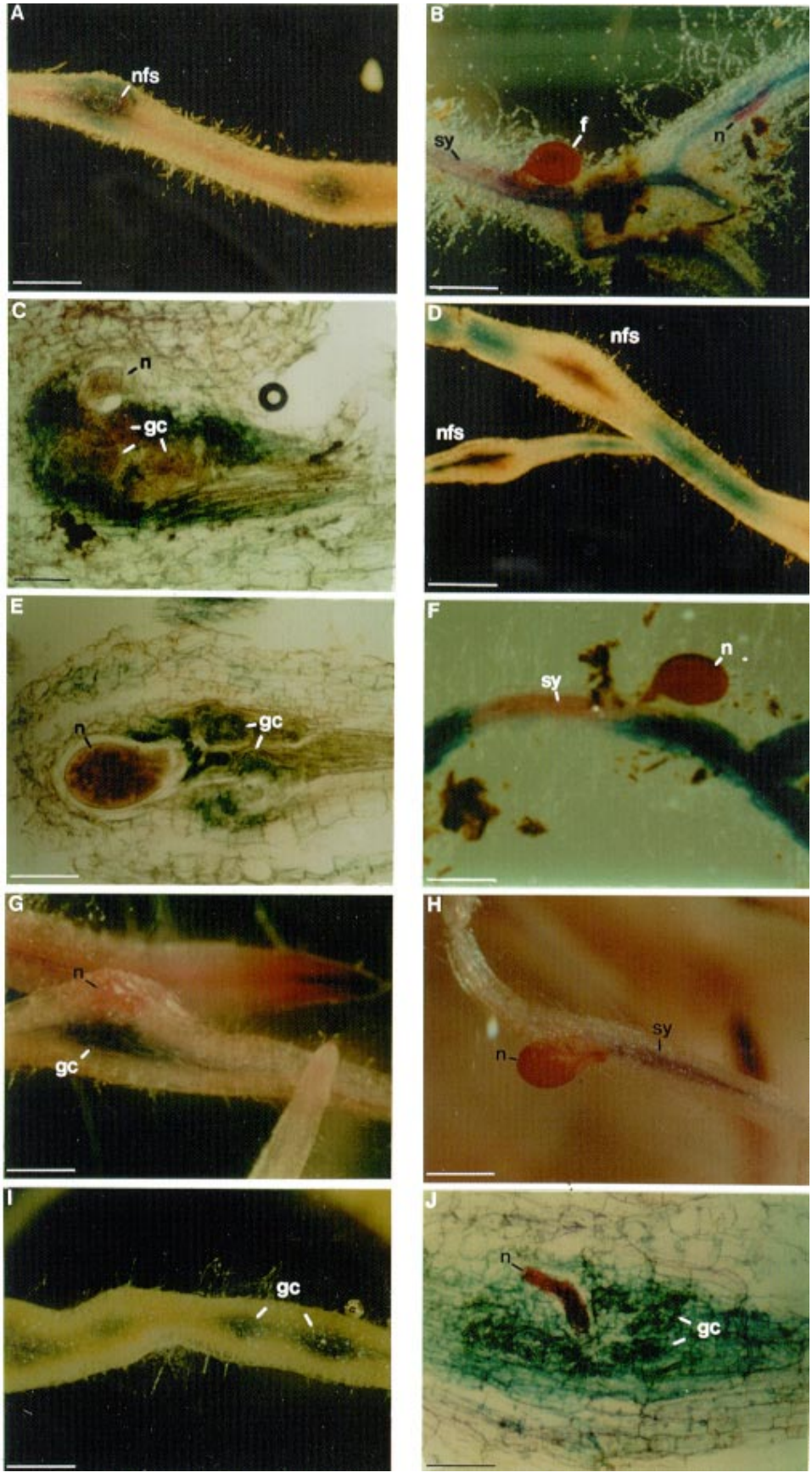
B domain + (–72).

This promoter is similar in sequence to the entire 35S (–343 to +8) promoter (Fig. 1) but, significantly, it lacks the *as*-1 binding site considered necessary for efficient root expression (Lam et al. 1989). Nevertheless, this construct still resulted in GUS activity in roots but the activity was weaker and more erratic than with the entire 35S promoter. The nematode-responsive nature of B+(–72) is similar to the pattern of the entire 35S activity in syncytia but results in fewer giant cells showing GUS activity: 63% for B+(–72) compared with 90% for the entire 35S promoter (Table 1).

Table 1. Number of nematode feeding sites (NFS) showing *uid*A expression driven by the 35S promoter and its subdomain

		Meloidogyne incognita: **Giant cell complexes**				***Globodera tabacum*** **subsp. *tabacum:* Syncytia**			
Construct	**Control[u]**	**GUS (+) NFS[v]**	**GUS (–) NFS[w]**	**Total sites observed**	**GUS (+) NFS (%)**	**GUS (+) NFS[v]**	**GUS (–) NFS[w]**	**Total sites observed**	**GUS (+) NFS (%)**
35S (–343 to +8)	**C S A**	90	10[x]	100	90	65	175[x]	240	27
B+(–72) (no *as*-1)	C S A	74	44[x]	118	63	42	85[x]	127	33
–46 (TATA)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
A (–90) domain[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB1+(–46)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB2+(–46)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB3+(–46)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB4+(–46)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB5+(–46)[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB1+A	S a	112	104	216	52	0	44	44	0
4xB2+A[y]	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .	. . .
4xB3+A	C S a	99	63[x,z]	162	61	2	64[x]	66	3
4xB4+A	s a	253	33	286	88	14	122	136	10
4xB5+A	s a	298	2	300	99	12	69	81	15

[u] Expression observed in uninfected roots. C = cortex, S = stele, A = root apex. Letters in bold uppercase indicate strong, essentially consistent expression; uppercase letters indicate less strong and/or inconsistent expression; lowercase letters indicate erratic and/or weak expression.
[v] Number of NFS showing β-glucuronidase (GUS) activity.
[w] Number of NFS showing no GUS activity.
[x] GUS expression down-regulated against the local background (pale feeding sites in blue roots).
[y] No GUS expression in roots of infected or uninfected plants.
[z] GUS expression down-regulated in all galls examined.


A
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4xB1+A.

Plants harboring the 4xB1+A construct expressed GUS in a small proportion of root tips and inconsistently in the stele. In whole-mount root segments approximately 50% of the feeding sites induced by *M. incognita* showed GUS activity associated with the central giant cell complex. No staining was seen in any of the galls examined. Cryo-sections revealed the GUS staining to be localized immediately around the giant cells and attendant nematodes with little activity inside the giant cells themselves (Fig. 2C). In contrast, there was no *uid*A expression in or around any of the syncytial feeding sites examined. In roots where GUS activity in the stele was evident, down-regulation of GUS could be seen where the stele ran close to any syncytial feeding sites.

4xB3+A.

Uninfected roots showed GUS staining in the root cortex and in a proportion of root tips. Expression was also evident within the central stele of the roots. However, upon infection with *M. incognita* there was no GUS activity in the gall tissue and adjacent areas of root (Fig. 2D). In these areas, *uid*A expression was strongly down-regulated against the local background. This demonstrates that the regulatory influence of the nematode extends beyond the immediate feeding cells. Expression of *uid*A in association with the giant cells themselves was inconsistent but the majority (61%) showed at least some GUS staining (Table 1). Cryo-sections confirmed that the GUS activity originated inside as well as immediately around the giant cells (Fig. 2E).

Unlike the giant cell complexes that showed some GUS activity, the syncytial feeding sites induced by *G. tabacum* subsp. *tabacum* showed virtually no GUS staining (Table 1). Again, marked repression of 4xB3+A was evident in the cortex cells around and adjacent to the feeding sites (Fig. 2F). This down-regulation of *uid*A was evident early in the infection process (approximately 4 to 6 days post infection) and became stronger as the feeding sites matured.

4xB4+A.

4xB4+A resulted in little background expression in roots but some GUS staining could be seen occasionally in the stele and a small proportion of root tips. Upon infection with *M. incognita*, 88% of the feeding sites showed strong GUS activity in and immediately around the giant cells (confirmed by cryo-sectioning). This was particularly marked because of the otherwise largely unstained root background (Fig. 2G). No expression was observed in the bulk of the gall tissue. GUS activity was also observed in the syncytial feeding sites of *G. tabacum* subsp. *tabacum* (Fig. 2H) but this was weaker and much less frequent (10% of syncytia) than in giant cells.

4xB5+A.

Background GUS expression driven by the 4xB5+A construct was similar to that described for 4xB4+A. However, the response of 4xB5+A to infection by *M. incognita* was striking, with 298 feeding sites out of 300 observed showing strong GUS activity inside the giant cells and cells immediately adjacent (Fig. 2I and J). No expression was seen in gall tissue. The response of 4xB5+A to *G. tabacum* subsp. *tabacum* was considerably less than with *M. incognita*. Only 15% of the syncytia showed GUS activity and in these *uid*A expression was apparently confined to the syncytium alone.

Nematode-responsive changes in root tip expression.

Uninfected plants harboring the constructs 4xB1+A, 4xB4+A, or 4xB5+A showed a low frequency of background GUS activity in root tips (Fig. 3). However, upon infection with nematodes the frequency of GUS activity in root tips increased significantly, even in roots distant from nematode infection/feeding sites. This effect was more marked after infection by *G. tabacum* subsp. *tabacum* than by *M. incognita*, especially in the case of the construct 4xB5+A, which increased from 1.3% of root tips showing GUS activity in uninfected plants to 42.8% GUS positive after infection.

DISCUSSION

The activity of the CaMV 35S (–343 to +8) promoter and various combinations of its subdomains has been analyzed in roots infected with a cyst and a root-knot nematode species. The results demonstrate a mixture of activities of the promoter constructs in association with NFS, ranging from strong and consistent GUS activity (4xB5+A with *M. incognita*) to apparent near total silencing (4xB3+A with *G. tabacum* subsp. *tabacum*). This work is timely because it serves to clarify the conflicting published and anecdotal reports regarding the nematode-responsive nature of this important promoter. Goddijn et al. (1993) and Sijmons et al. (1994b) reported the 35S promoter in *Arabidopsis thaliana* was strongly silenced in the feeding sites of both cyst and root-knot nematodes. Since the 35S promoter is used widely in biotechnology this observation has great relevance to those attempting to engineer nematode

Fig. 2. Histochemical staining for β-glucuronidase (GUS) activity in transgenic tobacco roots infected with cyst and root-knot nematodes. **A and B,** Tobacco line harboring the entire 35S (–343 to +8)-*uid*A construct infected with **(A)** *Meloidogyne incognita* or **(B)** *Globodera tabacum* subsp. *tabacum*. **A,** The root section illustrated shows little background GUS activity but staining is evident associated with the feeding sites (nfs) induced by *M. incognita*. **B,** The syncytial feeding site (sy) induced by the mature swollen female (f) of *G. tabacum* subsp. *tabacum* shows down-regulation of GUS activity relative to root background. In contrast, the feeding site of the immature nematode (n) is associated with little or no down-regulation. **C,** Cryo-section of GUS-stained feeding site induced by *M. incognita* in a 4xB1+A-*uid*A tobacco line. GUS activity is predominantly around the central giant cells (gc). **D and E,** 4xB3+A-*uid*A construct in tobacco roots infected with *M. incognita*. **D,** Nematode feeding sites show pronounced down-regulation of *uid*A expression but GUS activity was sometimes still evident in the central giant cells. **E,** Cryo-sectioning confirmed that GUS activity was predominantly within the nematode-induced giant cells. **F,** 4xB3+A-*uid*A construct in tobacco roots infected with *G. tabacum* subsp. *tabacum*. Down-regulation of GUS activity in and around the syncytial feeding site of the mature swollen female. **G and H,** 4xB4+A-*uid*A construct in tobacco roots infected with **(G)** *M. incognita* and **(H)** *G. tabacum* subsp. *tabacum*. GUS activity can be seen in and immediately around the giant cells induced by *M. incognita* and the syncytium induced by *G. tabacum* subsp. *tabacum*. Only a small proportion of syncytia show this up-regulation. **I and J,** Tobacco line harboring the 4xB5+A-*uid*A construct infected with *M. incognita*. **I,** GUS staining associated with the central giant cells. **J,** Confirmed by cryo-sectioning. Scale bars on plates, approximately: **A, D, and I** = 1 mm; **B, F, G, and H** = 500 µm; **J** = 200 µm; **C and E** = 100 µm.

resistance in transgenic plants (e.g., Atkinson et al. 1995; Burrows and de Waele 1997). In contrast to Goddijn et al. (1993), other investigators have not found strong down-regulation of the 35S promoter. Early indications that this promoter probably has at least some activity in NFS is provided by the observation that certain proteinase inhibitors driven by the 35S promoter in transgenic roots conferred enhanced levels of resistance to *M. incognita* and *G. pallida* (Atkinson et al. 1995). Recently, more direct evidence has come from a study by Urwin et al. (1997), who used a *gfp* marker gene to show clear activity of the 35S promoter in the syncytial feeding sites of *Heterodera schachtii* in *A. thaliana*.

The results obtained here with a –343 to +8 35S construct demonstrated unequivocally that the 35S promoter is active in feeding sites, but considerably more so in the giant cells induced by *M. incognita* than in the syncytia of *G. tabacum* subsp. *tabacum*. Indeed, there was even an indication of up-regulation of the 35S promoter relative to local root background in association with *M. incognita* feeding sites, although this may be due to the increased metabolic activity and cytoplasmic density in these areas. Although some *G. tabacum* subsp. *tabacum* syncytia showed expression of *uid*A this was in only approximately 30% of the feeding sites examined. In the remaining 70% the 35S promoter was inactive (or at least below the threshold of detection) against the local background. Furthermore, in agreement with Urwin et al. (1997), there was a marked tendency for decreasing activity of the 35S promoter in syncytia as the feeding sites mature.

It is possible to hypothesize that the 35S promoter contains multiple *cis* elements that interact with and respond to nematode infection to give the pattern of activity seen in NFS. In the case of syncytia the balance of activity is shifted toward silencing of expression. Goddijn et al. (1993) suggested that down-regulation of the 35S was correlated to it containing an *as*-1 site in the A domain. The *as*-1 site, which binds the transcription factor ASF-1 in tobacco nuclear extracts (Lam et al. 1989), is considered necessary for efficient expression of genes in roots and, importantly, several promoters that are active in syncytia conspicuously lack an *as*-1 site (Sijmons et al. 1994b). The implication was that *as*-1 is involved in a negative regulatory capacity to repress the promoter in feeding sites. However, replacement or mutation of *as*-1 in the 35S promoter did not release it from down-regulation and it was concluded that repression in feeding sites could not be attributed to *as*-1 (Sijmons et al. 1994b). Our results with the B+(–72) construct, which lacks the *as*-1 site, shed new light on the possible involvement of *as*-1 and provide circumstantial evidence that the *as*-1 site could, after all, act as a weak negative regulating element within syncytial feeding sites. Compared with the entire 35S construct, B+(–72) leads to a decrease in the frequency of GUS activity in giant cells that is roughly proportional to the decrease in background activity in whole roots, but it has no effect on syncytial expression; there might even be a slight increase (Table 1). In other words, deleting 18 base pairs (–73 to –90) containing most of the *as*-1 site leads to a proportional increase (relative to root background) in the frequency of *uid*A expression in syncytia. Re-evaluation of the data provided by Sijmons et al. (1994b) adds further independent evidence in support of this hypothesis; replacement or mutation of *as*-1 from a 35S construct in *A. thaliana* increased the frequency of GUS activity in syncytia from 5 to 20%. Clearly, more work is needed to determine if this effect is repeatable and whether it is related specifically to the removal of *as*-1.

The binding of *trans* factors to *cis* elements to turn off genes is a well-documented method of gene regulation in prokaryotes but, although recognized in eukaryotes, it is less well studied (Imagawa 1996). However, there is an indication that another nematode-responsive promoter could be down-regulated by negative regulation. Goddijn et al. (1993) showed that although the *rol*C promoter was repressed in NFS a 3′ truncation of this promoter was found to be active, possibly

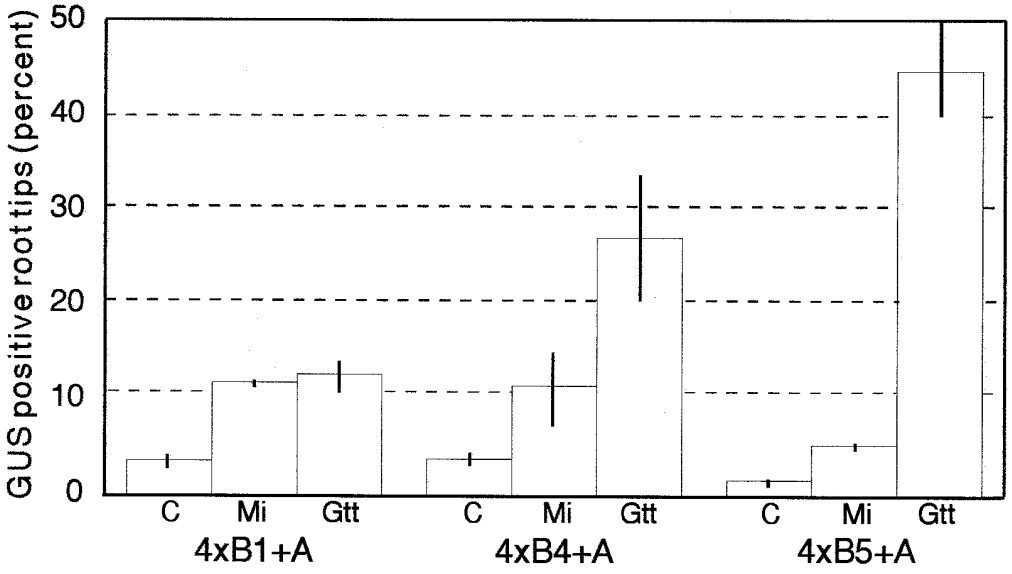

Fig. 3. Frequency of β-glucuronidase (GUS) activity in transgenic tobacco root tips of uninfected plants, compared with those infected by cyst and root-knot nematodes. Mean percentage (with standard error bars) of root tips examined that were GUS positive (*y* axis) is shown for each of three constructs (*x* axis). Uninfected (control) roots (C) are compared with those infected with root-knot nematode *Meloidogyne incognita* (Mi) and cyst nematode *Globodera tabacum* subsp. *tabacum* (Gtt), 25 and 35 days after inoculation, respectively.

indicating that a negative regulating *cis* element at the 3′ end of the *rol*C promoter was removed by the deletion.

Promoter silencing associated with NFS is, however, unlikely to be caused by a single mechanism and possibly also reflects a shift in the local "expression environment" in nematode-affected cells. For example, 4xB3+A drives strong expression of *uid*A in the cortex of roots and presumably the transcription factor(s) necessary for this are present in cortex cells. If the general expression environment in cortex cells adjacent to the feeding sites (and therefore under nematode influence) changes to become increasingly "uncortex-like" then availability of appropriate transcription factors may decrease and the promoter may fall silent.

In addition to down-regulation, a number of constructs used here showed up-regulation in or around NFS. Four constructs—4xB1+A, 4xB3+A, 4xB4+A, and 4xB5+A—all gave GUS activity associated with giant cells induced by *M. incognita*, with the latter two especially showing the strongest and most frequent activity. However, only the 4xB4+A and 4xB5+A constructs gave any GUS activity in syncytia and this tended to be weak and inconsistent. Some transcription factors that bind to the B subdomains have been previously identified. For example, the GATA site in B1 binds GATA 1, the B3 domain binds a factor called CAF, and MNF-1 binds to a region within the B4 domain (Benfey and Chua 1990; Lam and Chua 1990; Yanagisawa and Izui 1992). However, whether any of these are relevant to nematode-responsive activity is currently unknown.

Different domains of the 35S promoter are known to act synergistically in normal, uninfected plants (Benfey et al. 1990a, 1990b). Similarly, this study shows synergism in and around NFS. Neither the A (–90) domain alone nor any of the B subdomains fused to the minimal –46 (TATA) promoter were capable of driving nematode-responsive expression. However, when various B subdomains and the A domain were combined, synergism was evident, resulting in GUS activity in and/or around feeding sites. Furthermore, synergy also occurred between the B subdomains, none of which alone show activity in gall tissue; yet, when they were combined in the entire 35S promoter, GUS activity was readily observed in the galls.

One of the most striking findings of this study was the great differences in activity of the constructs between the galls/giant cells induced by *M. incognita* compared with the syncytial feeding sites of *G. tabacum* subsp. *tabacum*. In every case, where there was nematode-responsive expression, it was stronger and more consistent with the root-knot nematode. For some constructs, such as 4xB1+A, root-knot responsive expression could be observed whereas the cyst nematode gave none. Similar results have been observed with other promoters (Goddijn et al. 1993; Opperman et al. 1994; Barthels et al. 1997) and the molecular interactions between nematodes and host that are responsible for the bias toward *Meloidogyne* spp.-induced expression can only, as yet, be a matter of speculation. Perhaps the molecular routes used by these two nematode genera to induce their respective feeding sites are more divergent than has been appreciated previously.

The response of the 35S promoter to nematode infection has important implications for research efforts aimed at engineering nematode resistance in transgenic crops (Atkinson et al. 1995; Burrows and de Waele 1997). In many of the resistance strategies currently under investigation it is necessary to express inhibitory or nematicidal gene products inside the feeding cells. From our results, it seems that the –343 to +8 version of the 35S would be suitable for this task in the case of *M. incognita* giant cells, although constructs based on 4xB5+A should be considerably better. The 35S promoter had some activity in the syncytial feeding sites of *G. tabacum* subsp. *tabacum* but it was inconsistent, and most feeding sites seemed to escape detectable GUS activity. Selecting a suitable promoter to drive good levels of transgene expression in the syncytial feeding sites of cyst nematodes remains problematic. Nevertheless, the combinatorial model of eukaryotic promoters suggests that we may be able improve on syncytial expression by fusing combinations of 35S subdomains. Ni et al. (1995) assembled different domains of mannopine synthase (mas2) and octopine synthase (ocs) promoters to create a chimeric promoter capable of driving strong constitutive expression in plants. In a similar way, a combination B4 and B5 subdomains of the 35S promoter, perhaps with the –72 domain, could result in stronger/more frequent syncytial expression than the other constructs tested during this work.

During the course of this work two striking and surprising observations were made. First, the frequencies of root tip expression changed upon infection by nematodes. This may reflect a change in general root physiology related to local damage or pathogen detection. That the effect is more pronounced with cyst nematodes, which wound plant tissues more than do root-knot nematodes (Hansen et al. 1996), adds weight to this hypothesis. Second, promoter activities within NFS were found to be inconsistent. Why one feeding site should facilitate activity of a promoter, when another of apparently similar age did not, is unknown. There is currently insufficient data to judge how widespread this phenomenon is, but, from the few nematode-responsive promoters studied so far, at least one other (truncated *rol*C promoter) shows a similar effect (Goddijn et al. 1993).

In conclusion, this study has shown that a –343 to +8 version of the 35S promoter is active in the feeding sites of root-knot nematodes and to a lesser extent the syncytial cells induced by cyst nematodes. The subdomains of this promoter as described by Benfey et al. (1990a) show various degrees of nematode-responsive activity, from strong up-regulation to almost complete silencing. This work serves to clarify the confusing and contradictory literature surrounding the nematode-responsive nature of the 35S promoter. However, care should be take in extrapolating these results to other 35S constructs such as "double enhancer" versions or those containing other additions/truncations of the basic –343 to +8 sequence. The effects (if any) of such differences are hard to predict.

MATERIALS AND METHODS

Transgenic plants.

The plants used in this study were grown from seed derived from the transgenic tobacco plants described by Benfey et al. (1990a, 1990b). Fourteen different constructs were used (Fig. 1), most of which were based on tetramers of B subdomains: 4xB1, 4xB2, 4xB3, 4xB4, and 4xB5 in combination with either the minimal –46 (TATA) promoter or the entire A (–90) domain. In addition, lines of plants harboring the entire 35S promoter (–343 to +8), the 35S promoter with a deleted *as*-1 site and the minimal –46 (TATA) promoter were studied. At

least three plants from two or more different transgenic lines were examined for all constructs.

Growth and inoculation of plants.

Seeds were sown in open-ended transparent plastic growth chambers (1 × 3 × 10 cm) in a 1:1 mixture of sieved steam sterilized loam and peat. Plants were inoculated with either *G. tabacum* subsp. *tabacum*, which induces syncytial feeding sites, or *M., incognita*, which induces giant cell feeding sites. Infection of plants in soil with nematodes is not synchronous, and usually nematodes at different developmental stages are present on the same infected root system. To ensure that a good range of developmental stages (J3 to adult) was present, we carried out some preliminary tests to determine inoculation conditions and harvest times. The following procedures were found to be satisfactory, and were used for this study. Ten cysts (each containing approximately 200 eggs) of *G. tabacum* subsp. *tabacum* were added to the soil in each growth chamber, 1 to 2 cm below the tobacco seed, at the time of sowing. For *M. incognita*, approximately 500 eggs were injected to a depth of 2 cm into the soil around the roots of tobacco seedlings at the four-leaf stage. Control plants were not inoculated. Plants were harvested at 35 days post sowing for *G. tabacum* subsp. *tabacum* or 25 days post inoculation for *M. incognita*.

Analysis of nematode-infected roots.

Soil was washed gently from plant roots before staining for GUS activity essentially as described by Jefferson et al. (1987). The concentration of the chromogenic GUS substrate (5-bromo-4-chloro-3-indolyl β-D-glucuronic acid; X-gluc) used was 1 mM, and staining was carried out at 25°C overnight. To help in the visualization of nematodes and feeding sites, some root samples were also stained with acid fuchsin (Southey 1986). In the first instance, stained roots were observed with a binocular microscope and the background GUS activity noted in infected and uninfected roots. Following this, each infected root mass was systematically searched and NFS were counted and scored for apparent GUS activity.

In most cases, where blue feeding sites were observed, it was not possible with a binocular microscope to distinguish between GUS activity immediately around the outside of the feeding site and GUS activity inside. For this reason, where appropriate, cryo-sections of NFS were made, mounted in distilled water and observed at ×100 to ×400 magnification with a compound microscope. Photomicrographs were taken with Ektachrome ISO 400 film (Kodak, Rochester, NY).

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