A Role for AtWRKY23 in Feeding Site Establishment of Plant-Parasitic Nematodes[1][W]

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During the interaction between sedentary plant-parasitic nematodes and their host, complex morphological and physiological changes occur in the infected plant tissue, finally resulting in the establishment of a nematode feeding site. This cellular transformation is the result of altered plant gene expression most likely induced by proteins injected in the plant cell by the nematode. Here, we report on the identification of a *WRKY* transcription factor expressed during nematode infection. Using both promoter-reporter gene fusions and in situ reverse transcription-polymerase chain reaction, we could show that *AtWRKY23* is expressed during the early stages of feeding site establishment. Knocking down the expression of *WRKY23* resulted in lower infection of the cyst nematode *Heterodera schachtii*. *WRKY23* is an auxin-inducible gene and in uninfected plants WRKY23 acts downstream of the Aux/IAA protein SLR/IAA14. Although auxin is known to be involved in feeding site formation, our results suggest that, during early stages, auxin-independent signals might be at play to activate the initial expression of *WRKY23*.

Sedentary plant-parasitic nematodes (e.g. *Heterodera* and *Meloidogyne* ssp.) have developed a fascinating way to parasitize plants. After locating and migrating toward their host, they penetrate the root and find their way to the vascular tissue. There they manipulate plant root cells, resulting in the establishment of highly specialized nematode feeding sites (NFS). Depending on the nematode species, the initial feeding cell develops into either a syncytium (for cyst nematodes such as *Heterodera* spp.) or a system of giant cells (for the root-knot nematodes such as *Meloidogyne* spp.; Vanholme et al., 2004). The nematodes depend strictly on these feeding cells for food supply during their entire life cycle. Although it is not yet understood how nematodes establish syncytia or giant cells, glandular secretions produced by the nematode and injected into plant cells are suspected of interacting directly or indirectly with the plant nuclear genome, causing a whole cascade of altered gene expression that ultimately results in the complex NFS (Vanholme et al., 2004). To study how NFS become established, different molecular approaches were used to identify plant genes/promoters that are induced upon nematode infection. Previously, the promoter-tagging strategy was used, which led to the identification of six Arabidopsis (*Arabidopsis thaliana*) lines exhibiting GUS activity in nematode feeding structures (Barthels et al., 1997). A detailed molecular analysis of one of these lines (Att0001, ARM1) revealed that the tagged promoter belongs to a member of the WRKY transcription factor family (this study). In Arabidopsis, there are 72 expressed *WRKY* genes (Eulgem and Somssich, 2007) and the majority are transcriptionally inducible upon pathogen infection and other defense-related stimuli. One common feature of all WRKY proteins is the occurrence of a conserved WRKY domain of approximately 60 amino acids. The WRKY domain contains a highly conserved group of seven invariant amino acids WRKYGQK followed by a Cys-2His-2 or Cys-2His-Cys zinc-finger binding motif. Additional conserved domains and the type of the zinc-finger motif divide the family in different subgroups (Eulgem et al., 2000). During the past 10 years, a large body of evidence has accumulated implicating WRKY proteins in the transcriptional reprogramming during plant defense responses. For example, loss-of-*AtWRKY33* function rendered plants susceptible to infection by *Botrytis cinerea* and *Alternaria brassicicola* (Zheng et al., 2006), whereas overexpressing *AtWRKY18* resulted in plants with activated pathogenesis-related (PR) gene expression and enhanced resistance to the bacterial pathogen *Pseudomonas syringae* (Chen and Chen, 2002). However, the role of WRKY proteins seems to be far

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more complicated because *AtWRKY7*, *AtWRKY11*, and *AtWRKY17*, members of the IIb subgroup, can act as negative defense regulators and loss-of-function mutants showed increased resistance toward avirulent and virulent *P. syringae* strains. Moreover, members of subgroup IIa can play both positive and negative roles in plant defense and multiple studies have demonstrated interactions of WRKY proteins with either their own promoters or those of other family members. All these studies clearly show that WRKY proteins act in a complex transcriptional network, which is often referred to as the WRKY web (Eulgem, 2006). *WRKY* expression is, however, not exclusively associated with pathogen attack. It has been shown that *WRKY* genes were also up-regulated upon other stresses, such as wounding (Hara et al., 2000; Cheong et al., 2002), low temperatures (Huang and Duman, 2002), and during senescence (Hinderhofer and Zentgraf, 2001). Interestingly, WRKY proteins have been characterized with a defense-independent role in plant growth and development. *TTG2* encodes AtWRKY44 and functions in the development of trichomes and root hair cells (Johnson et al., 2002; Ishida et al., 2007), whereas *AtWRKY10*, identified through the *miniseed3* mutant, is reported as a regulator of seed size (Luo et al., 2005). In this study, we report on the involvement of WRKY23 during plant-parasitic nematode infection. The expression of *WRKY23* is up-regulated almost immediately upon nematode infection and *WRKY23* knock-down lines show a decreased sensitivity toward the cyst nematode *Heterodera schachtii*. Furthermore, we show that the expression of *WRKY23* is inducible by the plant hormone auxin, a known player in the plant-nematode interaction. However, the early *WRKY23* up-regulation upon infection seems to be independent from auxin signaling.

RESULTS

Up-Regulation of a WRKY Transcription Factor upon Infection with Plant-Parasitic Nematodes

A fundamental question in studying the plant-nematode interaction is how nematodes alter plant gene regulation in a cell-specific manner. To gain insight into this process, we previously used a promoter trap strategy in the model plant Arabidopsis (Barthels et al., 1997). One line, Att0001, showed very strong GUS activity in giant cells during the early stages of root-knot nematode (*Meloidogyne incognita*) infection (Barthels et al., 1997; Fig. 1, A and C). Similar results were obtained with the cyst nematode *H. schachtii* (Fig. 1B). Southern analysis of the Att0001 plants, using the *GUS*- or the *NPTII*-coding region as probe, showed that two T-DNAs were inserted as an inverted repeat over the right border (Supplemental Fig. S1A). Analysis of the T-DNA flanking sequences isolated from the tagged locus revealed that the tagged promoter belonged to *AtWRKY23* (At2g47260), a member of the WRKY transcription factor family. The WRKY23 protein consists of 337 amino acids from three exons encoding 146, 48, and 143 amino acids, respectively, and is a typical group-II WRKY protein, containing only one WRKY domain and having a Cys-2His-2 zinc-finger motif (Fig. 1F). Due to the presence of the conserved A-motif and the KAKKxxQK sequence, which probably functions as a nuclear localization signal, WRKY23 is categorized in the IIc subgroup (Eulgem et al., 2000). A WRKY23-GFP fusion protein construct driven by the 35S promoter in tobacco (*Nicotiana tabacum*) BY-2 cells localized to the nucleus, consistent with the assumed function of WRKY23 as a transcription factor (Fig. 1G). The full-length *WRKY23* promoter of 3.2 kb was isolated and *WRKY23*::*GUS* and *WRKY23*::*GFP* lines confirmed the strong activity of the promoter in NFS as initially observed in the tagged line (Fig. 1, D and E; Supplemental Fig. S1, B and C). This was further analyzed at the mRNA level using in situ reverse transcription (RT)-PCR. Transcripts of *WRKY23* strongly accumulated in young syncytia (Fig. 1H), whereas only weak staining could be observed in 10-d-old syncytia (Fig. 1I). No *WRKY23* product was detected with the control reactions without Taq polymerase (Fig. 1, K–M). These in situ RT-PCR results entirely mimicked the promoter activity studies.

Expression of *WRKY23* Is Inducible by Auxin in an IAA14-Dependent Way

WRKY proteins are well known for their involvement in biotic and abiotic stress responses and one plausible reason for the up-regulation of *WRKY23* in NFS could be an induction by a wound response mechanism. To investigate this, wounding experiments were performed on leaves and hypocotyls of *WRKY23*::*GUS* seedlings. When leaves were transversely dissected in an apical and a basal half, GUS staining could only be detected at the cutting edge of the apical part and not in the basal part (Fig. 2B). The same was observed when hypocotyls were cut (Fig. 2C). The plant hormone auxin is known to be transported throughout the plant in a basipetal way. As a result of cutting experiments, the auxin flow is disrupted, resulting in an accumulation of auxin at the basal side of the cut tissue. Auxin is reported as a major player during NFS establishment (Goverse et al., 2000); therefore, we questioned whether WRKY23 is an auxin-responsive transcription factor and thus whether the strong GUS activity at the basal end of the cut segment would be auxin related. To sort this out, we repeated the above-mentioned experiment using *WRKY23*::*GUS* seedlings treated with the auxin transport inhibitor 2,3,5-triiodobenzoic acid (TIBA) and observed an obvious reduction in GUS accumulation at the basal side of the injured segment (Fig. 2, D and E), indicating that the activation of the *WRKY23* promoter is due to auxin accumulation instead of being a response to wounding. The expression of

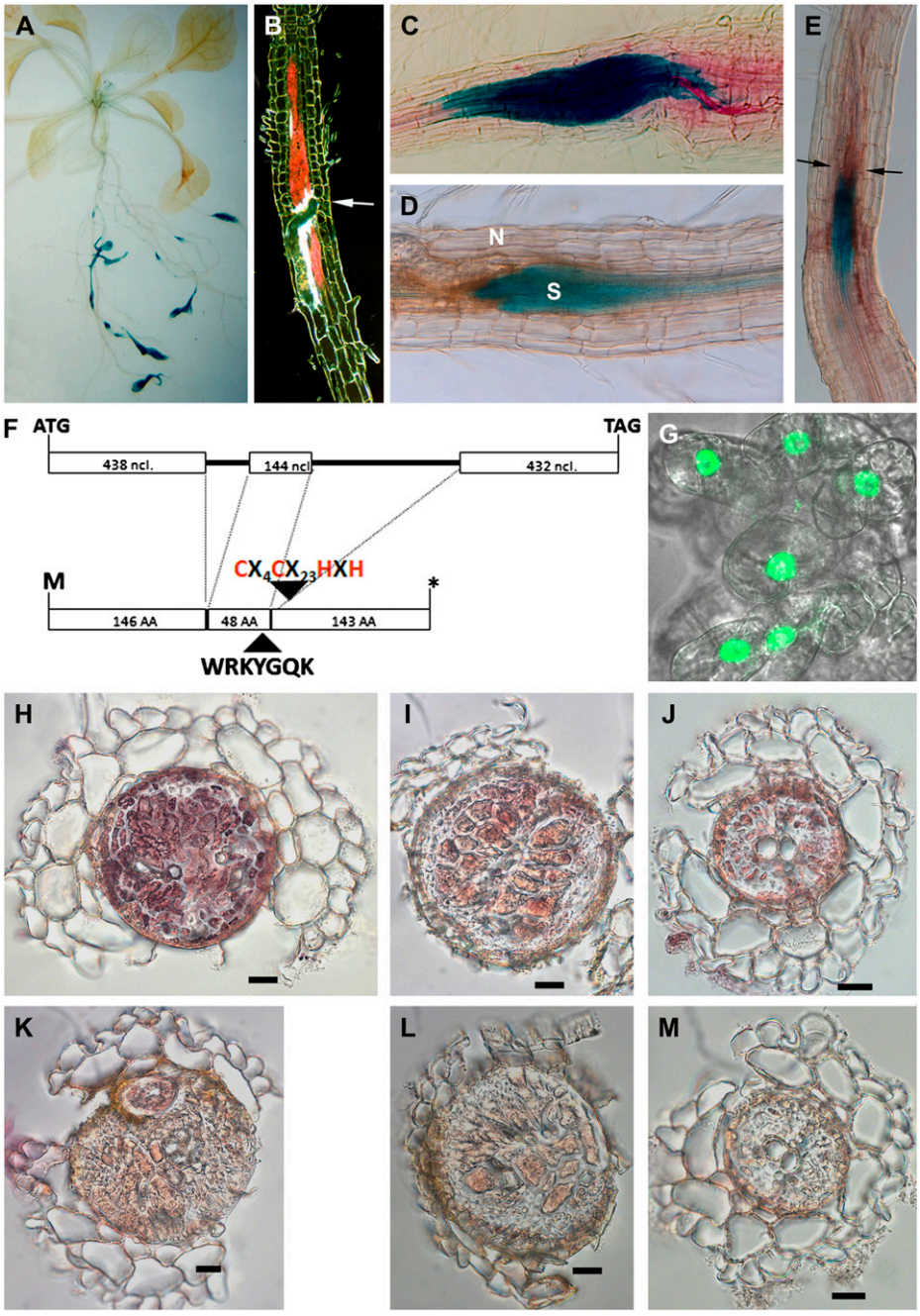

Figure 1. Expression of *AtWRKY23* during nematode infection. A to E, Infection of the promoter trap line Att0001 with *M. incognita* (A and C) and *H. schachtii* (B) and of *WRKY23*::*GUS* seedlings with *H. schachtii* at 2 dpi (E) and 5 dpi (D). All images show high *WRKY23* promoter activity in the NFS as can be seen by intense blue (A, C–E) or red (B, dark field) staining. Arrows indicate the head of the nematode. F, Schematic representation of the *WRKY* gene structure and specific protein elements. G, Nuclear localization of WRKY23-GFP fusion proteins in a stably transformed BY-2 culture. H to M, In situ RT-PCR analysis of *AtWRKY23* on sections of Arabidopsis-infected roots with *H. schachtii* at 5 dpi (H and K) and 10 dpi (I and L). K to M, Control reactions without Taq polymerase. J and M, Control reactions on root sections above the syncytium. N, Nematode; S, syncytium.

AtWRKY23 in response to auxin was further tested at the mRNA level in a time course experiment using semiquantitative RT-PCR. An increase of *WRKY23* transcript level was detected within 4 h of auxin treatment and from 8 h the *WRKY23* transcript reached a very high level (Fig. 2A). A detailed promoter analysis also revealed the presence of four elements that have been shown to be involved in auxin-regulated expression (Supplemental Fig. S1A). Next, the auxin-induced activation of the *WRKY23* promoter was compared to that of the auxin response marker DR5 (Ulmasov et al., 1997). Seeds were germinated on 1-*N*-naphthylphthalamic acid (NPA) and 72 h postgermination transferred to 10 μM naphthaleneacetic acid (NAA; according to Himanen et al., 2002). Germination on NPA ensures auxin depletion in the root and results in the absence of DR5 and auxin-responsive promoter activity at the onset of the auxin treatment. Two hours after transfer to auxin, DR5 activity was strongly induced, whereas the *WRKY23* promoter was only slightly activated at 6 h after transfer (Fig. 2, F–M). This suggests that WRKY23 acts downstream of the primary auxin response.

Auxin-responsive genes are controlled by the auxin response factors (ARFs) that activate or repress their expression and the Aux/IAA proteins that negatively

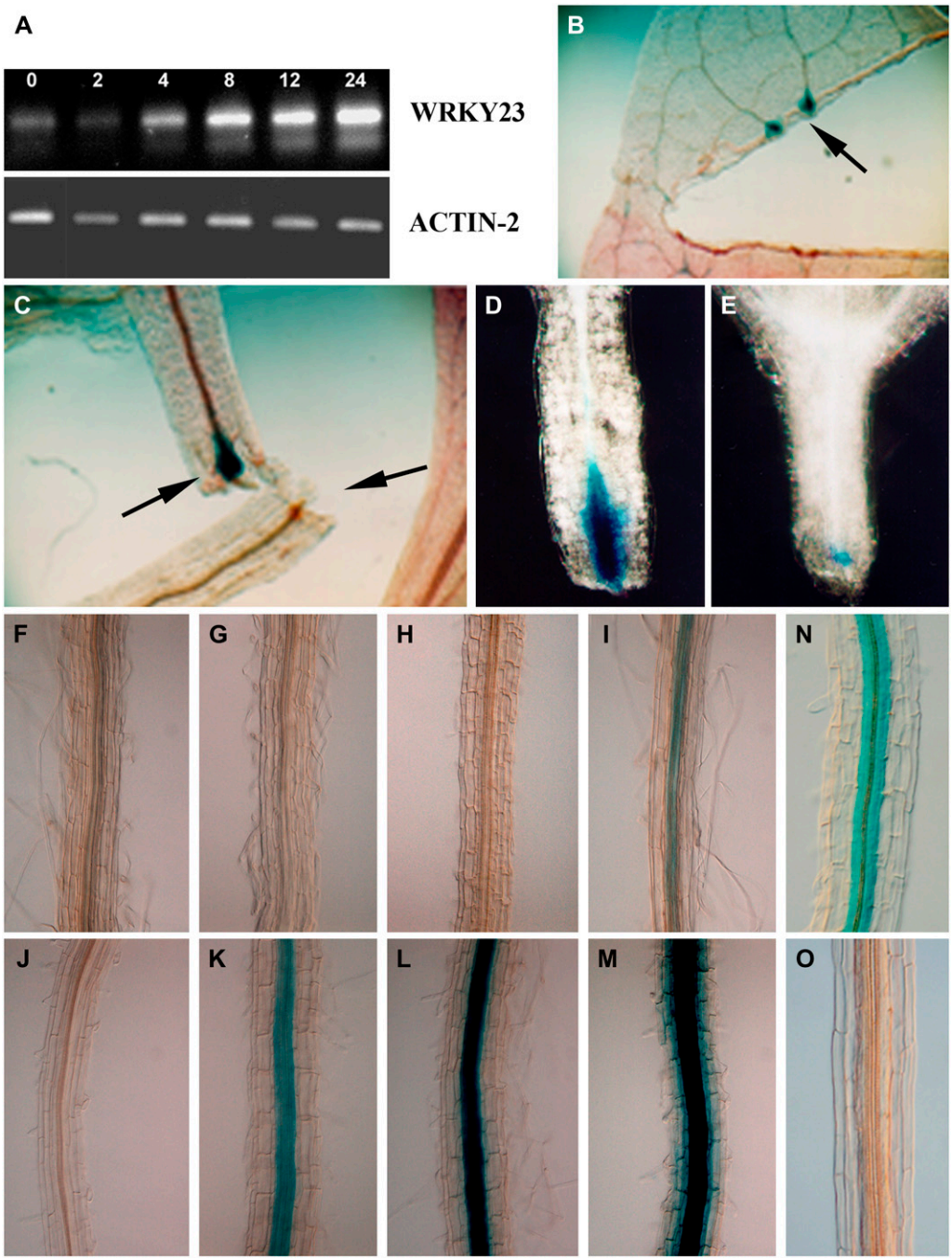

Figure 2. Auxin-inducible expression of *WRKY23*. A, Semiquantitative RT-PCR analysis of *WRKY23* using 10-d-old seedlings. Seedlings were treated for 0, 2, 4, 8, 12, and 24 h with 10 μM NAA. B to E, Wounding experiment on leaf (B) and hypocotyls (C–E) of *WRKY23*::*GUS* seedlings revealed intense *WRKY23* promoter activity at the basal side of the cut (B–D), which could be strongly reduced using 50 μM TIBA (E). No GUS staining could be detected at the apical side of the cut (B and C). F to M, Comparison of auxin-inducible activity of *DR5* (J–M) and *WRKY23* (F–I) promoters upon mock (F and J), 2-h (G and K), 4-h (H and L), and 6-h (I and M) auxin treatment. N and O, *WRKY23* expression in mature root tissue of *WRKY23*::*GUS* (N) and *WRKY23*::*GUS* × *slr* seedlings (O) upon 24-h auxin treatment.

regulate the ARFs at low auxin concentrations. To examine whether *WRKY23* expression depends on the Aux/IAA-ARF control mechanism, we checked the auxin inducibility of *WRKY23* in a *solitary root1* (*slr1*) background. *slr1* gain-of-function mutants produce a stabilized mutant IAA14 protein resulting in a complete lack of lateral roots (Fukaki et al., 2002). *WRKY23*::*GUS* was introduced in the *slr1* background and the F1 seedlings were treated with auxin. In contrast to *WRKY23*::*GUS* plants, no GUS staining

could be detected in the pericycle and vascular tissue of *WRKY23*::*GUS* × *slr1* roots (Fig. 2, N and O), strongly suggesting that *WRKY23* is responsive to auxin in an IAA14-dependent way.

Knocking Down *WRKY23* Expression Decreases Susceptibility to *H. schachtii*

To examine further the role WRKY23 might play during nematode parasitism, we screened all public available T-DNA insertion databases for *wrky23* knockout mutants. All five insertions found were situated in the promoter region and, unfortunately, the respective homozygous T-DNA insertion lines showed no or only a slight reduction in *WRKY23* transcript levels. In an attempt to obtain knock-down plants, *WRKY23* RNAi lines were generated based on a hairpin construct of a 108-bp *WRKY23*-specific fragment. Several lines with a clear reduction in *WRKY23* expression were obtained, but no obvious phenotypical differences compared to wild type were retrieved. Two lines with a 50% reduction in *WRKY23* transcript levels were infected with the cyst nematode *H. schachtii*. Interestingly, both lines reproducibly showed a 20% to 30% reduction in nematode infection (Fig. 3), indicating that an up-regulation of *WRKY23* might be required for successful induction of the NFS.

NFS-Specific *WRKY23* Up-Regulation Is Independent of Auxin

WRKY23 is highly expressed during early NFS establishment and it was demonstrated that auxin accumulates in nematode-infected cells that develop into NFS (Karczmarek et al., 2004). In uninfected plants, the expression of *WRKY23* is auxin inducible in an IAA14-dependent way. Therefore, we questioned whether *WRKY23* induction upon nematode infection might also be controlled by an Aux/IAA-ARF-dependent mechanism. *IAA14* promoter activity was analyzed during the early stages of feeding site induction. At both 2 and 5 d postinfection (dpi), strong GUS staining could be observed in the syncytium (Fig. 4, A and B), indicating that *IAA14* is expressed during feeding site establishment. To investigate the involvement of IAA14 in nematode infection in more detail, the *slr1* mutant was infected with *H. schachtii* and 6 weeks postinoculation the number of cysts was recorded both in wild-type and mutant plants. Consistent with previous observations that highlighted the importance of efficient auxin signaling for feeding site establishment (Goverse et al., 2000), the *slr1* mutant showed more than 60% reduction in infection (Fig. 4C). To further address the question of whether WRKY23 acts downstream of IAA14 in NFS, *WRKY23* expression was analyzed in the *slr/IAA14* mutant background upon nematode infection. Surprisingly, in all syncytia observed, the *WRKY23* promoter could still be activated by the nematodes in the presence of a stabilized Aux/IAA14 protein (Fig. 4, D and E). To further evaluate whether the induction of *WRKY23* upon infection could occur independently from Aux/IAA signaling, the exact timing of auxin response upon infection was compared to the temporal expression pattern of *WRKY23* in a time course experiment using *DR5*::*GUS* and *WRKY23*::*GUS* plants (Fig. 5). The earliest *DR5* promoter activity could be observed 21 h postinoculation (hpi; Fig. 5I), which is consistent with previous reports by Karczmarek et al. (2004). The GUS staining remained in the developing syncytium until 2 d upon inoculation. At later time points, the GUS staining became less specific to the syncytium and was more pronounced in the periphery of the NFS (Fig. 5O). Consistent with the above-mentioned results, the *WRKY23* promoter did not follow the *DR5* promoter activity and was already activated at 12 hpi (Fig. 5D). Moreover, at 5 dpi, *WRKY23* expression could still be observed in the NFS (Fig. 5P). These results show that, during nematode infection, *WRKY23* is activated prior to the enhanced auxin response reported by the *DR5*::*GUS* construct, whereas in uninfected plants the auxin-induced *WRKY23* expression is significantly later than the enhanced DR5 activity (Fig. 2, F–M). In the above-mentioned wounding experiments, application of a polar auxin transport inhibitor resulted in strong reduction of the *WRKY23* promoter activity at the cutting edges (Fig. 2, D and E). If the expression of *WRKY23* during nematode infection would be due to auxin accumulation in the initial feeding site, a similar reduction should be expected when infecting NPA-treated *WRKY23*::*GUS* plants. However, examining NFS at 18 hpi and 2 dpi revealed that NPA treatment did not influence *WRKY23* expression (Fig. 4, F–I). All

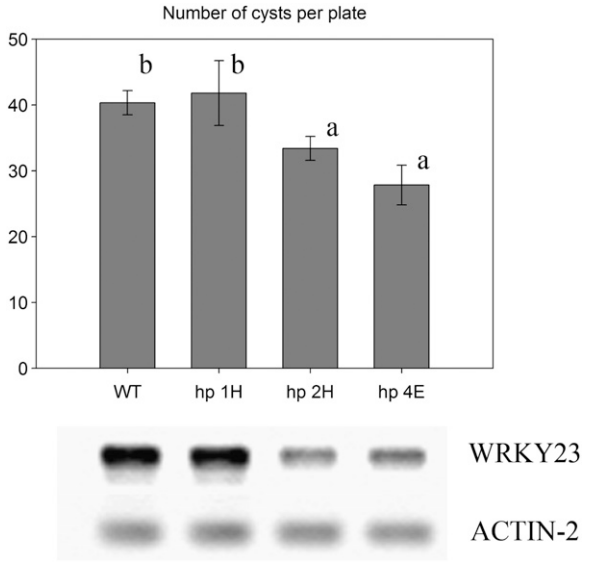

Figure 3. Infection of *WRKY23* knock-down lines. Top graph shows the number of cysts per plate counted 6 weeks postinoculation with *H. schachtii*. Bars with different letters denote a significant difference at $P < 0.05$. Bottom image shows the expression of *WRKY23* in the lines used for the infection experiment. *ACTIN-2* was used as internal control.

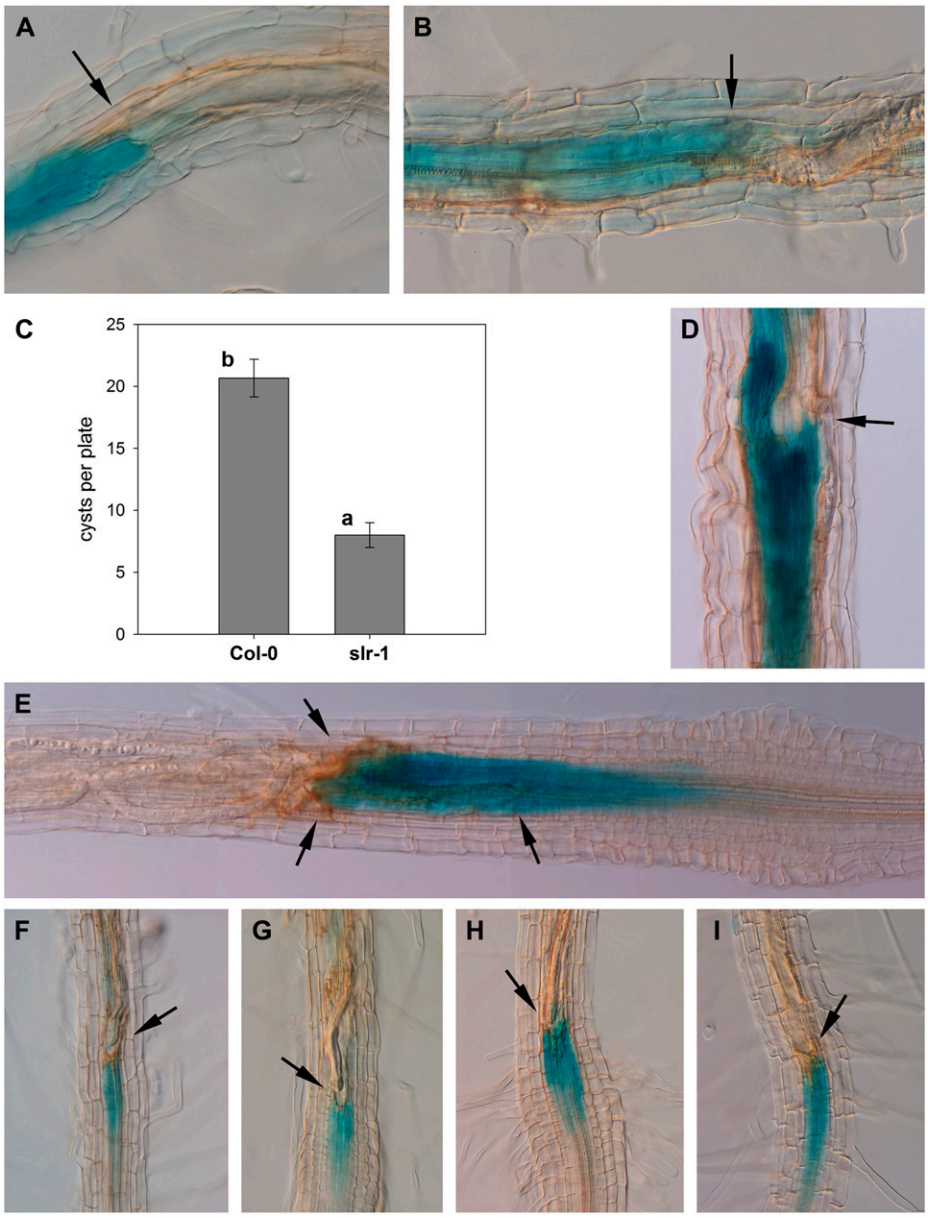

Figure 4. *WRKY23* promoter activation in syncytia is sustained in *slr* background. A and B, *IAA14* promoter activity at 2 dpi (A) and 4 dpi (B) using *H. schachtii*. C, Susceptibility against *H. schachtii* of wild type compared to *slr/IAA14*. Numbers of cysts per plate are plotted. Bars with different letters denote a significant difference at $P < 0.01$. D and E, GUS staining of *WRKY23::GUS* × *slr* seedlings upon *H. schachtii* infection at 2 dpi (E) and 5 dpi (D). F to I, *WRKY23* expression upon *H. schachtii* inoculation at 18 hpi (F and G) and 2 dpi (H and I) of NPA-treated (G and I) and untreated (F and H) *WRKY23::GUS* seedlings. Arrows indicate head of nematodes.

these data together strongly indicate that accumulation of auxin in the initial NFS cannot be solely responsible for *WRKY23* up-regulation and imply the existence of other nematode-dependent signals in regulating *WRKY23* expression.

DISCUSSION

A WRKY Protein of the IIc Subgroup Is Involved in Nematode Infection

Proteins belonging to the WRKY family of transcription factors are known to regulate a multiplicity of biotic and abiotic stress responses. They have been implicated in the regulation of genes involved in pathogen-induced stress, as well as drought and cold stresses (Dong et al., 2003; Pnueli et al., 2002; Rizhsky et al., 2002). Some WRKY proteins also play an important role during senescence (Miao et al., 2004) and plant development (Johnson et al., 2002). Their regulatory effect is primarily through their binding with conserved W-box elements present in the promoters of specific genes (Ulker and Somssich, 2004; Supplemental Fig. S1A). Here, we report the involvement of WRKY23 in nematode infection. Using promoter::GUS/GFP fusions and in situ RT-PCR, we could show that its expression is rapidly and highly induced upon infection with both root-knot and cyst nematodes. At later stages of the infection (10 dpi), the expression fades away, hinting at a role for WRKY23 during the early stages of nematode infection. The up-

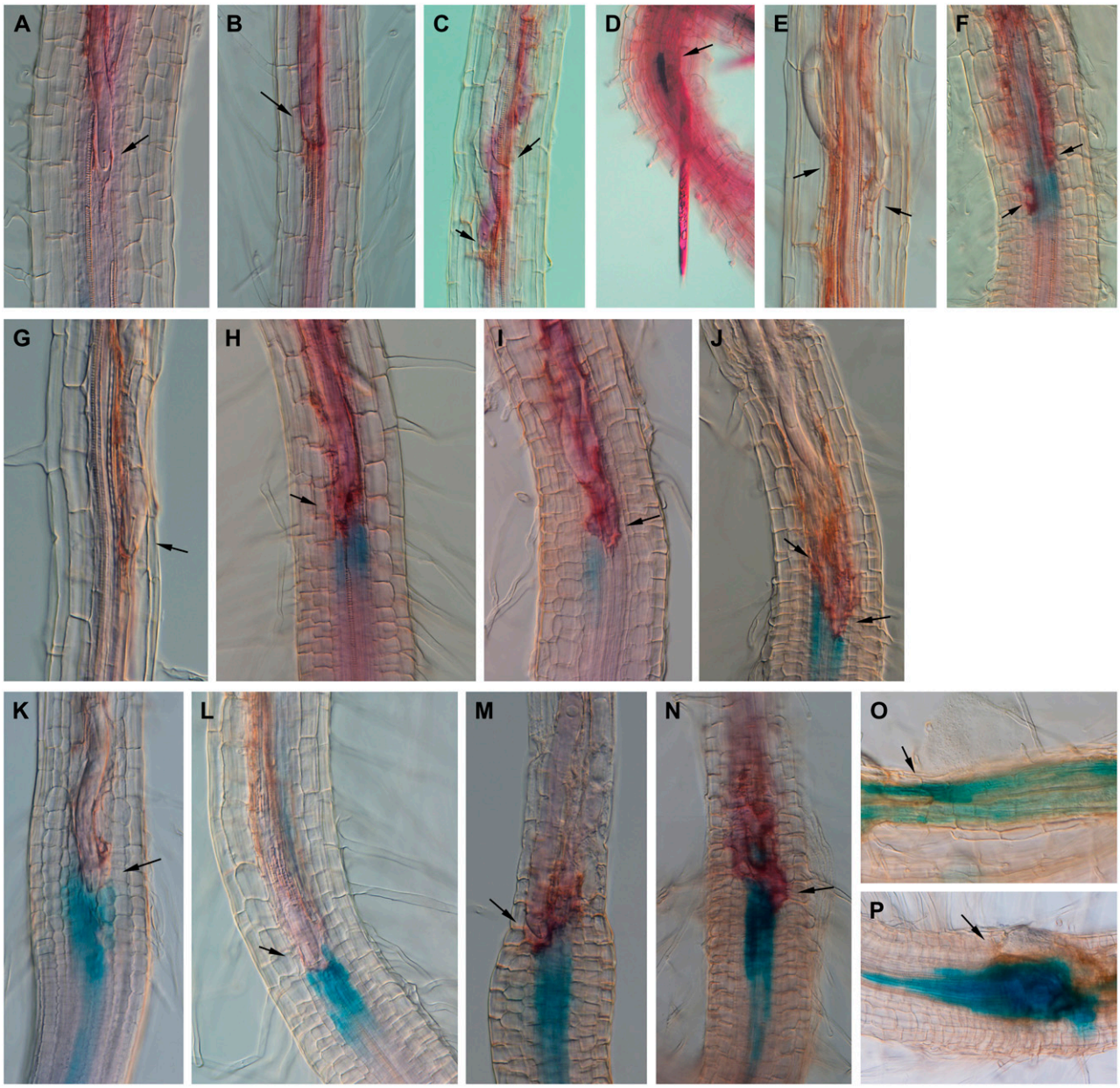

Figure 5. *WRKY23* promoter is activated in syncytia prior to the auxin response visualized by *DR5* promoter activity. Time course experiment comparing *WRKY23* (B, D, F, H, J, L, N, and P) and *DR5* (A, C, E, G, I, K, M, and O) promoter activity during cyst nematode infection at 9 hpi (A and B); 12 hpi (C and D); 15 hpi (E and F); 18 hpi (G and H); 21 hpi (I and J); 24 hpi (K and L); 48 hpi (M and N); and 5 dpi (O and P).

regulation of *WRKY23* could be confirmed by several transcript-profiling studies. Ithal et al. (2007) analyzed the expression of soybean (*Glycine max*) genes in feeding sites of *Heterodera glycines* using laser capture microdissection. The soybean probe sets Gma.8336 and Gma.16547 both showed high sequence similarity with *AtWRKY23* and were 7- to 17-fold up-regulated in syncytia (2 dpi), respectively (M. Mitchum, personal communication). *WRKY23* transcripts were also found to be up-regulated in microaspirated cytoplasm of syncytia at 3 dpi (K. Wieczorek and F. Grundler, unpublished data).

WRKY23 is a member of the subgroup IIc. Little is known about this rather large subgroup of 17 WRKY proteins. The only report so far is the functional characterization of AtWRKY75, which suggests that it acts as a modulator of phosphate starvation responses (Devaiah et al., 2007). To find regulatory elements in the promoters of group-IIc *WRKY* genes that might be shared, we performed a phylogenetic

footprinting. However, except for the W-box elements, which are characteristic for the promoters of *WRKY* genes, we did not find any elements specific for group-IIc WRKYs (data not shown).

Is WRKY23 a Negative Regulator of Basal Defense or Is Its Expression Hijacked by Nematodes?

Sedentary plant-parasitic nematodes are able to influence their host's gene expression to establish feeding sites. For example, the cyst nematode *H. schachtii* induces the expression of several expansin genes of its host during syncytium formation (Wieczorek et al., 2006). Expansins are cell wall-loosening proteins and enable the cyst nematode to install its feeding site by cell wall degradation and fusion of root cells. Besides this manipulation by the nematode, the infection process coincides with a plethora of plant defense responses. Genes encoding proteins related to stress and defense responses, such as PR proteins, are consistently up-regulated during all infection stages (Ithal et al., 2007). Also during the migration through the root and during expansion of the feeding site, nematodes cause wounding of the plant tissue. By performing wounding experiments on *WRKY23::GUS* seedlings, we were able to ensure that WRKY23 is not a part of the wound response accompanying the infection process. Also, cold treatments did not influence WRKY23::GUS activity (data not shown). Paradoxically to the role of WRKY proteins in plant defense, *WRKY23* knock-down lines showed reduced infection of the cyst nematode *H. schachtii*. This paradox might be explained by two different trains of thought. First, WRKY23 could act as a negative regulator of basal defense. Plant defense responses need to be under tight and fine-tuned regulation. Defense genes should only be activated during pathogen attack because constitutively activated plant defenses are energy consuming and hypersensitive responses, often accompanying plant defense, reduce the fitness of the plant. Thus, one hypothesis could be that WRKY23 tones down the expression of plant defense responses upon pathogen infection. Reducing the expression of *WRKY23* could therefore increase the resistance to cyst nematodes. Other *WRKY* genes have been reported with a similar function. *AtWRKY11* and *AtWRKY17* are both induced upon infection with *P. syringae* pv *tomato*, whereas *wrky11wrky17* double mutants showed increased resistance toward virulent and avirulent strains (Journot-Catalino et al., 2006). Also, WRKY7 acts as a negative regulator of plant defense toward *P. syringae* as could be demonstrated by the enhanced susceptibility of *WRKY7*-overexpressing plants and enhanced resistance of loss-of-function mutants (Kim et al., 2006).

The molecular mechanisms controlling plant defense responses are exceedingly complex. Necrotrophic pathogens, which benefit from host cell death, rather activate jasmonic acid (JA) signaling, whereas the salicylic acid (SA)-signaling pathway is generally effective against biotrophic pathogens that feed on living host tissue (Glazebrook, 2005). Considerable genetic evidence suggests that SA- and JA-signaling pathways are antagonistic (Gupta et al., 2000; Kloek et al., 2001). Consistent with their role in plant defense, at least 36 *AtWRKY* genes were differentially regulated upon SA treatment (Dong et al., 2003) and, interestingly, at least some WRKY proteins play crucial roles at the convergence of SA and JA. Overexpression of *WRKY70* results in constitutive SA signaling, whereas knocking down the expression results in activation of the JA pathway (Li et al., 2006). Plant pathogens can take advantage of this plant defense antagonism as could be demonstrated by the coronatine-producing bacterial plant pathogen *P. syringae*. Coronatine is a phytotoxin that activates JA signaling, thereby inhibiting SA-mediated defenses that normally limit growth of *P. syringae* within host tissue (Laurie-Berry et al., 2006). Because plant-parasitic nematodes depend on a nutrient-delivering host, it is tempting to speculate that their hosts' SA-signaling pathway would be activated. Recently, Wubben et al. (2008) could show enhanced susceptibility of SA-deficient mutants to *H. schachtii*, whereas SA-treated wild-type plants showed decreased susceptibility. It will be very interesting to investigate whether the JA defense pathway is also involved in nematode infection and whether WRKY23 or other members of the WRKY family would have a role in the interplay between both hormones. However, the findings that application of SA does not change *WRKY23* mRNA levels (Dong et al., 2003) or WRKY23::GUS activity (data not shown) argue against a defense-related role of WRKY23. Moreover, in a transcript-profiling search for JA-responsive genes, *WRKY23* transcript levels also did not change upon methyl jasmonate treatment (Pauwels et al., 2008; L. Pauwels and A. Goossens, personal communication).

A second hypothesis to explain the enhanced resistance of *WRKY23* knock-down lines to *H. schachtii* might be the hijacking of *WRKY23* by the nematode. It has been proposed that plant-parasitic nematodes use the developmental programs of their hosts to induce the NFS (Bird, 1996). In an attempt to do this, the nematode manipulates the plant gene expression for its own benefit. *WRKY23* is an auxin-inducible gene and in uninfected plants the auxin-inducible expression of *WRKY23* is controlled by the Aux/IAA protein SLR/IAA14. Our observations are consistent with a transcript-profiling study set up to identify auxin-inducible genes downstream of Aux/IAA14 (Vanneste et al., 2005; Supplemental Fig. S1D). Auxin is involved in almost every aspect of plant development and is the rooting hormone par excellence (for review, see Grunewald et al., 2007). The idea that auxin could be involved in the nematode infection process was launched as early as the 1960s (Balasubramanian and Rangaswami, 1962; Bird, 1962; Yu and Viglierchio, 1964; Cutler and Krusberg, 1968). Since then, an increasing amount of information points toward an important role for auxin in NFS establishment (Kochba and Samish, 1971; Glazer et al., 1986; Goverse et al.,

2000). More recently, in Arabidopsis, the up-regulation of the auxin-responsive DR5 reporter could be demonstrated shortly after nematode infection (Karczmarek et al., 2004; this study). Whether this local auxin accumulation is due to auxin directly secreted by the nematode (De Meutter et al., 2005) and/or is a result of directional auxin transport toward the initial feeding cell is still an unsolved question. Notwithstanding the importance of auxin, our results suggest that *WRKY23* can be activated by the nematode in an auxin-independent way. In the *slr1* mutant carrying a dominant-negative mutation that inhibits auxin-inducible expression of *WRKY23* in uninfected plants, the *WRKY23* promoter could still be activated in syncytia. *WRKY23* promoter activity could also be detected in NFS upon treatment with NPA, inhibiting polar auxin transport along the root and thus toward the initial feeding sites. This demonstrates that, during the infection process, *WRKY23* is not induced solely by the auxin accumulating in NFS. Moreover, a time course experiment revealed that the *WRKY23* promoter is activated prior to the auxin response visualized using the *DR5* promoter. One plausible explanation could be that the nematode is capable of bypassing the auxin-induced WRKY23 pathway to initiate its feeding site more rapidly. A detailed study on the endogenous role of WRKY23 in the future should clarify this problem. Using all publicly available mutant databases, we were not able to find knockout lines and the RNAi lines only showed <50% reduction. The lack of a viable full knockout might suggest that *WRKY23* is indeed an important developmental gene.

During *H. schachtii* infection, *WRKY23* is activated within the first 12 hpi. Given that, during this period, the nematode must penetrate its host's root, migrate toward the vascular tissue, and select an initial feeding cell, the activation of the *WRKY23* promoter can be considered as extremely fast. This observation propounds the idea that *WRKY23* could be directly activated by the nematode secretions. Recently, a CLE peptide with a function similar to CLAVATA3 of Arabidopsis has been characterized from the soybean cyst nematode *H. glycines* (Wang et al., 2005). The HgCLE peptide is able to complement the Arabidopsis mutant *clv3* and was shown to be involved in root development. However, when *WRKY23*::*GFP* seedlings were treated with HgCLE peptides, the GFP signal did not change (data not shown). It will be a challenge for the future to sort out whether nematode-secreted proteins can stimulate the *WRKY23* promoter by bypassing the rigid Aux/IAA-mediated control on auxin response to initiate NFS formation.

MATERIALS AND METHODS

Plant Lines and Growth Conditions

The tagged line Att0001 of Arabidopsis (*Arabidopsis thaliana* L. Heyhn.) ecotype C24 was transformed by the promoter trap vector pΔgusBin19 (Topping et al., 1991). *slr1* (Col-0 background) and *IAA14*::*GUS* (Col-0 background) lines were described by Fukaki et al. (2002), and *DR5*::*GUS* (Col-0 background) by Ulmasov et al. (1997). For in vitro growth studies, seeds were vernalized, surface sterilized (2 min in 70% ethanol and 10 min in 5% sodium hypochlorite), and plated on Murashige and Skoog medium (0.5× Murashige and Skoog; 1% Suc; 0.8% agar; pH 5.7). In general, plants were grown in vitro at 21°C under a 16-h-light/8-h-dark photoperiod. Wounding experiments were done on 10-d-old seedlings. For wounding experiments, hypocotyls and leaf blades of *WRKY23*::*GUS* (C24 background) plants were cut and plant segments were placed on solidified Murashige and Skoog medium. GUS assays were performed on samples 2 d after treatment. For auxin transport inhibition experiments, 10-d-old *WRKY23*::*GUS* seedlings were transversally cut at the hypocotyl and incubated upside down (leaf surface touching the agar) on solidified B5 medium with or without 50 μM TIBA. The GUS histochemical assay was performed after 2 d of incubation in the growth room. NPA treatments prior to *Heterodera schachtii* inoculations were done according to Goverse et al. (2000).

Nematode Culture and Infection Tests

Cultures of the root-knot nematode *Meloidogyne incognita* were maintained in vitro on roots of tomato (*Lycopersicon esculentum* 'Marmande') transformed with *Agrobacterium rhizogenes* and grown on Gamborg's B5 medium (Duchefa) at 28°C. Stage-2 juveniles (J2s) were obtained from egg masses hatched in sterile water 6 to 8 weeks after inoculation. Cyst nematodes (*H. schachtii*) were grown in vitro on roots of mustard (*Sinapis alba*) on Knop medium (Sijmons et al., 1991). J2s were obtained from cysts hatched in 3 mM $ZnCl_2$ 6 to 8 weeks after inoculation. For infection tests, 2-week-old seedlings were transferred to Knop medium on 12 × 12 cm^2 petri dishes (Greiner), resulting in five plants/plate and six replica plates/line. The petri dishes were placed slightly tilted to promote unidirectional root growth. One week later, two isolated root tips of each plant were inoculated with 5 μL of *H. schachtii* or *M. incognita* juveniles, with a total of about 200 J2s per plate. Therefore, the hatched nematodes were collected in 50-mL falcon tubes and centrifuged 3 min at 1,000 rpm. The supernatant was removed and the nematodes were resuspended in sterile 0.3% low-melting-point agarose. The solution was diluted until the desired nematode concentration (20 J2s/5 μL) was reached. For the first 2 weeks, the plates were sealed with urgopore and afterward parafilm was used. Six weeks after infection, the number of cysts (in the case of *H. schachtii*) or galls and egg masses (in the case of *M. incognita*) was counted on each plate and compared with the control lines. The data were statistically analyzed in SPSS (version 12.1) with the Levene test for homogeneity of variance and the independent sample *t* test to compare means.

Identification of WRKY23 and Isolation of *WRKY23* cDNA

To search for fragments containing T-DNA/plant DNA, we first carried out a Southern-blot analysis using the *GUS*-coding region as a probe. Therefore, 3 μg of purified DNA of the Att0001 line was digested with a range of restriction enzymes and separated in a 0.8% agarose gel. According to the Southern analysis, the *Ssp*I and *Eco*RI digests resulted in T-DNA/plant DNA fragments and were chosen for an inverse PCR (iPCR). The iPCR was performed using 2 μg of digested DNA and the fragments were ligated overnight at 14°C by 50 units of T4 ligase in a volume of 1 mL to generate monomeric circles. Ligated DNA was extracted with phenol-chloroform, then precipitated in ethanol, and resuspended in 100 μL of distilled water. PCR was performed using primer 1 (5′-CCAGCGTGGACCGCTTGCTGGACC-3′) and primer 2 (5′-GTATTGCCAACGAACCGGATACCCG-3′) for the *Ssp*I circle and primer 1 and primer 3 (5′-CCCAGTCACGACGTTGTAAAAC-3′) for the *Eco*RI circle. Both iPCR products were cloned into pGEMT (Promega) (ARM1-a and ARM1-b), sequenced, and used to screen a genomic library of Arabidopsis. Four positive plaques out of 10^5 were identified (Ch\ARM1-A, Ch\ARM1-B, Ch\ARM1-C, and Ch\ARM1-D) and, after purification, they were mapped based on restriction and hybridization analysis. The Ch\ARM1-D3500, a subclone from Ch\ARM1-D containing the insertion site of the T-DNAs, was sequenced and used in a BLAST program to screen the Arabidopsis database.

To obtain *WRKY23* cDNA, a cDNA library from *M. incognita*-infected Arabidopsis roots was screened using PCR. Two primers from the cloning region of the phage were used (forward 5′-GGTGGCGACGACTCCTG-GAGCCCG-3′, reverse 5′-TTGACACCAGACCAACTGGTAATG-3′) in combination with primer 4 (5′-ACAATGGAGTTTACAGATTTC-3′) and primer 5 (5′-GCGTGGCTATTAAGGTACT-3′). Amplified bands were cloned into

pGEMT (Promega) according to the manufacturer's instruction, and clones with either the 5′ end or the 3′ end of the cDNA (reverse/primer 4 and forward/primer 5, respectively) were identified. To obtain the full-length cDNA of *WRKY23*, a clone containing the 3′ end and a clone containing the 5′ end were digested with *Cla*I (cuts the insert only once and has no recognition site in the vector) and *Spe*I from the polycloning site of pGEMT. Digests were separated on a 0.8% gel and vector fragments containing either the 3′ or the 5′ end of the cDNA were isolated and purified using a gel extraction kit (Qiagen). Purified fragments were ligated to obtain the full-length *WRKY23*.

Generation of Constructs and Transgenic Lines

For isolation of the *WRKY23* promoter, DNA of the plaque genomic clone Ch\ARM1-D was digested by *Xba*I and the resulting 6.7-, 3.5-, 2.3-, and 0.7-kb fragments were cloned into pBluescriptII KS− (Ch\ARM1-D6663, Ch\ARM1-D3500, Ch\ARM1-D2299, and Ch\ARM1-D688). PCR was done on Ch/ARM1-D3500 DNA and the PCR product was cloned. The cloned fragment that contained 975 bp from the 3′ end of the *WRKY23* promoter was sequenced (pW975). The pW395 construct was made from pW975 by removing all DNA 5′ from the *Bsa*I site. The pW3195 was constructed by fusion of the pW395 fragment to the DNA 5′ of the *Bsa*I site of Ch\ARM1-D3500. The promoter fragment from the pW3195 construct was fused to the coding sequence of *GUS* in the pBI101 binary vector to generate the WRKY23::GUS construct.

To construct the WRKY-GFP fusion, the full length of *WRKY23* cDNA was amplified using forward and reverse primers from *WRKY23* extended with the attB1 and attB2 recombination sequences of the Gateway system. The PCR product was recombined with the pDONOR201 to generate pW23ENTRY. The pW23ENTRY clone was recombined to the pK2GWF7 to create pK2W23F7, a C-terminal *GFP* fusion construct. The CATMA program was used to make a gene-specific tag (GST) for *WRKY23*. A 108-bp-long fragment of the *WRKY23* open reading frame was amplified using primer W23GS5 (5′-TCCACCGTCAGAGCAATTAGT-3′) and primer W23GS3 (5′-AAGCAGACGAAATCGATGA-3′). The PCR product was recombined into pK2GWIWG7 vector to generate pK2WIW7 for RNAi-mediated gene silencing. Using the three-parental method, the vectors were transferred into *Agrobacterium tumefaciens* strain C58C1 (pMP90) and the pK2W23F7 into *A. tumefaciens* strain LBA4404. Arabidopsis plants were transformed by the floral-dip method (Clough and Bent, 1998) using ecotype Col-0 for the RNAi construct (pK2WIW7 vector) and ecotype C24 for the *WRKY::GUS* and *WRKY::GFP* constructs.

GUS Histochemical Assay and in Situ RT-PCR

For histochemical localization of GUS activity, plants were incubated in 90% acetone for 30 min at 4°C and then washed twice with NT buffer (100 mM Tris HCl/50 mM NaCl solution, pH 7.5). Subsequently, samples were incubated at 37°C in NT buffer supplemented with 2 mM ferricyanide. After 30 min, the GUS staining reaction was performed at 37°C in fresh NT buffer supplemented with ferricyanide and X-Glu (26.1 mg dissolved in dimethyl sulfoxide for 25 mL staining solution). After the GUS staining, the samples were washed in NT buffer, cleared using lactic acid, and analyzed using a digital interference contrast light microscope (Olympus) and photographed using a Nikon digital camera. The in situ RT-PCR procedure was performed according to Wieczorek et al. (2006). Syncytia at 5 and 10 dpi, as well as root fragments above syncytia, were dissected from roots and immediately put into cold fixation solution (63% ethanol [v/v]; 2% formalin [v/v]). After 48 h, syncytia were embedded in 4% low melting agarose and 25-μm-thick sections were prepared using a vibratome (VT 100; Leica). Further, a RT-PCR with specific primers and digoxigenin-labeled dUTP was carried out. After staining reaction with NBT/BCIP substrate, cross sections were photographed under an inverted microscope (Axiovert 200 M; Zeiss) with an integrated camera (AxioCam MRc5; Zeiss).

RNA Extraction, cDNA Synthesis, and RT-PCR

Ten-day-old seedlings were ground in liquid N_2 and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was prepared from 2 μg total RNA sample in a volume of 40 μL using a Superscript II reverse transcriptase first-strand cDNA synthesis kit (Invitrogen). Primers used to detect the endogenous *WRKY23* gene are primer WRKY_ssp5 and primer 3′-UTRB (5′-CTAGATTAAAACTATAAGACTATAT-3′). The *ACTIN-2* gene was used as a control, with primers Act2F (5′-GTTGCACCACCTGAAAGGAAG-3′) and primer Act2R (5′-CAATGGACTAAAACGCAAAA-3′). Various PCR cycles were tested to verify that the PCR conditions were not saturated. For auxin treatments, 10-d-old seedlings were transferred to Murashige and Skoog medium supplemented with 10 μM NAA (Sigma-Aldrich) for 2, 4, 8, 12, and 24 h. For *WRKY23* amplification, RT-PCR was performed using 24 cycles and for *ACTIN-2* using 22 cycles.

Localization in Tobacco BY-2 Cells

WRKY23 cDNA was cloned in the pK7WGF2 and pK7FWG2 vectors (Karimi et al., 2002) using the Gateway cloning facilities (Invitrogen). The obtained vectors were stably transformed in BY-2 cells using the *A. tumefaciens* strain LBA4404 harboring the construct. Four milliliters of a 3-d-old BY-2 suspension culture was mixed with 100 μL from an overnight grown *Agrobacterium* suspension and incubated for 3 d at 28°C. After incubation, the mixture was plated on BY-2 medium (4.302 g Murashige and Skoog, 0.2 g KH_2PO_4, 30 g Suc, 6.6 g agar, 0.02 mg, 2,4-dichlorophenoxyacetic acid [auxin], 0.05 mg thiamine, 5 mg/L myoinositol, pH 5.8) containing carbenicillin (500 mg/L) and kanamycin (100 mg/L). Growing calli were subsequently screened for fluorescence and the selected calli were imaged using a Zeiss LSM 510 confocal microscope.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_130234 (WRKY23) and NM_117535 (IAA14).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Schematic representation of the *WRKY23* promoter, which is activated by plant-parasitic nematodes as well as by auxin.

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