The *Arabidopsis* F-box/Kelch-Repeat Protein At2g44130 Is Upregulated in Giant Cells and Promotes Nematode Susceptibility

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We report that the F-box/Kelch-repeat protein At2g44130 is specifically induced by the root-knot nematode *Meloidogyne incognita* during the initial stages of the initiation and maintenance of the feeding site. In addition, we show that the expression of this gene promotes susceptibility of infection because knocking down the F-box gene (At2g44130) drastically reduces nematode attraction to and infection of roots. In contrast, F-box overexpressing (OE) lines had a hypersusceptible phenotype, with an increase of 34% in nematode attraction and 67% in nematode infection when grown in soil. This hypersusceptibility might be the result of an increased attraction of the second-stage juveniles toward root exudates of the F-box OE, which would suggest that the blend of compounds in the root exudates of the OE line was somewhat different from the ones present in the root exudates of the wild type and the F-box knockout and tilling lines. Although the function of the F-box/Kelch-repeat protein (At2g44130) is not known, we postulate that its activation by nematode effectors released during the infection process leads to the formation of SCF^(At2g44130) (Skp1-Cullin1-F-box protein) complexes, which are involved in facilitating successful infection by the nematode through targeting specific proteins for degradation.

Plant-parasitic nematodes have evolved sophisticated strategies to form permanent feeding sites essential for their survival. The economically most damaging plant nematodes—root-knot nematodes (RKN) and cyst nematodes (CN)—rely on a single feeding site for all the nutrients required for the development to the adult stage. Therefore, they need to keep these feeding structures alive for a period of up to 6 weeks, which requires the suppression of plant defenses by the nematodes (Smant and Jones 2011). The complex mechanisms used by plant nematodes to redifferentiate plant cells into morphologically distinct feeder cells have long fascinated researchers but still remain largely unresolved (Bellafiore and Briggs 2010; Curtis 2007). It is hypothesized that so-called effector molecules, contained in the secretion of the esophageal glands of the nematodes, are released into the host target cells through the stylet

and are responsible for the manipulation of key host cell mechanisms. Over recent years, a number of secreted nematode effectors have been identified with very diverse functions (Davis et al. 2009). Prominent among them are those involved in plant cell-wall softening and degradation; however, effectors involved in ubiquitination, suppression of plant defense, or interference with the plant signaling or hormone pathways have also been described (Haegman et al. 2012).

The ubiquitin-proteasome system (UPS) is involved in the regulation of many cell signaling and regulation processes and contributes significantly to the regulation of plant defenses through the selective degradation of proteins. Proteins are marked for degradation by the proteasome by polyubiquitination through a series of enzymes: E1 ubiquitin activating enzymes, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases. The E3 ubiquitin ligases are a multiprotein complex consisting of a cullin, Skp1, RBX1, and F-box protein, with the F-box conferring substrate specificity (Ciechanover et al. 2000). Several examples have been reported where bacterial and eukaryotic pathogens interfere and manipulate the host ubiquitination system in order to facilitate successful infection. For example, it was shown that the effector protein AvrPtoB released by *Pseudomonas syringae* pv. *tomato* DC3000 contains a C-terminal E3 ubiquitin ligase domain which was demonstrated to also have ubiquitin ligase activity (Janjusevic et al. 2007). It was subsequently shown that one target of this ligase was Fen, a kinase involved in the activation of programmed cell death which is responsible for Rsb immunity in tomato (Rosebrock et al. 2007). Through the ubiquitination of Fen by AvrPtoB and targeting it for degradation via the host 26S proteasome complex, *P. syringae* facilitates its colonization of the host by inhibiting the activation of the hypersensitive response. In *Arabidopsis*, AvrPtoB was shown to add ubiquitin to the membrane-resident receptor kinase FLS2, a receptor activated by the pathogen-associated molecular pattern-containing molecule flagellin, which is involved in triggering innate (basal) immunity, thereby eliciting its degradation via the host 26S proteasome complex. By eliminating this sensor from the cell periphery, *P. syringae* is capable of enhancing its own proliferation (Goehre et al. 2008). A different strategy is employed by *Agrobacterium tumefaciens*: in order to facilitate successful integration of the T-DNA into the host genome, the proteins that coat the T-DNA and which are necessary for the transport of the T-DNA complex into the host cell nucleus (VirD2, VirE2, and the plant-derived VIP1) need to be degraded. For this purpose, *A. tumefaciens* translocates the F-box protein VirF into host cells. There, it localizes to the plant cell nucleus where it forms a functional Skp1-Cullin1-F-box pro-

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tein (SCF^{VirF}) complex which is required for the degradation of the VirE2 and VIP1 proteins (Tzfira et al. 2004).

Like other plant pathogens, nematodes are thought to interfere with these processes by secreting effectors which perturb the host ubiquitination pathways; however, thus far, only circumstantial evidence exists to support this hypothesis. For example, in a cDNA library prepared from the esophageal gland cells of the soybean CN *Heterodera glycines*, genes encoding potentially secreted components of the ubiquitin proteasome pathway, a S-phase kinase-associated protein 1 (SKP1) and a RING-H2 zinc finger protein, could be identified (Abad et al. 2008; Gao et al. 2003). In addition, genes encoding a novel type of ubiquitin extension proteins secreted by the dorsal esophageal gland from *H. schachtii* were identified by Tytgat and associates (2004). A more recent investigation of the secretome of *Meloidogyne incognita* using a proteomics approach identified 486 secreted proteins, among them several which could be involved in the perturbation of the ubiquitination system of the plant cell, such as an SKP1 homolog featuring a nuclear localization signal (Bellafronte et al. 2008, Tytgat et al. 2004). The most persuasive experiments thus far were performed by Sindhu and associates (2009), where the RNAi-mediated silencing of three esophageal secreted proteins with possible functions in the ubiquitination pathway (i.e., proteins with homology to plant SKP proteins and plant ubiquitins and similarity to plant ring H2 zinc finger) led to a significant decrease in the number of developing females of *H. schachtii* in *Arabidopsis*, with the greatest effect (64%) reported for the SKP homolog.

In the examples given above, nematode-encoded proteins were described which, due to their homologies to plant proteins with known function in the ubiquitination pathway, are thought to disrupt and manipulate the host ubiquitination process to their own advantage. However, plant genes involved in the ubiquitination pathway and responsive to nematode infection have not been described thus far. Here, we report that the F-box/Kelch-repeat protein At2g44130 is specifically induced by the RKN *M. incognita* and we provide evidence that suggests that this F-box protein plays a prominent role during the infection of *Arabidopsis thaliana* by the RKN *M. incognita*.

RESULTS

Expression profile of *A. thaliana* F-box/Kelch-repeat protein (Atg244130) during nematode infection.

We recently described an *Arabidopsis* reporter line where the promoter of a F-box/Kelch-repeat protein (Atg244130) has been fused to β -glucuronidase (GUS) (Matthes et al. 2010). This line shows constitutive expression of the GUS reporter only in the abscission zones of the flower organs but the GUS expression can be induced by *cis*-jasmonate, where its expression is restricted to the midrib of the leaves. This line was subjected to several biotic stresses such as feeding by the larvae of *Spodoptora littoralis* and *Plutella xylostella*, infestation with *Myzus persicae*, and infection with *Pseudomonas syringae* but none of the tested pathogens was capable of inducing the re-

Fig. 1. β -Glucuronidase (GUS) expression in roots of *Arabidopsis thaliana* during infection with root-knot nematodes (RKN) and cyst nematodes. GUS::F-box/Kelch-repeat (Atg244130) GUS activity during a time course of infection with *Meloidogyne incognita*. Images show high F-box/Kelch-repeat promoter activity in the giant cells (intense blue), and nematodes are stained pink after acid-fuchsin staining. **A**, At 3 days postinfection (DPI), GUS staining was observed in the vascular cylinder during migration of second-stage juveniles (J2) of *M. incognita*; **B** and **C**, GUS activity in the giant cells induced by RKN at 7 DPI with parasitic J2 arrowed; **D**, GUS activity in the galls induced by RKN after 14 DPI; **E** and **F**, GUS activity in the giant cells and gall tissue induced by RKN after 14 DPI with fourth-stage juveniles arrowed; **G**, cross-section of a gall formed after 14 DPI with RKN; **H** and **I**, GUS activity in giant cells and gall tissue after 21 DPI with *M. incognita*. GC = giant cells; arrows indicate the nematode head. Scale bar = 10 μ m.

porter gene (data not shown). However, when we tested the roots of *Arabidopsis* plants which had been infected with the RKN *Meloidogyne incognita*, strong GUS expression was detected in the galls formed by the nematode. Therefore, we performed a time course experiment in order to investigate the temporal and spatial expression of the GUS reporter induced by *M. incognita* infection. In two independent experiments, the compost surrounding the plants was inoculated with *M. incognita* second-stage juveniles (J2) and the roots assayed for GUS expression at 3, 7, 14, and 21 days postinfection (DPI). No GUS staining was observed at 3 DPI, suggesting that this gene is not induced in the root during invasion or migration of the nematodes (not shown). However, at 7 DPI, when the migratory phase of the nematode is over, GUS staining is detectable where the nematode is about to establish or has already established its feeding site (Fig. 1A). The intensity of the GUS staining is increased after 14 DPI (Fig. 1B), with up to 90% of galls showing strong GUS activity; in contrast, at 21 DPI, GUS activity becomes very patchy (Fig. 1C) and the proportion of stained galls decreased considerably (data not shown). In order to correlate the expression of GUS with the localization of the nematodes, the samples were subjected to staining with acid fuchsin, which gives the nematode a bright red color and makes its visualization possible. GUS expression at 7 DPI is restricted to tissue surrounding the “head” of the nematode (Fig. 1D and E), which would suggest that the induction of the F-box occurs at the establishment of the feeding site and during giant-cell formation. At 14 DPI, when the nematode had undergone two molts and developed into a third-stage juvenile or a fourth-stage female with spike tail, the GUS staining was intense in the giant cells. There was an increased galling of the cortical tissue but the increase of the GUS activity seemed to be restricted to the giant cells (Fig. 1F and G, a cross section). At 21 DPI, nematodes had developed further into young or adult females and there was a reduction of GUS staining surrounding the giant cells suggesting that, at this stage, the expression of the F-box gene was no longer required in the giant cells (Fig. 1H and I).

The same transgenic GUS reporter lines were used to assess whether the F-Box gene could also be induced during feeding site establishment by CN such as *H. schachtii* and *H. cajani*, for which *Arabidopsis* is a suitable host, or the potato CN *Globodera pallida* which, although able to infect and initiate feeding sites, is unable to complete its life-cycle in *Arabidopsis*. No GUS staining was observed in the syncytia formed by these nematodes. However, occasional GUS activity was observed in the vascular cylinder of roots infected with *G. pallida* (Supplementary Fig. S1). Therefore, it seems that the *A. thaliana* F-box/Kelch-repeat protein (At2g44130) is specifically upregulated by RKN during establishment of their feeding sites but not by CN.

Knocking down the F-box/Kelch-repeat protein (At2g44130) expression decreases susceptibility to *M. incognita*.

In order to address the question of whether the activation of the F-box/Kelch-repeat protein could be correlated with a functional role during the infection of *Arabidopsis* by *M. incognita*, two *Arabidopsis* lines harboring nonfunctional alleles of the gene (T-DNA knockout [KO] and tilling) (Supplementary Fig. S2A to C) were obtained and overexpressing (OE) lines were generated. The effect that these gene perturbations might have on the ability of *M. incognita* to form its feeding site and develop inside these *Arabidopsis* lines was investigated. Assays monitoring the formation of nematode-induced giant cells were performed on plants grown in either sandy compost or axenic cultures. After 21 DPI, roots were stained with acid fuchsin and the number of nematodes inside the roots of each infected *Arabidopsis* plant was counted. In plants grown axenically, nematode parasitism of the F-box KO, tilling, and OE lines is significantly different from wild-type (WT) plants ($P < 0.05$) (Fig. 2A). Equally, when plants were grown in soil, the F-box OE was significantly different from the F-box KO and tilling lines ($P < 0.05$) and the F-box KO line was marginally significantly different ($P = 0.08$) from WT plants (Fig. 2B). For both plant cultivation systems, all nema-

Fig. 2. A, Nematode development after 21 days postinfection (DPI) in the *Arabidopsis* lines maintained in axenic cultures. The number of parasitic nematodes inside the roots was highly significantly different ($P < 0.001$, F test) between the lines. Means on square root scale for statistical comparison (replication): F-box knockout (KO) 0.88 ($n = 7$), overexpressing (OE) 13.40 ($n = 7$), tilling 0.61 ($n = 9$), and wild type (WT) 8.16 ($n = 3$); maximum standard error of the difference (SED) = 2.177 on 22 degrees of freedom (df), least significant difference (LSD) (5%) = 4.516. The F-box (Atg244130) KO, OE, and tilling lines were significantly ($P < 0.05$, LSD) different from WT plants. Compared with the WT plants, there was a reduction of 92 and 95% in the number of nematodes that developed inside the F-box KO and tilling lines, respectively, and an increase of 67% was observed in the number of females inside the roots of the F-box OE line. **B,** Nematode development after 21 DPI in the *Arabidopsis* lines maintained in soil. The number of parasitic nematodes inside the roots was significantly different ($P = 0.043$, F test) between the lines. Means on square root scale for statistical comparison (replication): F-box KO 3.43 ($n = 2$), OE 10.52 ($n = 6$), tilling 6.26 ($n = 4$), and WT 8.35 ($n = 3$); maximum SED = 2.603 on 11 df, LSD (5%) = 5.729. The F-box (Atg244130) OE was significantly different from the F-box KO ($P < 0.05$, LSD) and tilling ($P < 0.05$, LSD) lines. The F-box KO was marginally different ($P = 0.08$, post-analysis of variance t test) from the WT. Compared with the WT plants, there was a reduction of 66 and 40% in the number of females in the F-box KO and tilling lines, respectively, and an increase of 72% in the number of females inside the F-box OE line.

todes inside the roots developed into young or adult females in the lines tested, and consistently more females were counted in the roots of the F-box OE line than in the roots of the WT; however, due to some variability between the OE plants, these data were not statistically significant ($P = 0.303$). In comparison with WT plants, there were 67% (soil) and 72% (axenic cultures) more females inside the F-box OE line, indicating that this *Arabidopsis* line is hypersusceptible to the nematodes. By contrast, when grown axenically, a reduction in parasitism of 92 and 95% was observed in the F-box KO and tilling lines, respectively, compared with the WT. In compost-grown plants, 66 and 40% fewer females were observed in the F-box KO and tilling lines, respectively, than in the WT, indicating that only a small number of RKN could successfully develop in the *Arabidopsis* lines with reduced or no expression of the F-box (At2g44130). There was no obvious difference in the size or morphology of the galls formed in the KO, tilling, or OE lines relative to the WT Col-0 and no obvious differences in their phenotypes or the root weight ($P = 0.383$, F test) or root length ($P = 0.891$, F test) of the *Arabidopsis* WT and the KO, tilling, and OE lines (data not shown).

Clearly, knocking down the F-box gene (At2g44130) expression drastically reduced nematode infection but did not affect nematode development because they matured into females. In order to investigate whether this silencing could interfere with nematode attraction or penetration into the roots, the infection cycle was observed at earlier time points. An attraction bioassay was carried out in pluronic gel in order to monitor nematode movement and attraction to *Arabidopsis* roots. Attraction does not indicate penetration and, even if root penetration occurs, it does not guarantee successful nematode development. In a time course sampling at 3, 6, and 18 h, we compared the attraction of *M. incognita* J2 to the root tips of *Arabidopsis* WT Col-0, the F-box KO and tilling lines, and the F-box overexpressor (Fig 3A). There was a highly significant ($P < 0.001$, F test) interaction between the *Arabidopsis* lines tested and time. No significant difference in nematode attraction to any of the lines was observed after 3 h. However, after 6 h, attraction

of the J2 to the F-box KO and OE lines was significantly different ($P < 0.05$) from WT plants and F-box KO line was significantly different ($P < 0.05$) from the tilling line. At 18 h, the F-box OE, KO, and tilling lines were significantly different ($P < 0.05$) from WT plants. Overall, after 18 h, 34% more *M. incognita* J2 were attracted to the roots of the F-box OE line than to the roots of the WT, whereas the roots of the F-box KO and the F-box tilling lines were considerably less attractive to the J2 than the WT (a decrease of 65 and 35%, respectively). This finding might suggest that the root exudate of the F-box OE could have greater concentration of compounds mediating attraction of RKN. There was a strong increasing trend in attraction for the WT plants and a burst in attraction between 6 and 18 h for the F-box OE line. Nematode attraction increased from 3 to 6 h for the F-box tilling line and remained stable to 18 h. Attraction was least in F-box KO line and there was no significant difference ($P < 0.05$) between 3, 6, and 18 h despite a slight increase. After 24 h, the *Arabidopsis* plants were removed from the pluronic gel and the number of J2 that had penetrated the roots was counted under a stereomicroscope. Significant differences ($P < 0.001$, F test) between lines were found for these counts. The nematode invasion of the F-box KO, tilling, and OE lines was significantly ($P < 0.05$) different from WT plants and that of F-box KO line differed significantly ($P < 0.05$) from F-box OE line (Fig. 3B). In comparison with WT plants, there was a reduction of 93 and 62% in the number of nematodes that invaded the F-box KO and tilling lines, respectively, after 24 h in pluronic gel. The J2 were attracted in greater numbers to the WT and F-box OE but penetrated the WT roots faster and, after 24 h, most J2 had already invaded the WT roots.

DISCUSSION

The results reported here show that the F-box/Kelch-repeat protein (At2g44130) is specifically induced in *A. thaliana* roots by the RKN *M. incognita* during the initial stages of the initiation and maintenance of the feeding site. At 21 DPI,

Fig. 3. A, Time course of attraction of *Meloidogyne incognita* second-stage juveniles (J2) toward roots of *Arabidopsis thaliana* in pluronic gel. The graph shows the number of *M. incognita* J2 touching *Arabidopsis* roots after 3, 6, and 18 h of incubation in pluronic gel. There was a highly significant ($P < 0.001$, F test) interaction between *Arabidopsis* F-box (Atg244130) knockout (KO), overexpressing (OE), and tilling lines and time. Replication: F-box KO, $n = 10$; OE, $n = 3$; tilling, $n = 6$; and wild type (WT), $n = 3$. Maximum standard error of the difference (SED) = 2.876 on 54 degrees of freedom (df), least significant difference (LSD) (5%) = 5.767 (between lines), 2.761 on 36df, LSD (5%) = 5.599 (within lines). Compared with WT plants, 34% more J2 of *M. incognita* were touching the roots of the F-box OE line and 53 and 65% less nematodes were attracted to the F-box KO and tilling lines, respectively, after 18 h in pluronic gel. **B**, Invasion of *A. thaliana* by *M. incognita* J2 after 24 h in pluronic gel. Significant differences ($P < 0.001$, F test) were found between lines. Replication: F-box KO, OE, and tilling, $n = 6$ and WT, $n = 3$; maximum SED = 1.359 on 17 df, LSD (5%) = 2.868. Nematode invasion of the F-box (Atg244130) KO, OE, and tilling lines was significantly ($P < 0.05$, LSD) different from WT plants, and the F-box KO line differed significantly ($P < 0.05$, LSD) from the F-box OE line. There was a reduction of 93 and 62% of the number of nematodes that invaded the roots of the F-box KO and tilling lines, respectively, after 24 h.

when the female has fully developed, the expression of this gene does not seem to be maintained because the GUS staining in the F-box::GUS reporter line became patchy and less intense. No such induction was detected in the syncytia formed by CN. Transcriptomic approaches have been performed thus far in order to understand the processes leading to feeding site establishment for both RKN and CN. The induction of a number of F-box genes has been shown to be mostly repressed in the giant cells at 3 DPI; however, the F-box (At2g22030) was shown to be induced in 3-day-old giant cells. As in our experiments after 21 DPI, most F-box genes were shown to be down-regulated. (Barcala et al. 2010; Fuller et al. 2007; Jammes et al. 2005). These results corroborate our findings and suggest that F-box genes repressed at earlier infection points might play a role in defense; however, the *Arabidopsis* At2g44130 has not been reported thus far. In addition, we demonstrated that the expression of this gene was paramount for successful infestation of *Arabidopsis* by RKN. When compared with the WT, tilling lines and T-DNA KO lines for this gene showed a significant decrease in fully developed females after 21 DPI which, in the most extreme case, amounted to 95% in axenically grown plants. OE lines, on the other hand, were able to support increased numbers (72%) of nematodes when compared with WT *Arabidopsis*. Because the nematodes that did infect were capable of developing successfully into females in the KO and tilling lines, we questioned whether the observed reduction in numbers was due to a decrease in attractiveness of these lines to the infective nematode J2 and whether the J2 could effectively penetrate the roots. Compared with WT *Arabidopsis*, attraction of J2 to roots of the T-DNA KO and tilling lines was significantly reduced. By contrast, a greater number of *M. incognita* J2 were attracted to the F-box OE than to the WT. This hypersusceptibility might be the result of an increased attraction of the J2 toward root exudates of the F-box OE plants, which would suggest that the blend of compounds in the root exudates of the OE line was somewhat different from the ones present in the root exudates of WT and the F-box KO and tilling lines. For example, an increased attraction of J2 of the sugar beet CN was observed to the roots of the *Arabidopsis* mutant *rhd1-4*, which, among other phenotypes, is characterized by the overproduction of ethylene. Twice as many J2 were found to be attracted to this mutant as to WT *Arabidopsis* (Wubben et al. 2001). In order for nematodes to find roots, they need to respond to stimulus gradients around roots that are unique in character, concentration, or combination. Through co-evolution with the host plants, the nematodes have developed a responsive behavior to products or byproducts of ubiquitous plant signals, and this confers evolutionary advantage to the nematodes. Host-finding behavior involves recognition of host signals by the nematode's sensilla (sense organs), which function as the conduit between stimulus, reception, and behavioral output (Curtis et al. 2009; Dutta et al. 2011). Nevertheless, little is known about the molecular mechanisms that make a particular plant a host or a non-host for a given plant-parasitic nematode species. Development of fundamental studies to understand the nature of exudates affecting nematode behavior and what causes a plant to be a host or non-host will reveal targets for chemical or genetic intervention to control RKN based on disruption of nematode behavior or adjustment of host response (Curtis et al. 2009).

The function of the F-box/Kelch-repeat protein (At2g44130) is not known. Staining of the F-box promoter::GUS reporter line showed that, in planta, this gene is constitutively expressed only in the abscission zones of flower organs and in the abscission zone of seed in the seed pods; however, the function of the gene in these areas remains unknown, because both KO and OE lines do not show an obvious phenotype. However, the

expression of the F-box is inducible by *cis*-jasmonate because exposure of the reporter line to *cis*-jasmonate leads to a strong GUS staining only in the vasculature of the midrib of leaves (Matthes et al. 2010). This observation might be of significance because, in the root, it is the vascular tissue where nematodes establish their feeding sites (Williamson and Husey 1996).

Taking into account the supposed working mechanisms of F-box proteins, our results would predict that the induction of the F-box/Kelch-repeat protein (At2g44130), presumably by an effector released from the esophageal glands of the nematode during the infection process, would lead to the formation of plant SCF^(At2g44130) complexes, which would target proteins involved in the modulation of cellular processes necessary for feeding site establishment. This modulation could be due to either the degradation of cellular regulators by polyubiquitination or the modification of protein functions by ubiquitination with nonproteolytic signaling properties (Craig et al. 2009). In this scenario, the F-box could be seen as a master regulator. It was shown recently that some bacterial effectors do target proteins with master regulatory properties, as in the case described for *Xanthomonas* spp., which release AvrBs3, an effector that localizes to the plant cell nucleus and is a transcription factor. There, it induces the transcription of *upa20*, presumably by binding to its 5' regulatory region, which has been shown to be a master regulator of cell size, because *Upa20* alone induces hypertrophy of mesophyll cells in *Nicotiana benthamiana* and other solanaceous plants (Kay et al. 2007). A similar role might be attributed to the transcription factor AtWRKY23. This transcription factor was shown to be induced during infection of *Arabidopsis* by the CN *H. schachtii*, and its knocking down by RNAi resulted in lower infection rates. Therefore, it seems that an upregulation of WRKY23 is necessary for the successful induction of the feeding site and that WRKY23 might be involved in the complex redifferentiation process of the host cells giving rise to these feeding structures. Alternatively, it was suggested that WRKY23 could be a negative regulator of the defense response and its activation by the nematode would be responsible for abolishing plant defense mechanisms otherwise activated by the nematodes (Grunewald et al. 2008). A role as negative regulator of defense could also be envisaged for the F-box protein described here by postulating that it might be involved in the degradation of proteins orchestrating the plant defense response. In this respect, it is noteworthy to mention that microarray analysis performed on RKN infestation sites revealed that a considerable number of genes, putatively involved in defense, are being downregulated during successful establishment of the RKN, including some F-box genes (Fuller et al. 2007; Jammes et al. 2005).

Until now, a multitude of studies has focused mainly on the search for plant resistance genes in order to engineer plants with resistance to parasitic nematodes. The best known example is the *Mi* gene in tomato, which confers resistance against RKN (Milligan et al. 1998). However, only a limited number of plant species are resistant to RKN and there are many crops for which appropriate resistance loci have not been identified, as is the case for *Arabidopsis* (Williamson and Kumar 2006). On the other hand, plant genes were identified recently whose presence is required for successful infestation by pathogens and which were subsequently referred to as "susceptibility" (*S*) genes. One such example is the *PMR6* gene in *Arabidopsis*, whose recessive loss-of-function mutant is completely resistant to powdery mildew infection (Vogel et al. 2002). The results we reported here suggest that the identified F-box/Kelch protein could be regarded as such an *S* gene for nematode infection, because its nonfunctionality led to a considerable reduction in nematode numbers in roots. Further work showing that the downstream gene expression of the ubiquina-

tion pathways is altered in the mutants would provide concrete evidence for the functional role of this gene in the plant–nematode interaction.

Our findings raise several points of paramount interest: what are the compounds or signals present in the root exudate of the F-Box OE, tilling, and KO lines responsible for the change in nematode behavior; what are the effectors released by the nematode responsible for the activation of the F-box protein; which genes are regulated by the SCF^(At2g44130) complex; what is the function of the F-box-gene in planta; and how can these findings be exploited in order to achieve nematode resistance?

MATERIALS AND METHODS

Nematode cultures.

Pure cultures of the RKN *M. incognita* (race 1 NCSU), were maintained on roots of tomato plants (*Solanum lycopersicum* 'Tiny Tim') grown in a mixture of sterilized Rothamsted weed mix and sand in a temperature-controlled glasshouse at 28°C. Freshly hatched J2 were obtained from egg masses or sterilized eggs as described by Curtis (1996). Potato CN, *G. pallida* (Woburn population), were cultured on *S. tuberosum* 'Maris Piper' in sterilized sand-loam soil mixture and grown in a temperature-controlled glasshouse at 18°C. Freshly hatched J2 were obtained by soaking the cysts first in tap water for 3 to 5 days to hydrate, and then in potato root diffusate diluted four times with water. They were then left at approximately 18°C (Clarke and Perry 1977). Hatched J2 were collected every 24 h and maintained at 4 to 5°C for 30 min, before being centrifuged at 1,000 × g for 5 min. They were then used immediately. *H. schachtii* and *H. cajani* were cultured in sterilized sand-loam soil on *Beta vulgaris* and *Vigna unguiculata*, respectively, and maintained in a temperature-controlled glasshouse at 20 and 25°C, respectively. The cysts were extracted from soil and freshly hatched J2 were obtained by soaking cysts in water.

Plant material and cultivation.

A. thaliana plants (ecotype Col, WT) were grown from our seed stock. The At2g44130 promoter::GUS reporter line has been described by Matthes and associates (2010). The tilling line (The Nottingham Arabidopsis Stock Center (NASC) ID N93037) and the T-DNA KO line (SALK_143644/NASC ID N643644) were obtained from NASC. Seed were germinated in Levingtons F2 compost and, after 10 days, individual plants were transferred into a 7-cm-diameter pot filled with a mixture of Rothamsted weed mix and Levingtons F2 (3:1), which facilitates the washing of the roots due to its sandy consistency. Plants were then cultivated in a controlled-environment cabinet under short-day conditions (8-h light period, 250 µE m⁻² s⁻¹). During the light period, the temperature was kept at 23°C, with a relative humidity of 75%. During the dark period, the temperature was 18°C, with a relative humidity of 80%. Axenic cultures of *A. thaliana* were maintained as described by Sijmons and associates (1991) and Curtis (1996) from surface-sterilized seed germinated on Gamborg's B-5 basal medium with minimal organics (Sigma Aldrich, St. Louis) containing 1.5% sucrose and 0.75% Phytigel in 55-mm plates. The plates were incubated for 48 h at 4°C and then transferred to 24 ± 2°C with 16 h of light and 8 h of darkness for 2 to 3 weeks before use.

Generation of homozygous lines.

The tilling line for the locus At2g44130 was obtained using the service provided by the Seattle tilling project using the primers F 5'-TCGCAACTTTCGTCAGTTTGTGTGT-3' and R 5'-ACGTGAAGATGACTGGCAAATGCTCTG-3', which covers the whole gene. Of 13 individuals harboring mutations in At2g44130, 1 mutation resulted in the introduction of a

premature stop codon instead of the amino acid W at position 179. This line was provided as a heterozygote by NASC (NASC ID N93037). In order to isolate homozygous plants, 15 individuals of this line were analyzed using derived cleaved amplified polymorphic sequence markers according to Neff and associates (1998). In brief, a 262-bp fragment was amplified with the primers F 5'-GTTCTGGAATTCGCCGAAGGAACTG-3' and R 5'-AACCGCTCAGCACGCAAAACCT-3', introducing a *BsrI* site into the WT sequence. Amplification following standard conditions given by Invitrogen was performed for 40 cycles, with each cycle including a 30-s melt at 94°C, 30 s of annealing at 60°C, and extension at 72°C for 45 s; followed by 2 min of primer extension at 72°C. After purification of the polymerase chain reaction (PCR) products over a Qiagen column, the PCR products were digested with *BsrI* (New England Biolabs, Beverly, MA, U.S.A.) and the fragments separated on a 2.5% agarose gel. KO mutants for the gene At2g44130 were searched using the T-DNA express service from SIGNAL. Only one line harboring the T-DNA insert 5' upstream of the gene of interest was identified (SALK_143644). Individuals grown from that seed stock were screened with primers designed using the iSct primer tool. In brief, in an initial screen, genomic DNA from 16 individuals was amplified with Lb1 (5'-GCGTGGACCGCTTGCTGCAACT-3') and SALK_143644 RP (5'-AAGCGTTAAACAAAAGTGCG-3') in order to identify those plants containing an insert (40 cycles with 30 s of melt at 94°C, 30 s of annealing at 60°C, and extension at 72°C for 60 s; with a final primer extension of 2 min). Seven plants were chosen from this screen. Genomic DNA from these seven individuals was then amplified using primers SALK_143644 LP2 (5'-CGGCGTTAGCGGTTGAACGA-3') and SALK_143644 RP, which amplify the WT gene without T-DNA insertion. Plants showing an amplification product for this primer combination were discarded as heterozygotes, which left four individuals as potential homozygous KO plants. Those plants were tested for At2g44130 expression after exposure to *cis*-jasmonate by Northern blots.

Northern blots.

Total RNA from leaves was prepared from *A. thaliana* ecotype Col (WT) and the homozygous T-DNA KO plants (SALK_143644/NASC ID N643644) not treated or treated with *cis*-jasmonate (Avocado Research Chemicals, Lancashire, U.K.) as described by Matthes and associates (2010) using Trizol reagent. RNA (7 µg) was electrophoretically separated on a 1% agarose/formaldehyde gel and blotted onto Hybond-N+ membranes (GE Healthcare Life Sciences Amersham, Little Chalfont, U.K.) by capillary transfer. The probe was prepared by PCR with At2g44130-specific primers (F 5'-CGAGAGACGTTTACGTTCTG-3'; R 5'-CATAACGTAGAGGAGCTTCT-3'). PCR products were labeled with [α -32P] dCTP prior to hybridization. Hybridization was performed according to Sambrook and associates (1989). Filters were washed twice at 65°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate for 20 min and autoradiographed at -80°C using two intensifying screens.

Nematode infection of plants.

Three-week-old plants of *A. thaliana* ecotype Col (WT), the At2g44130 promoter::GUS reporter line, the tilling line NASC ID N93037 (T), the T-DNA KO line SALK_143644/NASC ID N643644 (KO), and the OE line were maintained in pots filled with a soil mixture or in axenic cultures and inoculated with 400 or 200 J2 of *M. incognita* or *G. pallida*, respectively. Replication for each bioassay was variable from 2 to 10 plants per genotype, depending on availability, with two to three technical replicates being measured per plant. Plants were kept in

glasshouse compartments for 21 days under controlled environmental conditions at a temperature of 26°C for plants infected with *M. incognita* and 17°C for plants infected with *G. pallida*. The plants were watered 4 days after inoculation to synchronize the infection and maintained at 25°C during the day and at 23°C at night for plants infected with *M. incognita*, or at 17°C at night for plants infected with *G. pallida*. All plants were maintained under controlled glasshouse conditions with 16 h of artificial light (400-W SON-T agro bulbs, placed 1 m above pots). The pots of all the replicates of all plant species were positioned randomly. Roots were washed free of soil and the length and fresh weight of the roots was determined. The roots were then chopped into 1- to 2-cm segments and stained with acid fuchsin according to the protocol by Byrd and associates (1983), which allows detection of nematodes inside roots. After the procedure, nematodes are stained bright red in contrast with the clear root background. The number of nematodes inside the roots was counted using a stereomicroscope and the number of nematodes per gram of root tissue was calculated. A time course of infection of *M. incognita* was observed for the *Arabidopsis* At2g44130 promoter::GUS reporter line and roots were stained with GUS and acid fuchsin 3, 7, 14, and 21 DPI. Histochemical localization of GUS activity in infected and uninfected roots of the At2g44130 promoter::GUS reporter was performed with the substrate 5-bromo-4-chloro-3-indolyl D glucuronide (Melford Laboratories Ltd., Ipswich, U.K.) according to Jefferson and associates (1987). Then, 25 mg was dissolved in 1 ml of methylcellulose (Sigma Aldrich) and subsequently diluted to 0.25 mg/ml with 0.1 M NaHPO₄ (pH 7.0) buffer, 0.1% Triton X-100, and 0.5 mM potassium ferricyanide/ferrocyanide. The plant tissue was then incubated overnight at 37°C for the GUS reaction to occur. Root galls containing nematodes and exhibiting GUS staining were subjected to cryostat sectioning as described by Curtis (1996). Photographs of stained roots were taken using an Olympus inverted microscope with Openlab software v. 2.2.3 in an Apple Macintosh computer or a Leica M205FA microscope with Leica Application Suite software.

Attraction and invasion bioassays.

J2 were concentrated by centrifugation using 1,000 × *g* for 5 min at 4°C. Three milliliters of 23% pluronic gel F-127 powder (Sigma Aldrich) was evenly mixed with approximately 500 J2 of *M. incognita* onto each 35-by-10-mm FALCON EASY-GRIP petri dish. This bioassay, carried out in pluronic gel, resembles the three-dimensional natural soil environment and has proved particularly useful in investigating the short-distance attraction of plant-parasitic nematodes to host roots and their accumulation around certain sites on the roots. Monitoring of nematode movement around host roots is easy due to the excellent transparency of the gel (Dutta et al. 2011; Wang et al. 2009). Seven-day-old seedlings of *A. thaliana* were placed in the center of the gel and the plates were kept in a humid chamber, which was kept in an incubator at 27°C. The numbers of J2 of *M. incognita* touching the terminal 2 mm of the root tip were counted 3, 6, and 18 h after introduction of the *Arabidopsis* root into the pluronic gel. The *Arabidopsis* plants were removed from the pluronic gel after 24 h to assess nematode invasion. The roots were washed gently to remove any nematodes attached to the outside and stained with acid fuchsin. The number of nematodes that penetrated these roots was then counted under a stereomicroscope.

Statistical analysis.

Data on numbers of nematodes per gram of root tissue, root weight, number of nematodes touching root tips at different time points (attraction), and the number of nematodes that pene-

trated roots after 24 h were subjected to analysis of variance (ANOVA) to ascertain the statistical significance (using *F* tests) of time, *Arabidopsis* lines, and the interaction between these two factors for attraction and penetration data (split-plot in time ANOVA), and of lines only (one-way ANOVA) for the other variables. Due to some variance heterogeneity across the *Arabidopsis* lines, numbers of nematodes per gram of root tissue required square root transformation, and root weight and length required a more powerful log (to base *e*) transformation. Following the ANOVA, standard error of the difference values on the residual degrees of freedom were output, and the least significant difference values were used to make biologically meaningful comparisons of means at the 5% (*P* = 0.05) level of significance. The GenStat (Fourteenth Edition; VSN International Ltd., Hemel Hempstead, U.K.) statistical system was used for all analyses.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

SIGnal T-DNA Express: *Arabidopsis* gene mapping tool:
signal.salk.edu/cgi-bin/tdnaexpress