The Root-Knot Nematode Calreticulin Mi-CRT Is a Key Effector in Plant Defense Suppression

M. Jaouannet,^{1,2,3} M. Magliano,^{1,2,3} M. J. Arguel,^{1,2,3} M. Gourgues,^{1,2,3} E. Evangelisti,^{1,2,3} P. Abad,^{1,2,3} and M. N. Rosso^{1,2,3}

¹INRA, UMR 1355 Institut Sophia Agrobiotech, ²Université de Nice Sophia Antipolis, UMR Institut Sophia Agrobiotech, and ³CNRS, UMR 7254 Institut Sophia Agrobiotech, 400 route des Chappes F-06903 Sophia Antipolis, France

Submitted 23 May 2012. Accepted 24 July 2012.

Root-knot nematodes (RKN) are obligate biotrophic parasites that settle close to the vascular tissues in roots, where they induce the differentiation of specialized feeding cells and maintain a compatible interaction for 3 to 8 weeks. Transcriptome analyses of the plant response to parasitic infection have shown that plant defenses are strictly controlled during the interaction. This suggests that, similar to other pathogens, RKN secrete effectors that suppress host defenses. We show here that Mi-CRT, a calreticulin (CRT) secreted by the nematode into the apoplast of infected tissues, plays an important role in infection success, because Mi-CRT knockdown by RNA interference affected the ability of the nematodes to infect plants. Stably transformed *Arabidopsis thaliana* plants producing the secreted form of Mi-CRT were more susceptible to nematode infection than wild-type plants. They were also more susceptible to infection with another root pathogen, the oomycete *Phytophthora parasitica*. Mi-CRT overexpression in *A. thaliana* suppressed the induction of defense marker genes and callose deposition after treatment with the pathogen-associated molecular pattern elf18. Our results show that Mi-CRT secreted in the apoplast by the nematode has a role in the suppression of plant basal defenses during the interaction.

The success of host tissue colonization by biotrophic and hemibiotrophic pathogens depends on the modulation of plant defenses by these organisms. Pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), also known as microbe-associated molecular pattern (MAMP)-triggered immunity, is the first line of inducible defense against pathogens. It is activated by pattern recognition receptors (PRR), which interact directly with conserved PAMPs or MAMPs. For example, FLS2 and EFR are PRR that recognize flagellin and the elongation factor EF-Tu, respectively, these two proteins being widely conserved in bacteria. PTI does not always fully restrict pathogen proliferation and can result in weak or robust resis-

tance responses (Schwessinger and Zipfel 2008). PTI is characterized by cytosolic calcium influx, K⁺ and Cl⁻ efflux, membrane depolarization, the activation of mitogen-activated protein kinase cascades, the production of reactive oxygen species and ethylene (ET), and the rapid induction of defense-related genes (Asai et al. 2002; Zipfel et al. 2004). PTI also alters metabolite composition and the production of secondary defense compounds, such as the glucosinolates produced by plants of the Brassicaceae to limit fungal infection (Bednarek et al. 2009; Sana et al. 2010). Plants also strengthen their physical barriers, by closing their stomata and enhancing cell wall fortification, notably by callose deposition (Hématy et al. 2009).

Successful pathogens deploy an arsenal of effectors that attenuate or suppress PTI responses, allowing them to proliferate and cause disease (Dodds and Rathjen 2010). Root-knot nematodes (RKN) are obligate root parasites that infest more than 5,000 plant species worldwide (Blok et al. 2008). Second-stage juveniles (J2) penetrate root tips and migrate intercellularly to the vascular cylinder, where they settle in the intercellular space. They perforate the cell walls of five to seven parenchyma cells with a protrusible stylet, via which they inject secretions that induce the redifferentiation of the selected cells into hypertrophied, multinucleate, and metabolically hyperactive feeding cells called giant cells (Jones and Payne 1978; Wiggers et al. 1990). Giant-cell induction is accompanied by the hypertrophy and hyperplasia of adjacent parenchyma cells and the formation of a typical gall. The parasitic J2 then start feeding and develop into parasitic third- and fourth-stage juveniles (J3 and J4, respectively) and, finally, into adults. The interaction ceases only after females have laid eggs on the root surface and died. A fascinating and unique feature of RKN of the genus *Meloidogyne* is their ability to maintain a biotrophic interaction within the host for the 3 to 6 weeks necessary for the completion of their life cycle. Several lines of evidence suggest that nematodes have evolved effectors that modulate PTI during the interaction. Microarray analyses of infected root tissues and laser-dissected giant cells have shown that plant defense responses are finely balanced during the plant-nematode interaction (Barcala et al. 2010; Damiani et al. 2012; Jammes et al. 2005). For example, defense genes involved in the salicylic acid (SA)-, jasmonic acid (JA)- and ET-dependent signaling pathways are downregulated during the plant-nematode interaction. The genes encoding the pathogenesis-related (PR) proteins PR-1, PR-2, and PR-5 are strongly induced in *Arabidopsis* roots 9 days after infection (DAI) by *Meloidogyne incognita* (Hamamouch et al. 2011) but the microarray analysis of dissected feeding cells showed a reduction of the expression of PR-1 in the giant cells 3 DAI (Barcala et al. 2010).

Corresponding author: P. Abad; E-mail: abad@sophia.inra.fr

Current address of M. N. Rosso: Université Aix-Marseille, UMR1163 Biotechnologie des Champignons Filamenteux, F-13288 Marseille, France.

*The e-Xtra logo stands for “electronic extra” and indicates that five supplementary figures and one supplementary table are published online.

It has been suggested that effectors secreted by nematodes contribute to plant defense suppression during infection (Davis et al. 2008; Rosso et al. 2011). In planta immunolocalization studies have demonstrated that several proteins produced in the esophageal glands are injected into plant tissues via the nematode stylet (Doyle and Lambert 2002; Jaubert et al. 2005; Vieira et al. 2011). However, it remains unclear how RKN modulate plant defenses. There are several lines of evidence suggesting that chorismate mutase, a protein secreted by the RKN *M. javanica*, is involved in the suppression of plant defenses (Doyle and Lambert 2003). It has been suggested that the injection of chorismate mutase into the cytoplasm of plant cells by the nematode may lead to chorismate export from plastids, thereby reducing the pool of chorismate available in plastids for the synthesis of flavonoids, SA, phytoalexins and auxin (Doyle and Lambert 2003). Alternatively, the secreted chorismate mutase may modify the metabolic status of infected cells, as suggested

for the secreted chorismate mutase of the fungus *Ustilago maydis* (Djamei et al. 2011).

The calreticulin (CRT) Mi-CRT from *M. incognita* is synthesized in the subventral glands of preparasitic J2 and in the dorsal gland of parasitic stages. Mi-CRT is secreted into plant tissues via the stylet, and accumulates to high abundance at the cell wall of giant cells (Jaubert et al. 2005). In a previous work, the knock-down of Mi-CRT in preparasitic infective juveniles resulted in a reduced ability of nematodes to induce galls on tomato, indicating that preparasitic juveniles were affected in virulence (Dubreuil et al. 2009). This result highlighted the importance of Mi-CRT for the success of infection. CRT are calcium-binding proteins that are highly conserved in plants and animals. They are present mostly in the lumen of the endoplasmic reticulum (ER), where they act as Ca^{2+} -binding chaperones, regulating Ca^{2+} storage and signaling. CRT located at the nuclear envelope, in the cytoplasm, or at the cell surface regulate numerous cell functions, including gene expression, cell adhesion, and immunoregulation via calcium binding or direct interaction with signaling proteins (Gold et al. 2010; Michalak et al. 2009). Several animal parasites also secrete CRT into the host during infection, and the CRT secreted by *Trypanosoma* spp. and animal-parasitic nematodes have been shown to play a role in the modulation of host defenses (Naresha et al. 2009; Ramírez et al. 2011; Valck et al. 2010). In plants, CRT have been located primarily within the lumen of the ER and in the Golgi apparatus (Samaj et al. 2008). However, they have also been detected in the nuclear envelope (Napier et al. 1995) and plasmodesmata (Baluska et al. 1999; Chen et al. 1994) and at the plasma membrane (Sharma et al. 2004). CRT gene expression in plants is responsive to various stresses, including gravistimulation, cold, and drought (Jia et al. 2009). It has recently been shown in *Arabidopsis thaliana* that the CRT isoform At-CRT3 specifically recognizes the EF-Tu receptor EFR and participates in EFR quality control within the ER (Christensen et al. 2010).

In this study, we analyzed the role of Mi-CRT in the plant-nematode interaction and showed that the knock-down of Mi-CRT gene expression in nematodes decreased the ability of these parasites to infect plants. Conversely, plants overproducing Mi-CRT were more susceptible to infection with this nematode. We investigated the ability of Mi-CRT to suppress the PTI triggered by the PAMP elf18 in *A. thaliana*. Based on our findings, we suggest that Mi-CRT is involved in PTI suppression during the interaction.

RESULTS

Mi-CRT knockdown affects *M. incognita* virulence in *A. thaliana*.

We investigated the role of Mi-CRT during the interaction by analyzing the ability of nematodes to complete their biological cycle in planta after Mi-CRT gene knock-down in parasitic stages. We generated plants that produced hairpin-shaped double-stranded RNA homologous to the nucleotide 318-to-621 region of the Mi-CRT (Minc06693) open reading frame (hpMi-CRT). Three transgenic lines, each containing one copy of the hpMi-CRT transgene, were selected by segregation analysis on selective medium containing kanamycin. The reverse-transcription quantitative polymerase chain reaction (RT-qPCR) method was used for the quantitative determination of transgene expression in the transgenic lines (Supplementary Fig. S1). We selected the transgenic line with the highest level of expression, line 19-1, for the production of homozygous T4 plants, which were used for the following analysis.

The homozygous hpMi-CRT T4 plants had a root phenotype identical to that of wild-type plants (Supplementary Fig. S2).

Fig. 1. Effect of calreticulin (CRT) Mi-CRT knockdown on *Meloidogyne incognita* virulence. **A**, Expression levels of the Mi-CRT gene in the nematodes feeding on plants overproducing hpMi-CRT. **B**, Mean number of galls produced ($\pm$ standard error of the mean [SEM]) on infected wild-type (Wt) and hpMi-CRT plants. **C**, Mean number of egg masses per gall. Error bars: SEM. Kruskal-Wallis tests were used for statistical analysis (** indicates $P < 0.01$). Two independent biological replicates were carried out, with 24 plants per condition.

We checked for Mi-CRT knockdown in the feeding nematodes by inoculating hpMi-CRT plants with nematodes and quantifying Mi-CRT transcript levels in the nematodes embedded in galls 21 DAI. The primers used to quantify Mi-CRT transcripts were designed to be specific to Minc06693. We observed 62 and 75% depletion of Mi-CRT transcripts in the feeding nematodes in two independent experiments, respectively (Fig. 1A). We compared the ability of nematodes to induce gall formation and to produce eggs on wild-type and hpMi-CRT plants. Significantly fewer galls were observed on hpMi-CRT plants, suggesting that Mi-CRT knockdown in parasitic stages affected the capacity of the nematodes to initiate the feeding site (Fig. 1B). The egg mass/gall ratio was not affected by Mi-CRT knockdown (Fig. 1C), indicating that female development and egg production were not affected in nematodes that had established and formed a gall. Identical results were obtained for two independent biological replicates (Fig. 1B and C).

Mi-CRT expression

in *A. thaliana* increases susceptibility to *M. incognita*.

The Mi-CRT gene was overexpressed in *A. thaliana* plants, under the control of the constitutive *Cauliflower mosaic virus* 35S promoter. For this purpose, we deleted the ER retention site (His-Asp-Glu-Leu [HDEL]) from the coding sequence. We used a construct containing the Mi-CRT secretion signal peptide (Mi-CRT+SP) to mimic Mi-CRT secretion into the apoplast by the nematode during infection (Jaubert et al. 2005). A second construct, from which the secretion signal peptide was deleted (Mi-CRT-SP), was predicted to lead to a cytoplasmic distribution of Mi-CRT-SP.

The subcellular distribution of Mi-CRT+SP and Mi-CRT-SP in plant cells was determined with enhanced green fluorescent protein (eGFP) fusions and confocal microscopy imaging of agroinfiltrated tobacco leaf cells. Mi-CRT-SP fused to eGFP was located in the cytoplasm of agroinfiltrated tobacco leaf cells (Fig. 2A). A signal was also detected in the nuclei, probably due to passive diffusion of the fusion protein (predicted

molecular weight approximately 70 kDa) (Haasen et al. 1999, Jaouannet et al. 2012). Two lines of evidence suggested that Mi-CRT+SP was secreted into the apoplast of transformed tobacco leaf cells. Labeling of the plasma membrane with FM4-64 showed that Mi-CRT+SP fused to eGFP reached the cell periphery (Fig. 2E, arrows). Furthermore, the colocalization of the fusion protein with the ER (Fig. 2b to d) and Golgi markers (Fig. 2F to H) indicated that the protein entered the secretory pathway. These data demonstrated that the nematode secretion signal peptide was functional in plant cells and that Mi-CRT+SP fused to eGFP was secreted to the apoplast via the plant secretory pathway.

We generated plants that overproduced Mi-CRT with or without the signal peptide, under the control of the 35S promoter. For each construct, three transgenic lines, each containing one copy of the Mi-CRT transgene, were selected by segregation analysis on selective medium containing kanamycin. RT-qPCR was used to determine transgene expression levels in transgenic lines (Supplementary Fig. S3). The 35S::Mi-CRT+SP number 7 and 35S::Mi-CRT-SP number 3.4 transgenic lines, which had the highest levels of overexpression, were selected. We produced homozygous T4 lines that were used for further analysis. No significant differences in root length were observed between wild-type plants and Mi-CRT+SP or Mi-CRT-SP overexpression lines 2 weeks after germination (Supplementary Fig. S4).

We investigated the effect of Mi-CRT+SP or Mi-CRT-SP expression in *A. thaliana* on the susceptibility of the plants to infection with *M. incognita*. Plants overexpressing Mi-CRT+SP had more galls than wild-type plants, indicating that these plants were more susceptible to infection with *M. incognita* than wild-type plants (Fig. 3A).

Conversely, *A. thaliana* plants overexpressing Mi-CRT-SP were much less susceptible to infection with *M. incognita* (Fig. 3B). However, the egg mass/gall ratio was not affected in any of these transgenic plants (Fig. 3C and D). Similar results were obtained for two independent biological replicates.

Fig. 2. Subcellular localization of calreticulin (CRT) Mi-CRT in tobacco leaf cells. Single-plane confocal images of tobacco epidermal leaf cells infiltrated with *Agrobacterium tumefaciens* and producing Mi-CRT with or without peptide signal, fused to the enhanced green fluorescent protein (eGFP) reporter. Micrographs: **A**, **B**, and **F**, eGFP signal (green); **C**, monomeric red fluorescent protein (mRFP) signal (red) of endoplasmic reticulum (ER) or **G**, Golgi markers; **D** and **H**, merged images of the eGFP and mRFP signals. **A**, Mi-CRT-SP fused to eGFP accumulated in the cytoplasm (Cyt) and in the nuclei (N). **B** and **F**, Mi-CRT+SP fused to eGFP entered the secretory pathway, as demonstrated **C** and **D**, by its colocalization with ER and **G** and **H**, Golgi markers. **E**, Labeling of the plasma membrane with FM4-64 (red) showed that Mi-CRT+SP fused to eGFP (green) reached the cell periphery (arrows). Scale bars: 10 μ m.

Mi-CRT gene expression in *A. thaliana* increases susceptibility to the oomycete *Phytophthora parasitica*.

We investigated the effect of Mi-CRT overexpression on the susceptibility of *A. thaliana* to the hemibiotrophic pathogen *Phytophthora parasitica*. Plants that overexpressed Mi-CRT+SP were more susceptible than wild-type plants to *P. parasitica*, as shown by the faster colonization of root tissues by the pathogen (Fig. 4A). The differences between transgenic and wild-type plants following inoculation with *P. parasitica* were statistically significant. A *P* value of 0.00033 was obtained in Scheirer-Ray-Hare nonparametric two-way analysis

(ANOVA). By contrast, pathogen colonization was similar in plants overexpressing Mi-CRT-SP and wild-type plants (*P* = 0.14) (Fig. 4B).

Mi-CRT alters PTI in *A. thaliana*.

Because Mi-CRT+SP expression in *A. thaliana* increased plant susceptibility to both *M. incognita* and *P. parasitica*, we investigated the possible role of Mi-CRT+SP in host defense suppression during pathogen infection. We analyzed the expression of defense marker genes after the triggering of PTI by the PAMP elf18 in wild-type Wassilewska (Ws) seedlings and

Fig. 3. Effect of the overproduction of calreticulin (CRT) Mi-CRT on *Arabidopsis thaliana* susceptibility to *Meloidogyne incognita*. **A** and **B**, Mean number of galls produced by *M. incognita* ($\pm$ standard error of the mean [SEM]) on infected wild type (Wt) and Mi-CRT+SP (**A**) or Mi-CRT-SP (**B**) overexpression lines of *A. thaliana*. **C**, and **D**, Mean number of egg masses per gall. Error bars: SEM. Kruskal-Wallis tests were carried out for statistical analysis (** and * indicate *P* < 0.01 and 0.05, respectively). Two independent biological replicates were carried out, with 20 plants per condition.

Fig. 4. Effect of the overproduction of calreticulin (CRT) Mi-CRT on *Arabidopsis thaliana* susceptibility to *Phytophthora parasitica*. Disease progression on *A. thaliana* plants overproducing **A**, Mi-CRT+SP or **B**, Mi-CRT-SP inoculated with *P. parasitica*, as assessed by disease index (Attard et al. 2010). Differences between transgenic lines and wild-type plants on inoculation with *P. parasitica* were statistically significant, as determined by Scheirer-Ray-Hare nonparametric two-way analysis of variance for ranked data (*H* < 0.05). Two independent biological replicates were carried out, with 25 plants per condition.

seedlings overproducing Mi-CRT+SP. We compared the expression of the defense marker genes *FRK1*, *WRKY33*, *PDF1.2*, *CYP81F2*, *WRKY29*, *PAD4*, and *PR-1* by RT-qPCR with their expression levels in plants grown in the same conditions without elf18 treatment. As expected, elf18 strongly induced the expression of the marker genes analyzed in wild-type plants. By contrast, the *FRK1*, *WRKY33*, *PDF1.2*, *CYP81F2*, *WRKY29*, and *PAD4* genes were totally repressed in plants overproducing Mi-CRT+SP (Fig. 5A). Surprisingly, the induction of *PR-1* was not affected.

We also assessed callose deposition in wild-type plants and in plants overproducing Mi-CRT+SP after the triggering of PTI with elf18. As expected, elf18 treatment induced callose deposition in wild-type cotyledons (Fig. 5B). By contrast, we observed no callose deposition in the cotyledons of plants overproducing Mi-CRT+SP after treatment with elf18 (Fig. 5B). On the contrary, we observed no alteration in callose deposition in *A. thaliana* plants overexpressing Mi-CRT-SP (Supplementary Fig. S5). The absence of defense marker gene induction and the suppression of callose deposition in response to elf18 indicated that PTI was altered by the production of Mi-CRT+SP in *A. thaliana*.

DISCUSSION

RKN employ an arsenal of effector proteins to invade host plant roots and induce feeding sites (Bellafiore et al. 2008; Jaouannet et al. 2012; Jaubert et al. 2002). RKN are biotrophic pathogens and the success of infection depends on the ability of the pathogen to overcome PTI, as reported for other pathogens. Several type III secreted protein effectors and fungal effectors have been shown to contribute to pathogen virulence by suppressing PTI (Caldo et al. 2006; Göhre and Robatzek 2008; Kim et al. 2009). PTI involvement in plant–nematode interaction remains poorly documented. Yet, some cyst nematode effectors are suspected to suppress host defenses to establish host compatibility (Hamamouch et al. 2012; Hewezi et al. 2010; Patel et al. 2010; Rehman et al. 2009). Nematode-secreted effectors may interfere with plant defense in locally infected tissue or systemically, by modulating systemic signaling (Kyndt et al. 2011).

Mi-CRT is secreted by RKN throughout parasitism and accumulates to high abundance in the apoplast of infected tissues during giant-cell induction and maintenance (Bellafiore et al. 2008; Jaubert et al. 2002). Mi-CRT is synthesized in the esophageal glands of the nematode and has a secretion signal peptide to direct its secretion from the esophageal glands to the esophageal lumen. Like other secreted esophageal proteins, it is then transported through the pharynx and secreted outside the nematode via the stylet (Hussey 1989). Two hypotheses have been proposed concerning the role of Mi-CRT in parasitism (Dubreuil et al. 2009; Jaubert et al. 2002). First, Mi-CRT may act as a chaperone of secreted nematode proteins in the esophageal glands. In this case, the secretion of Mi-CRT in planta (Bellafiore et al. 2008; Jaubert et al. 2002) would be due to the binding of this protein to other stylet-secreted proteins, and Mi-CRT would have no direct role in the interaction. Alternatively, Mi-CRT may be directly involved in successful infection, through the modulation of plant responses, as suggested previously.

We show here that Mi-CRT plays a direct role in the success of infection. We knocked down Mi-CRT gene expression after nematode invasion of the root, by producing *A. thaliana* plants that overexpressed an Mi-CRT-specific hairpin RNA. Nematodes reared on these plants had already invaded the root tissues and initiated the induction of giant cells before the ingestion of the double-stranded hairpin RNA and triggering of Mi-CRT

knockdown. The smaller number of galls on plants producing the Mi-CRT hairpin RNA suggested a role for Mi-CRT in successful nematode establishment after root invasion and initiation of giant-cell differentiation. This conclusion was supported by data from infection assays on *A. thaliana* plants that produced Mi-CRT targeted to the apoplast. Plants overproducing Mi-CRT+SP were more susceptible than wild-type plants to nematode infection. Interestingly, plants overproducing Mi-CRT in the apoplast were also more susceptible to the hemibiotrophic root pathogen *P. parasitica*. Conversely, Mi-CRT overproduction in the cytoplasm of plant cells increased plant resistance to RKN but had no effect on infection

Fig. 5. Calreticulin (CRT) Mi-CRT+SP suppresses the defense triggered by elf18 in *Arabidopsis thaliana*. **A**, Wild-type plants (Wt) and plants from the Mi-CRT+SP overproducing line were grown for 9 days on Murashige and Skoog agar medium and treated with 1 μ M elf18. Induction of the defense marker genes *FRK1*, *WRKY33*, *PDF1.2*, *CYP81F2*, *WRKY29*, *PAD4*, and *PR-1* in response to elf18 was determined relative to plants grown without elf18 treatment, by reverse-transcription quantitative polymerase chain reaction. Three independent experiments were performed and gave similar results. **B**, Callose deposition in cotyledons of 9-day-old *A. thaliana* seedlings from Wt and Mi-CRT+SP overproducing line, 24 h after mock or 1 μ M elf18 treatment. Callose deposition was analyzed by staining with aniline blue, and the number of fluorescent spots was determined by UV epifluorescence microscopy. Values represent means ($\pm$ standard error of the mean; $n > 10$).

by *P. parasitica*. These plants were not altered in callose deposition after elf18 treatment. Together, these results suggest that Mi-CRT expressed into the plant cell cytoplasm has no effect on plant defense. Although the mode of action of the cytoplasmic Mi-CRT is beyond the scope of this study, it is tempting to suggest that it may alter intracellular calcium homeostasis or signaling in plant cells (Qiu et al. 2012), and thereby, disturb the formation, maintenance, or maturation of the giant cells. Obviously, further studies would be required to determine how the overproduction of Mi-CRT in the cytoplasm interferes with the plant response to the pathogen.

The greater susceptibility to *M. incognita* and *P. parasitica* results from the suppression of basal defenses in the roots of plants overproducing Mi-CRT+SP. We found that the production of Mi-CRT in the apoplasm of plant cells altered PTI induction after treatment with the EF-Tu-derived peptide elf18. The induction of marker defense genes was strongly suppressed in plants overproducing Mi-CRT in the apoplasm. In particular, the SA-dependent defense pathway was repressed by Mi-CRT, as demonstrated by the absence of *PAD4* transcript induction after elf18 treatment. SA-mediated signaling plays a significant role in the plant response to nematode infection, as shown by the greater susceptibility of *A. thaliana* plants with impaired SA biosynthesis or signaling to the cyst nematode *Heterodera schachtii*. By contrast, SA-treated wild-type plants were less susceptible to *H. schachtii* (Wubben et al. 2008). Thus, Mi-CRT may contribute to parasitism success by suppressing SA-mediated defenses. Mi-CRT also suppressed the induction of *PDF1.2*, a defense gene from the JA/ET pathway, suggesting that the secreted Mi-CRT may interfere with both SA and JA/ET defense signaling pathways during the interaction. Moreover, the SA and JA/ET pathways have been shown to play a major role in defense against the rice RKN, *M. graminicola* (Nahar et al. 2011).

Surprisingly, although *PAD4* induction was controlled by Mi-CRT, the induction of *PR-1* was not affected in Mi-CRT-producing plants. *A. thaliana pad4* mutants are deficient in SA accumulation and show very low levels of *PR1* after inoculation by the biotrophic fungal pathogen *Erysiphe cichoracearum* (Mang et al. 2009). *PR-1* was highly induced in *Arabidopsis* roots after *M. incognita* infection (Hamamouch et al. 2011) but the microarray analysis of dissected feeding cells showed a reduction in *PR-1* expression in dissected giant cells at 3 DAI (Barcala et al. 2010). In another study, the modulation of the JA pathway by the ET pathway led to downregulation of major defense-related genes such as *PR-1* in the early stages of RKN infection (1 to 3 DAI) (Nahar et al. 2011). These results demonstrate that the regulation of *PR-1* during infection is unsettled. Our results suggest that Mi-CRT could affect SA-accumulation via *PAD-4* but that *PR-1* could be regulated during RKN infection by the JA and ET pathways.

The mode of action of Mi-CRT is unknown. During nematode infection, Mi-CRT accumulates in the apoplasm of feeding cells (Jaubert et al. 2005), where it could interact with host proteins involved in immune defense, as shown for CRT secreted into the extracellular space by vertebrate parasitic nematodes (Ramírez et al. 2011). In *Arabidopsis*, AtCRT3 interacts within the ER with the EFR receptor of EF-Tu to control the proper folding and accumulation of the receptor (Li et al. 2009). Our attempts to identify a plant target for Mi-CRT with a yeast two-hybrid system have been unsuccessful, and further investigations based on strategies suitable for use with plant targets located at the surface of plant cells are required to elucidate the molecular mechanism of PTI suppression by Mi-CRT. Alternatively, Mi-CRT may chelate calcium in the apoplasm and prevent calcium influx, one of the primary signals for defense induction. The importance of Ca^{2+} in defense sig-

nal transduction is illustrated by bacterial pathogens that produce exopolysaccharides to chelate Ca^{2+} , as part of a virulence strategy for overcoming PTI (Aslam et al. 2008). It was recently shown that Ca^{2+} signaling regulates SA-mediated immunity in *Arabidopsis* (Qiu et al. 2012). The binding of Ca^{2+} /calmodulin to the transcription factor SIGNAL RESPONSIVE1 prevents accumulation of SA via the transcriptional repression of *EDS1* (Du et al. 2009).

In conclusion, the calcium-binding properties of Mi-CRT may play an important role in overcoming the basal defenses of *Arabidopsis*. The mechanism of defense suppression by Mi-CRT remains unclear but our findings provide the first evidence for manipulation of the plant basal immune response by a nematode effector secreted into the apoplasm.

MATERIALS AND METHODS

Plasmid constructions.

The full-length coding sequence of Mi-CRT (Minc06693/AF402771.1), deleted of the ER retention site (HDEL), with or without the nematode signal peptide sequence, was amplified from the cDNA clone with primers Mi-CRT+SP Fo or Mi-CRT-SP Fo and Mi-CRT Ba (Supplementary Table S1). The coding sequence of Mi-CRT, with or without the nematode signal peptide, was transferred from pDON207 to the Gateway pK7FGW2.0 binary overexpression vector (Karimi et al. 2002).

For RNA interference in planta, we amplified nucleotides 318 to 621 from the full-length Mi-CRT cDNA by PCR with the specific primers Cal2RF-attB1 and Cal2RR-attB2. This 304-bp fragment was then inserted into the Gateway donor vector pDON207 (Invitrogen, Karlsruhe, Germany) for transfer into the pK7GWIWG2D(II) expression vector (Karimi et al. 2002) with LR Clonase (Invitrogen).

A. thaliana Wassilewskia accessions (Ws-4) were transformed and transgenic lines were selected as described in "Plant culture and transformation".

Subcellular localization in plant cells.

For transient expression in plant cells, fully expanded leaves from 3- to 4-week-old *Nicotiana benthamiana* plants were infiltrated with a suspension of *Agrobacterium tumefaciens* GV3101 (Holsters et al. 1980) containing the vectors, diluted to an optical density at 600 nm of 0.5 in infiltration medium (10 mM MgCl_2 , 10 mM MES [pH 5.6], and 200 μM acetosyringone). We used an ER marker (*Arabidopsis thaliana* wall-associated kinase 2) fused at its C terminus to an ER retention site (HDEL) (Nelson et al. 2007) and to monomeric red fluorescent protein (mRFP). Golgi localization was controlled by the cytoplasmic tail and transmembrane domain (first 49 amino acids) of GmMan1, soybean α -1,2-mannosidase I, fused to mRFP (Nelson et al. 2007). For plasma membrane staining, leaf disks were vacuum infiltrated during 3 min with 1 μM FM4-64 dye (Molecular Probes, Eugene, OR, U.S.A.) (Caillaud et al. 2008). Infiltrated leaf patches were mounted in water and analyzed on an inverted confocal microscope (Axiovert 200 M, LSM510 META; Carl Zeiss GmbH, Jena, Germany). Sections were analyzed with LSM Image Browser software.

Plant culture and transformation.

Wild-type *A. thaliana* Wassilewskia accessions (Ws-4) were grown at 20°C under a cycle of 8 h of light and 16 h of darkness for 20 days, and were then transferred to conditions of 12 h of light and 12 h of darkness. For in vitro analyses, seed were surface sterilized by soaking for 5 min in ethanol and then for 5 min in 2.63% sodium hypochlorite. Seed were extensively washed with ethanol, dried, and dispensed onto petri dishes

containing Murashige and Skoog (MS) medium (Duchefa Biochemie B.V., Haarlem, Netherlands), 1% sucrose, and 0.7% agar (plant cell culture-tested) (Sigma-Aldrich, St. Louis), pH 6.5. Plates were sealed with Parafilm to prevent evaporation. *A. thaliana* was transformed as previously described (Zhang et al. 2006) with *Agrobacterium tumefaciens* GV3101 (Holsters et al. 1980).

Transformed T1 plants were selected on MS medium supplemented with 0.7% agar and kanamycin at 50 µg/ml and transferred to soil for seed production. Segregation analysis on plates containing kanamycin at 50 µg/ml was performed on T2 plants. Transgene expression in the single-copy transgenic lines was assessed by RT-qPCR. The transgenic lines with the highest levels of transgene expression were selected and the third (T3) and fourth (T4) generations were produced for these lines with stable transgene inheritance and expression.

Infection assays.

M. incognita nematodes from the Morelos population were reared on greenhouse-grown tomato (*Solanum lycopersicum* 'St. Pierre'). Egg masses were collected from infested tomato roots and eggs were separated from the gelatinous matrix by treatment in 0.5% sodium hypochlorite. The eggs were rinsed with distilled water and the density of the preparation was increased by centrifugation at $1,600 \times g$ for 2 min in a 30% sucrose solution at 4°C. The eggs were washed in distilled water, collected by centrifugation in a microcentrifuge, and stored at -80°C until RNA extraction. Freshly hatched J2 were collected as previously described (Rosso et al. 1999).

For nematode infection assays, *Arabidopsis thaliana* plants were grown in a soil mixture (3:1 mixture of soil and sand). Four-week-old plants were inoculated with 400 J2. Galls and egg masses were counted 8 weeks after inoculation. Statistical analyses were performed by Kruskal-Wallis analysis. Experiments were carried out in duplicate.

For oomycete infection assays, *A. thaliana* seed were sterilized as described in "Plant culture and transformation" and grown in sterile conditions in vitro. Four-week-old plants were inoculated with zoospores from *P. parasitica* 310, as previously described (Attard et al. 2010), except that 500 zoospores were used for inoculation. At least 25 seedlings were analyzed for each experiment. Experiments were carried out in duplicate. Statistical differences were determined by Scheirer-Ray-Hare non-parametric two-way ANOVA for ranked data ($H < 0.05$).

RNA isolation and RT-qPCR analysis.

Selection of transgenic lines. For RT-qPCR, total RNA was extracted from frozen *Arabidopsis* seedlings with TRIzol reagent (Invitrogen). Total RNA was suspended in 100 µl of RNase-free water and treated with RQ1 RNase-free DNase (Promega, Heidelberg, Germany). We added one unit of DNase per microgram of RNA. The first-strand cDNA was synthesized from 1 µg of total RNA, with the Superscript III Reverse Transcriptase Kit (Invitrogen) for gene-specific RT. Real-time RT-qPCR was performed on a Chromo4 System (Bio-Rad, Hercules, CA, U.S.A.) with Opticon 3.1 software, as follows: 95°C for 10 min followed by 44 cycles of 95°C for 15 s and 60°C for 1 min, with the qPCR MasterMix Plus for SYBR green I and fluorescein (Eurogentec, Seraing, Belgium). Each sample reaction was run in duplicate. Cycle threshold (Ct) values were normalized to the Ct value for the housekeeping gene, NADH dehydrogenase (AT1G02020), and the oxidase OXA1 (AT5G62050) was used as a control for normalization. Expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method (Applied Biosystems, Foster City, CA, U.S.A.).

Level of Mi-CRT gene expression in the parasitic stages of *M. incognita*. We collected galls 21 days after the infection of

the transgenic lines overproducing hpMi-CRT. Mi-CRT transcript levels in the feeding nematodes were determined by RT-qPCR on total RNA extracted from galls, evenly distributed over the plant roots, with the primers Cal2RF-qpcr and Cal2RR-qpcr. The *M. incognita* 18S ribosomal cDNA was used as a reference (GenBank accession number U81578) in reactions with gene-specific primers.

For RT-qPCR, total RNA was extracted from frozen galls with TRIzol reagent (Invitrogen). Total RNA was suspended in 50 µl of RNase-free water and treated with RQ1 RNase-free DNase (Promega). RT-qPCR was performed as previously described. Each sample reaction was run in triplicate. Ct values were normalized to the Ct value for the 18S of *M. incognita*. Expression levels were calculated by the $2^{-\Delta\Delta C_t}$ method (Applied Biosystems).

Defense suppression test.

Analysis of defense gene expression. Ten-day-old seedlings were transferred to liquid MS medium with 0.5% sucrose and MES (pH 5.6) at 0.5 g liter⁻¹ and treated with 1 µM elf18 (acetyl-MSKEKFERTKPHVNVGTI). The expression of the defense marker genes *FRK1*, *WRKY33*, *PDF1.2*, *CYP81F2*, *WRKY29*, and *PAD4* was determined by RT-qPCR after 30 min of treatment. The induction of PR-1 was determined after 12 h of treatment. For RT-qPCR, total RNA was extracted from 10 mg of frozen seedlings with TRIzol reagent (Invitrogen). Gene-specific RT and RT-qPCR were done as described above. Each sample reaction was run in triplicate. Ct values were normalized to the Ct value for the *A. thaliana* ubiquitin carboxyl-terminal hydrolase 22. Normalized transcript levels in elicitor-treated samples were compared with normalized transcript levels in samples obtained from plants incubated in the same conditions without elf18, and the fold-changes in expression level were calculated by the $2^{-\Delta\Delta C_t}$ method (Applied Biosystems).

Analysis of callose deposition. Ten-day-old seedlings were transferred to hydroponic liquid MS medium with 0.5% sucrose and MES (pH 5.6) at 0.5 g liter⁻¹. Seedlings were treated with 1 µM elf18 for 24 h. The cotyledons were collected and stained with 0.01% aniline blue in phosphate-buffered saline, pH 7.5. After 15 to 30 min, we viewed at least 20 cotyledons per condition under UV light with an epifluorescence microscope (Microscope Fluo Axioplan II; Carl Zeiss GmbH). Callose deposition was quantified from digital photographs with ImageJ (Abramoff et al. 2004).

ACKNOWLEDGMENTS

We thank B. Favery and M. Quentin (INRA PACA, Sophia-Antipolis, France) for discussions and helpful comments on the manuscript, and C. Zipfel (The Sainsbury Laboratory, John Innes Centre, Norwich, United Kingdom) for fruitful discussions on the role of Mi-CRT in the plant defense suppression. This work was supported by INRA and the University of Nice Sophia-Antipolis and benefited from interactions stimulated by COST Action 872. M. Jaouannet received a grant from INRA and the Region Provence-Alpes-Côte d'Azur.

LITERATURE CITED

- Abramoff, M., Magelhaes, P., and Ram, S. 2004. Image processing with ImageJ. *Biophoton. Int.* 11:36-42.
- Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W., Gomez-Gomez, L., Boller, T., Ausubel, F. M., and Sheen, J. 2002. MAP kinase signalling cascade in *Arabidopsis* innate immunity. *Nature* 415:977-983.
- Aslam, S., Newman, M., Erbs, G., Morrissey, K., Chinchilla, D., Boller, T., Jensen, T., De Castro, C., Ierano, T., Molinaro, A., Jackson, R., Knight, M., and Cooper, R. 2008. Bacterial polysaccharides suppress induced innate immunity by calcium chelation. *Curr. Biol.* 18:1078-1083.
- Attard, A., Gourgues, M., Callemeyn-Torre, N., and Keller, H. 2010. The immediate activation of defense responses in *Arabidopsis* roots is not

- sufficient to prevent *Phytophthora parasitica* infection. *New Phytol.* 187:449-460.
- Baluská, F., Samaj, J., Napier, R., and Volkmann, D. 1999. Maize calreticulin localizes preferentially to plasmodesmata in root apex. *Plant J.* 19:481-488.
- Barcala, M., García, A., Cabrera, J., Casson, S., Lindsey, K., Favory, B., García-Casado, G., Solano, R., Fenoll, C., and Escobar, C. 2010. Early transcriptomic events in microdissected *Arabidopsis* nematode-induced giant cells. *Plant J.* 61:698-712.
- Bednarek, P., Pislewska-Bednarek, M., Svatos, A., Schneider, B., Doubek, J., Mansurova, M., Humphry, M., Consonni, C., Panstruga, R., Sanchez-Vallet, A., Molina, A., and Schulze-Lefert, P. 2009. A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323:101-106.
- Bellafiore, S., Shen, Z., Rosso, M. N., Abad, P., Shih, P., and Briggs, S. P. 2008. Direct identification of the *Meloidogyne incognita* secretome reveals proteins with host cell reprogramming potential. *PLoS Pathog.* 4:e1000192. Online publication.
- Blok, V. C., Jones, J. T., Phillips, M. S., and Trudgill, D. L. 2008. Parasitism genes and host range disparities in biotrophic nematodes: The conundrum of polyphagy versus specialisation. *BioEssays* 30:249-259.
- Caillaud, M. C., Lecomte, P., Jammes, F., Quentin, M., and Pagnotta, S. 2008. MAP65-3 microtubule associated protein is essential for nematode-induced giant cell ontogenesis in *Arabidopsis*. *Plant Cell* 20:423-437.
- Caldo, R. A., Nettleton, D., Peng, J., and Wise, R. P. 2006. Stage-specific suppression of basal defense discriminates barley plants containing fast- and delayed-acting *Mla* powdery mildew resistance alleles. *Mol. Plant-Microbe Interact.* 19:939-947.
- Chen, F., Hayes, P., Mulrooney, D., and Pan, A. 1994. Identification and characterization of cDNA clones encoding plant calreticulin in barley. *Plant Cell* 6:835-843.
- Christensen, A., Svensson, K., Thelin, L., Zhang, W., Tintor, N., Prins, D., Funke, N., Michalak, M., Schulze-Lefert, P., Saijo, Y., Sommarin, M., Widell, S., and Persson, S. 2010. Higher plant calreticulins have acquired specialized functions in *Arabidopsis*. *PLoS One* 5:e11342. Online publication.
- Damiani, I., Baldacci-Cresp, F., Hopkins, J., Andrio, E., Balzergue, S., Lecomte, P., Puppo, A., Abad, P., Favory, B., and Hérouart, D. 2012. Plant genes involved in harbouring symbiotic rhizobia or pathogenic nematodes. *New Phytol.* 194:511-522.
- Davis, E. L., Hussey, R. S., Mitchum, M. G., and Baum, T. J. 2008. Parasitism proteins in nematode-plant interactions. *Curr. Opin. Plant Biol.* 11:360-366.
- Djamei, A., Schipper, K., Rabe, F., Ghosh, A., Vincon, V., Kahnt, J., Osorio, S., Tohge, T., Fernie, A. R., Feussner, I., Feussner, K., Meinicke, P., Stierhof, Y.-D., Schwarz, H., Macek, B., Mann, M., and Kahmann, R. 2011. Metabolic priming by a secreted fungal effector. *Nature* 478:395-398.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11:539-548.
- Doyle, E., and Lambert, K. 2002. Cloning and characterization of an esophageal-gland-specific pectate lyase from the root-knot nematode *Meloidogyne javanica*. *Mol. Plant-Microbe Interact.* 15:549-556.
- Doyle, E., and Lambert, K. 2003. *Meloidogyne javanica* chitinase mutase 1 alters plant cell development. *Mol. Plant-Microbe Interact.* 16:123-131.
- Du, L., Ali, G., Simons, K., Hou, J., and Yang, T. 2009. Ca^{2+} /calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457:1154-1158.
- Dubreuil, G., Magliano, M., Dubrana, M., Lozano, J., Lecomte, P., Favory, B., Abad, P., and Rosso, M. 2009. Tobacco rattle virus mediates gene silencing in a plant parasitic root-knot nematode. *J. Exp. Bot.* 60:4041-4050.
- Göhre, V., and Robatzek, S. 2008. Breaking the barriers: Microbial effector molecules subvert plant immunity. *Annu. Rev. Phytopathol.* 46:189-215.
- Gold, L., Eggleton, P., Sweetwyne, M. T., Van Duyn, L. B., Greives, M. R., Naylor, S. M., Michalak, M., and Murphy-Ullrich, J. E. 2010. Calreticulin: Non-endoplasmic reticulum functions in physiology and disease. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 24:665-683.
- Haasen, D., Köhler, C., Neuhaus, G., and Merkle, T. 1999. Nuclear export of proteins in plants: AtXPO1 is the export receptor for leucine-rich nuclear export signals in *Arabidopsis thaliana*. *Plant J.* 20:695-705.
- Hamamouch, N., Li, C., Seo, P. J., Park, C. M., and Davis, E. L. 2011. Expression of *Arabidopsis* pathogenesis-related genes during nematode infection. *Mol. Plant Pathol.* 12:355-364.
- Hamamouch, N., Li, C., Hewezi, T., Baum, T. J., Mitchum, M. G., Hussey, R. S., Vodkin, L. O., and Davis, E. L. 2012. The interaction of the novel 30C02 cyst nematode effector protein with a plant beta-1,3-endoglucanase may suppress host defence to promote parasitism. *J. Exp. Bot.* 63:3683-3695.
- Hématy, K., Cherk, C., and Somerville, S. 2009. Host-pathogen warfare at the plant cell wall. *Curr. Opin. Plant Biol.* 12:406-413.
- Hewezi, T., Howe, P. J., Maier, T. R., Hussey, R. S., Mitchum, M. G., Davis, E. L., and Baum, T. J. 2010. *Arabidopsis* spermidine synthase is targeted by an effector protein of the cyst nematode *Heterodera schachtii*. *Plant Physiol.* 152:968-984.
- Holsters, M., Silva, B., Van Vliet, F., Genetello, C., De Block, M., Dhaese, P., Depicker, A., Inzé, D., Engler, G., Villarreal, R., Van Montagu, M., and Schell, J. 1980. The functional organization of the nopaline *A. tumefaciens* plasmid pTiC58. *Plasmid* 3:212-230.
- Hussey, R. S. 1989. Disease-inducing secretions of plant parasitic nematodes. *Annu. Rev. Phytopathol.* 27:123-141.
- Jammes, F., Lecomte, P., de Almeida-Engler, J., Bitton, F., and Martin-Magniette, M. L. 2005. Genome-wide expression profiling of the host response to root-knot nematode infection in *Arabidopsis*. *Plant J.* 44:447-458.
- Jaouannet, M., Perfus-Barbeoch, L., Deleury, E., Magliano, M., Engler, G., Vieira, P., Danchin, E. G. J., Da Rocha, M., Coquillard, P., Abad, P., and Rosso, M. N. 2012. A root-knot nematode-secreted protein is injected into giant cells and targeted to the nuclei. *New Phytol.* 194:924-931.
- Jaubert, S., Laffaire, J. B., Pottier, C., Abad, P., Rosso, M. N., and Ledger, T. N. 2002. Direct identification of stylet secreted proteins from root-knot nematodes by a proteomic approach. *Mol. Biochem. Parasitol.* 121:205-211.
- Jaubert, S., Milac, A. L., Petrescu, A. J., de Almeida-Engler, J., Abad, P., and Rosso, M. N. 2005. In planta secretion of a calreticulin by migratory and sedentary stages of root-knot nematode. *Mol. Plant-Microbe Interact.* 18:1277-1284.
- Jia, X. Y., He, L. H., Jing, R. L., and Li, R. Z. 2009. Calreticulin: Conserved protein and diverse functions in plants. *Physiol. Plant.* 136:127-138.
- Jones, M. G. K., and Payne, H. L. 1978. Early stages of nematode-induced giant cell formation in roots of *Impatiens balsamina*. *J. Nematol.* 10:70-84.
- Karimi, M., Inzé, D., and Depicker, A. 2002. GATEWAYTM vectors for *Agrobacterium*-mediated plant transformation. *Trends Plant Sci.* 7:193-195.
- Kim, J. G., Li, X., Roden, J. A., Taylor, K. W., Aakre, C. D., Su, B., Lalonde, S., Kirik, A., Chen, Y., Baranage, G., McLane, H., Martin, G. B., and Mudgett, M. B. 2009. *Xanthomonas* T3S effector XopN suppresses PAMP-triggered immunity and interacts with a tomato atypical receptor-like kinase and TGT1. *Plant Cell* 21:1305-1323.
- Kyndt, T., Nahar, K., Haegeman, A., De Vleeschauwer, D., Höfte, M., and Gheysen, G. 2011. Comparing systemic defence-related gene expression changes upon migratory and sedentary nematode attack in rice. *Plant Biol. (Stuttg.)* 14:73-82.
- Li, J., Zhao-Hui, C., Batoux, M., Nekrasov, V., Roux, M., Chinchilla, D., Zipfel, C., and Jones, J. D. G. 2009. Specific ER quality control components required for biogenesis of the plant innate immune receptor EFR. *Proc. Natl. Acad. Sci. U.S.A.* 106:15973-15978.
- Mang, H. G., Laluk, K. A., Parsons, E. P., Kosma, D. K., Cooper, B. R., Park, H. C., AbuQamar, S., Bocconcelli, C., Miyazaki, S., Consiglio, F., Chilosi, G., Bohnert, H. J., Bressan, R. A., Mengiste, T., and Jenks, M. A. 2009. The *Arabidopsis* RESURRECTION1 gene regulates a novel antagonistic interaction in plant defense to biotrophs and necrotrophs. *Plant Physiol.* 151:290-305.
- Michalak, M., Groenendyk, J., Szabo, E., Gold, L. I., and Opas, M. 2009. Calreticulin, a multi-process calcium-buffering chaperone of the endoplasmic reticulum. *Biochem. J.* 417:651-666.
- Nahar, K., Kyndt, T., De Vleeschauwer, D., Höfte, M., and Gheysen, G. 2011. The jasmonate pathway is a key player in systemically induced defense against root-knot nematodes in rice. *Plant Physiol.* 157:305-316.
- Napier, R. M., Treman, S., Henderson, J., Boyce, J. M., Hawes, C., Fricker, M. D., and Venis, M. A. 1995. Purification, sequencing and functions of calreticulin from maize. *J. Exp. Bot.* 46:1603-1613.
- Naresha, S., Suryawanshi, A., Agarwal, M., Singh, B. P., and Joshi, P. 2009. Mapping the complement C1q binding site in *Haemonchus contortus* calreticulin. *Mol. Biochem. Parasitol.* 166:42-46.
- Nelson, B. K., Cai, X., and Nebenführ, A. 2007. A multicolored set of in vivo organelle markers for co-localization studies in *Arabidopsis* and other plants. *Plant J.* 51:1126-1136.
- Patel, N., Hamamouch, N., Li, C., Hewezi, T., Hussey, R. S., Baum, T. J., Mitchum, M. G., and Davis, E. L. 2010. A nematode effector protein similar to annexins in host plants. *J. Exp. Bot.* 61:235-248.

- Qiu, Y., Xi, J., Du, L., Roje, S., and Poovaiah, B. W. 2012. A dual regulatory role of *Arabidopsis* calreticulin-2 in plant innate immunity. *Plant J.* 69:489-500.
- Ramírez, G., Valck, C., Ferreira, V. P., López, N., and Ferreira, A. 2011. Extracellular *Trypanosoma cruzi* calreticulin in the host-parasite interplay. *Trends Parasitol.* 27:115-122.
- Rehman, S., Postma, W., Tytgat, T., Prins, P., Qin, L., Overmars, H., Vossen, J., Spiridon, L. N., Petrescu, A. J., Govers, A., Bakker, J., and Smant, G. 2009. A secreted SPRY domain-containing protein (SPRYSEC) from the plant-parasitic nematode *Globodera rostochiensis* interacts with a CC-NB-LRR protein from a susceptible tomato. *Mol. Plant-Microbe Interact.* 22:330-340.
- Rosso, M. N., Favery, B., Piotte, C., Arthaud, L., De Boer, J. M., Hussey, R. S., Bakker, J., Baum, T. J., and Abad, P. 1999. Isolation of a cDNA encoding a beta-1,4-endoglucanase in the root-knot nematode *Meloidogyne incognita* and expression analysis during plant parasitism. *Mol. Plant-Microbe Interact.* 12:585-591.
- Rosso, M. N., Hussey, R. S., Davis, E. L., Smant, G., Baum, T., Abad, P., and Mitchum, M. G. 2011. Nematode effector proteins: Targets and functions in plant parasitism. Pages 327-354 in: *Effectors in Plant-Microbe Interactions*. F. Martin and S. Kamoun, eds. Wiley-Blackwell, Oxford.
- Samaj, J., Salaj, J., Obert, B., Baluska, F., Menzel, D., and Volkmann, D. 2008. Calreticulin mRNA and protein are localized to protein bodies in storage maize callus cells. *Plant Cell Rep.* 27:231-239.
- Sana, T., Fischer, S., Wohlgemuth, G., Katrekar, A., Jung, K., Ronald, P. C., and Fiehn, O. 2010. Metabolomic and transcriptomic analysis of the rice response to the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*. *Metabolomics* 6:451-465.
- Schwessinger, B., and Zipfel, C. 2008. News from the frontline: Recent insights into PAMP-triggered immunity in plants. *Curr. Opin. Plant Biol.* 11:389-395.
- Sharma, A., Isogai, M., Yamamoto, T., Sakaguchi, K., Hashimoto, J., and Komatsu, S. 2004. A novel interaction between calreticulin and ubiquitin-like nuclear protein in rice. *Plant Cell Physiol.* 45:684-692.
- Valck, C., Ramírez, G., López, N., Ribeiro, C. H., Maldonado, I., Sánchez, G., Ferreira, V. P., Schwaebler, W., and Ferreira, A. 2010. Molecular mechanisms involved in the inactivation of the first component of human complement by *Trypanosoma cruzi* calreticulin. *Mol. Immunol.* 47:1516-1521.
- Vieira, P., Danchin, E. G. J., Neveu, C., Crozat, C., Jaubert, S., Hussey, R. S., Engler, G., Abad, P., de Almeida-Engler, J., Castagnone-Sereno, P., and Rosso, M.-N. 2011. The plant apoplast is an important recipient compartment for nematode secreted proteins. *J. Exp. Bot.* 62:1241-1253.
- Wiggers, R. J., Starr, J. L., and Price, H. J. 1990. DNA content and variation in chromosome number in plant cells affected by *Meloidogyne incognita* and *M. arenaria*. *Phytopathology* 80:1391-1395.
- Wubben, M. J., Jin, J., and Baum, T. J. 2008. Cyst nematode parasitism of *Arabidopsis thaliana* is inhibited by salicylic acid (SA) and elicits uncoupled SA-independent pathogenesis related gene expression in roots. *Mol. Plant-Microbe Interact.* 21:424-432.
- Zhang, X., Henriques, R., Lin, S. S., Niu, Q. W., and Chua, N. H. 2006. *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. *Nat. Protoc.* 1:641-646.
- Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., and Jones, J. D. G. 2004. Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 428:764-767.