A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants

Shahid Siddique[a], Zoran S. Radakovic[a], Carola M. De La Torre[b,1], Demosthenis Chronis[b,1,2], Ondřej Novák[c], Eswarayya Ramireddy[d], Julia Holbein[a], Christiane Matera[a], Marion Hütten[a], Philipp Gutbrod[a], Muhammad Shahzad Anjam[a], Elzbieta Rozanska[e], Samer Habash[a], Abdelnaser Elashry[a], Miroslaw Sobczak[e], Tatsuo Kakimoto[f], Miroslav Strnad[c], Thomas Schmülling[d], Melissa G. Mitchum[b], and Florian M. W. Grundler[a,3]

[a]Rheinische Friedrich-Wilhelms-University of Bonn, Department of Molecular Phytomedicine, D-53115 Bonn, Germany; [b]Division of Plant Sciences and Bond Life Sciences Center, University of Missouri, Columbia, MO 65211; [c]Laboratory of Growth Regulators, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University and Institute of Experimental Botany Academy of Sciences of the Czech Republic, CZ-78371 Olomouc, Czech Republic; [d]Institute of Biology/Applied Genetics, Dahlem Centre of Plant Sciences, Freie Universität Berlin, D-14195 Berlin, Germany; [e]Department of Botany, Warsaw University of Life Sciences, PL-02787 Warsaw, Poland; and [f]Department of Biology, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

Edited by Paul Schulze-Lefert, Max Planck Institute for Plant Breeding Research, Cologne, Germany, and approved August 26, 2015 (received for review February 21, 2015)

Sedentary plant-parasitic cyst nematodes are biotrophs that cause significant losses in agriculture. Parasitism is based on modifications of host root cells that lead to the formation of a hypermetabolic feeding site (a syncytium) from which nematodes withdraw nutrients. The host cell cycle is activated in an initial cell selected by the nematode for feeding, followed by activation of neighboring cells and subsequent expansion of feeding site through fusion of hundreds of cells. It is generally assumed that nematodes manipulate production and signaling of the plant hormone cytokinin to activate cell division. In fact, nematodes have been shown to produce cytokinin in vitro; however, whether the hormone is secreted into host plants and plays a role in parasitism remained unknown. Here, we analyzed the spatiotemporal activation of cytokinin signaling during interaction between the cyst nematode, *Heterodera schachtii,* and *Arabidopsis* using cytokinin-responsive promoter:reporter lines. Our results showed that cytokinin signaling is activated not only in the syncytium but also in neighboring cells to be incorporated into syncytium. An analysis of nematode infection on mutants that are deficient in cytokinin or cytokinin signaling revealed a significant decrease in susceptibility of these plants to nematodes. Further, we identified a cytokinin-synthesizing isopentenyltransferase gene in *H. schachtii* and show that silencing of this gene in nematodes leads to a significant decrease in virulence due to a reduced expansion of feeding sites. Our findings demonstrate the ability of a plant-parasitic nematode to synthesize a functional plant hormone to manipulate the host system and establish a long-term parasitic interaction.

Arabidopsis thaliana | cell cycle | cytokinin | cyst nematode | IPT

Plant-parasitic nematodes are a significant threat to almost all economically important crops. International surveys revealed an average annual crop yield loss of more than 10% due to nematode infestation and up to 20% for certain crops, e.g., bananas (1). Most of this damage is caused by the sedentary root-knot (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.). Infective second-stage juveniles (J2) of both root-knot nematodes (RKNs) and cyst nematodes invade plant roots near the tip and move toward the vascular cylinder. On reaching the vascular cylinder, RKNs induce the formation of several giant cells, whereas cyst nematodes induce the formation of a syncytium. These feeding sites serve as the nematode's sole source of nutrients throughout its life cycle for several weeks. Cyst nematodes are dimorphic, but the mechanism of sex determination is not clearly understood. It has, nonetheless, been observed that the environment strongly influences the outcome of the sex ratio in cyst nematodes. Under favorable conditions with plenty of nutrients, the majority of juveniles develop into females. However, when the juveniles are exposed to adverse conditions, as seen in resistant plants, the percentage of males increases considerably.

The mechanism by which nematodes transform root cells into highly active feeding sites is not clearly understood. One of the first events induced by both RKNs and cyst nematodes during feeding site development is cell cycle activation (2–5). The feeding cells induced by RKNs hypertrophy and contain multiple enlarged nuclei. The multinucleation occurs as a result of repeated cycles of mitosis and endo-reduplication. In contrast, the syncytium forms through the local dissolution of cell walls accompanied by the hypertrophy of incorporated cells and the division of neighboring cells (2, 5). An expression analysis of key cell cycle genes and cell cycle inhibitors demonstrated that mitosis is also essential for syncytium development (5).

Cytokinin is an adenine derivative carrying an isoprenoid or aromatic side chain that regulate numerous developmental and physiological responses in plants, including cell division, sink

Significance

Sedentary plant-parasitic cyst nematodes are microscopic roundworms that cause significant yield losses in agriculture. Successful parasitism is based on the formation of a hypermetabolic feeding site in host roots from which the nematodes withdraw their nutrients. The host cell cycle is activated at the site of infection and contributes to the formation of the syncytium. Here, we provide genetic evidence that nematode-derived cytokinin is involved in activating the host cell cycle during infection. Our findings show the ability of an animal to synthesize and secrete a functional plant hormone to establish long-term parasitism.

Author contributions: S.S., M.G.M., and F.M.W.G. designed research; S.S., Z.S.R., C.M.D.L.T., D.C., O.N., J.H., C.M., M.H., P.G., M.S.A., E. Rozanska, S.H., A.E., M. Sobczak, T.K., M. Strnad, and M.G.M. performed research; E. Ramireddy and T.S. contributed new reagents/analytic tools; S.S., Z.S.R., C.M.D.L.T., D.C., O.N., A.E., M. Sobczak, M. Strnad, T.S., and M.G.M. analyzed data; and S.S., M. Sobczak, T.S., M.G.M., and F.M.W.G. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. KT717690 and KT726964).

[1]C.M.D.L.T. and D.C. contributed equally to this work.

[2]Present address: Department of Plant Pathology and Plant-Microbe Biology, Cornell University, Ithaca, NY 14853.

[3]To whom correspondence should be addressed. Email: grundler@uni-bonn.de.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1503657112/-/DCSupplemental.

tissue establishment, root growth, branching, senescence, seed germination, and stress responses (6, 7). Plants have two classes of isopentenyltransferase (IPT) enzymes that act on adenine: ATP/ADP-IPTs and tRNA-IPTs. In *Arabidopsis*, ATP/ADP-IPTs are encoded by seven genes (*AtIPT1*, *AtIPT3*, *AtIPT4*, *AtIPT5*, *AtIPT6*, *AtIPT7*, and *AtIPT8*) and contribute to the bulk of cytokinin synthesis. tRNA-IPTs are encoded by two genes (*AtIPT2* and *AtIPT9*), and they are primarily responsible for producing the *c*Z-type cytokinin (8). Cytokinin signaling is mediated by a complex two-component system in which the signal is perceived by membrane-located histidine kinases (AHKs) and transmitted to the nucleus through a phosphorelay system via phosphotransmitter proteins. Inside the nucleus, transcription factors known as type B response regulators (ARRs) become activated and induce the transcription of cytokinin target genes. In *Arabidopsis thaliana*, cytokinin receptor kinases are encoded by three genes: *AHK2*, *AHK3,* and *CRE1/AHK4*. *AHK4* is predominantly expressed in roots, whereas *AHK2* and *AHK3* are more abundant in aboveground plant parts (9–12).

A few studies have explored the role of cytokinin in plant interactions with the RKN *Meloidogyne incognita* (13–15). However, the mechanism underlying their role is still not well understood. Nevertheless, because giant-cell formation involves the reentry of plant cells into the cell cycle, the role of cytokinin in this aspect of the plant-nematode interaction has long been suspected. Similarly, the ability of cytokinin to mobilize nutrients and delay senescence led to the hypothesis that cytokinin may also be involved in the maintenance and function of nematode feeding sites as sinks (16, 17). It has also been speculated that nematodes produce cytokinin that might be injected into plants to establish parasitism (18). In contrast to RKNs, no studies have investigated the involvement of cytokinin in cyst nematode feeding site development. In the present study, we investigated the contribution of cytokinin to the formation of the syncytium induced by *Heterodera schachtii* in *Arabidopsis*.

Results

Cytokinin Signaling Is Activated at Nematode Feeding Sites. To assess whether cytokinin signaling is activated in *Arabidopsis* in response to sugar beet cyst nematode infection, we used the recently described *Two Component signaling Sensor new:Green Fluorescent Protein* (*TCSn*:*GFP*), which reflects the activity of type B response regulators (19). *TCSn*:*GFP* plants were grown in 35-mm glass bottom microwell dishes and infected with J2 nematodes of *H. schachtii,* and the fluorescence of *GFP* was analyzed in a time-course analysis at 4, 6, and 10 d after inoculation (dai) representing J2, J3, and J4 stages of nematode development, respectively. We observed a strong GFP signal in the developing feeding sites at all time points, which extended into immediately surrounding cells (Fig. 1*A*), indicating that cytokinin signaling is specifically and early activated during the plant-nematode interaction.

To investigate which of the three receptors (AHK2, AHK3, AHK4) mediates cytokinin signaling during nematode infection, we analyzed the activity of the cytokinin-sensitive marker construct *ARR5*:*GUS* in Col-0 and in three different double receptor mutant backgrounds (*ahk2/3, ahk2/4*, and *ahk3/4*) (10, 20). We found that a majority of root infection zones (~70%) exhibited GUS staining at 1 dai in Col-0 (*SI Appendix 1*, Fig. S1). A similar strong GUS expression was detected in almost all syncytia and neighboring areas at 3, 5, and 10 dai (Fig. 1*B* and *SI Appendix 1*, Figs. S1 and S2). In comparison with Col-0, there was a reduction in the number of positively stained infection sites in *ahk2/4* lines at all time points examined (*SI Appendix 1*, Fig. S1). However, the strongest reduction was observed in *ahk3/4* lines, where GUS expression was absent (Fig. 1*B* and *SI Appendix 1*, Figs. S1 and S2). The expression of *ARR5:GUS* in uninfected control roots was the same as described before (*SI Appendix 1*, Fig. S3) (21). These results suggest that cytokinin signaling is activated during the early stages of feeding site development and that this activation is mainly mediated by AHK3 and AHK4.

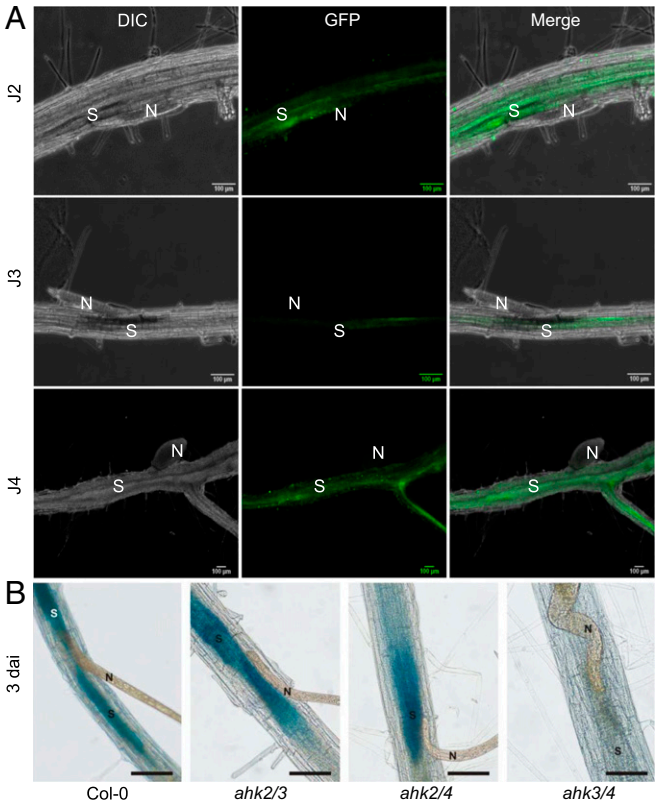

Fig. 1. Activation of cytokinin signaling in *Arabidopsis* roots on nematode infection. (*A*) Cytokinin-dependent expression of *TCSn:GFP* in Col-0 plants on *H. schachtii* infection at 4 (J2), 6 (J3), and 10 (J4) dai, respectively. (*Left*) Differential interference contrast (DIC). (*Center*) GFP. (*Right*) Merged image of DIC and GFP. (*B*) Cytokinin-dependent expression of *ARR5:GUS* in *Arabidopsis* roots on *H. schachtii* infection at 3 dai. N, nematode; S, syncytium. (Scale bar, 100 μm.)

To determine whether cytokinin AHK receptors are expressed in syncytia, previously described transgenic *Arabidopsis AHK2:GUS*, *AHK3:GUS*, and *AHK4:GUS* reporter lines were analyzed in response to *H. schachtii* infection at 3, 5, and 14 dai (11). Infected *AHK2:GUS* seedlings showed weak GUS activity at the feeding site as early as 3 dai, which gradually declined as the parasitic nematodes reached third (J3) and fourth (J4) developmental stages at 5 and 14 dai, respectively (*SI Appendix 1*, Fig. S4). *AHK3:GUS* and *AHK4:GUS* seedlings showed *GUS* expression in syncytia at 3 and 5 dai (*SI Appendix 1*, Fig. S4), with reduced levels by 14 dai, as the nematodes reached the fourth juvenile stage (J4) (*SI Appendix 1*, Fig. S4).

Cytokinin Signaling Is Required for Nematode Development. To characterize the role of cytokinin in cyst nematode parasitism, we screened loss-of-function cytokinin receptor double mutants (*ahk2/3*, *ahk2/4*, and *ahk3/4*) in nematode infection assays (10). Plants were grown and infected with J2 nematodes, and the number of males and females was counted at 14 dai. Similarly, cysts were counted at 30 dai. We found no significant difference in the number of females per plant at 14 dai between Col-0, *ahk2/3* and *ahk2/4*. However, it was significantly decreased in *ahk3/4* at 14 dai compared with Col-0 plants (Fig. 2*A*). On the other hand, the average number of cysts was significantly decreased in all three mutants compared with Col-0 (*SI Appendix 1*, Fig. S5). There was also a significant decrease in the average size of female nematodes in *ahk3/4* plants, whereas this decrease did not occur in *ahk2/3* or *ahk2/4* plants (Fig. 2*B*). Moreover, the average size of the female-associated syncytia was significantly decreased in all three mutants compared with Col-0 plants (Fig. 2*C*). Thus, AHK-mediated

cytokinin signaling is activated during parasitism and is required for cyst nematode development and syncytium formation.

Cytokinin Degradation Leads to a Decrease in Nematode Susceptibility. Although the infection assays using the double-receptor mutants showed a decrease in female numbers, size, and associated syncytium size, one active receptor was still present in these plants, which might compensate for the loss of gene function. This finding is consistent with the *AHK:GUS* analysis showing expression of all three receptors at nematode feeding sites. Triple receptor KO mutants are extremely dwarf, precluding any meaningful analysis (10). Therefore, we examined transgenic lines overexpressing four different cytokinin oxidase genes (*CKXs*) driven by a constitutively expressed CaMV 35S promoter (*35S:CKX1*, *35S:CKX2*, *35S:CKX3*, and *35S:CKX4*). A transgenic line overexpressing the *CKX3* gene driven by a root-specific promoter (*PYK10:CKX3*) was also used (22, 23). These lines were subjected to the nematode infection assay, and we found a significant decrease in the susceptibility of various *CKX* lines compared with Col-0 (Fig. 2 *D–F* and *SI Appendix 1*, Fig. S6), supporting the idea that a threshold level of cytokinin is critical for nematode infection.

Cell Cycle Activation and Progression at Nematode Feeding Sites Is Mediated by Cytokinin. Cytokinin is a key regulator of cell cycle activation and progression. It has been previously shown that the cell cycle is activated during nematode infections, which contributes to the development of syncytia and galls (4). We therefore hypothesized that impairment in cytokinin signaling might influence the division of cells in and around the nematode feeding sites. To investigate this hypothesis, we examined the expression of the *cyclin B1;1* gene (*CycB1;1*), which is activated during G2 phase of the cell cycle and reaches its maximum at the G2-to-M phase transition. Therefore, *CycB1;1* expression occurs only in dividing cells.

Previously described *CycB1;1:GUS* lines as a marker of cell division were used to monitor cell cycle activation in response to *H. schachtii* infection via a time course analysis at 1, 3, 5, and 10 dai (24). No staining was detected at the infection region in *CycB1;1:GUS* transformed Col-0 roots at 1 dai. However, the majority of the infection sites showed positive GUS staining (~60%) at 3 dai. The number of GUS-positive infection sites increased to ~75% at 5 dai (*SI Appendix 1*, Figs. S7 and S8). We found that staining was not only present at the edges of developing syncytia; but the entire feeding site was also stained. These results confirm previous observations that cells around the syncytium actively divide to become incorporated into the expanding syncytium (4, 25). To determine whether this cell division was mediated by cytokinin, we introgressed the *CycB1;1:GUS* reporter line (24) into the cytokinin double receptor mutants. There was no change in the frequency or intensity of GUS staining in the *CycB1;1:GUS* transformed *ahk2/3* lines; however, both the intensity and occurrence were decreased in *CycB1;1:GUS* transformed *ahk2/4* and *ahk3/4* lines (*SI Appendix 1*, Figs. S7 and S8).

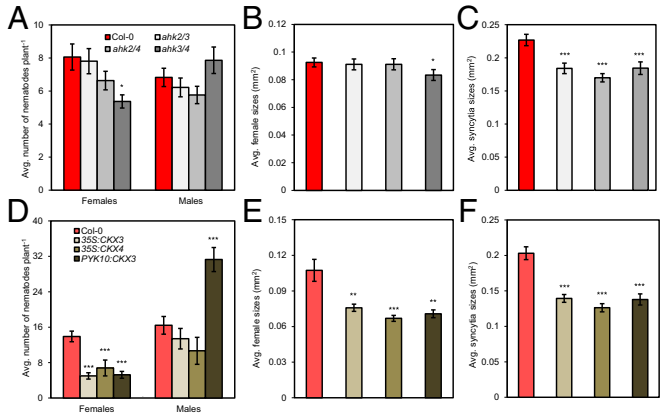

Fig. 2. Nematode infection assays in cytokinin receptor mutants and cytokinin oxidase overexpressing lines. (*A*) Average number of nematodes per plant present in Col-0 and *ahk* mutant lines at 14 dai. (*B* and *C*) Average sizes of female nematodes (*B*) and plant syncytia (*C*) in Col-0, *ahk* mutant lines at 14 dai. (*D*) Average number of nematodes per plant present in Col-0 and *CKX* lines at 14 dai. (*E* and *F*) Average sizes of female nematodes (*E*) and plant syncytia (*F*) in Col-0 and *CKX* lines at 14 dai. Bars represent mean ± SE for three independent biological replicates. Data were analyzed using single-factor ANOVA ($P < 0.05$) and Dunnett post hoc test. Asterisks represent statistically significant difference to corresponding Col-0. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

The Host Plant Is a Source of Cytokinin for Feeding Site Expansion. Our results to this point indicated that cytokinin signaling activates the host cell cycle during nematode infection and that this activation is vital for the expansion of the syncytium. However, it was not clear whether the host plants produced the cytokinin or if they were secreted by the nematodes. To clarify the source of the cytokinin during nematode infection, we infected *Arabidopsis* multiple-KO mutants of members of the ATP/ADP-IPT (*ipt3/5/7*) and tRNA-IPT (*ipt2/9*) gene families with *H. schachtii* (8). Our infection assay results showed that the average number of nematodes and the average size of females did not significantly differ between *ipt3/5/7, ipt2/9*, and Col-0 plants (Fig. 3 *A*, *B*, and *D*). However, the average size of syncytia was significantly reduced in *ipt3/5/7* compared with Col-0 plants (Fig. 3*C*). These results suggest that the cytokinin involved in the expansion of the nematode feeding site is partially produced by the host plants.

The Cytokinin Content of Plant Tissues Increases on Infection. To quantify and classify the cytokinin involved in nematode and syncytium development, root segments containing syncytia at 5 and 10 dai were dissected from Col-0, *ipt3/5/7*, and *PYK10:CKX3* plants, and their cytokinin content was compared (8). Obtained data showed that in Col-0 the total cytokinin, content increased significantly at 5 dai compared with control roots. There was a further sevenfold increase in the total cytokinin in root segments containing syncytia at 10 dai compared with 5 dai (Fig. 3*E*). A detailed look showed that the total cytokinin consisted of ~50% *tZ*-type, 25% *iP*-type, and 25% c*Z*+*DHZ*-type in all analyzed tissues (*SI Appendix 1*, Fig. S10). The same trend was observed for the levels of free bases *tZ*, *iP*, and *cZ*. However, the levels of *DHZ* were below the detection limit (*SI Appendix 1*, Fig. S10). We also found that, although the *ipt3/5/7* lines exhibited the lowest amount of total cytokinin in uninfected roots, the amount of cytokinin on infection was the lowest in the syncytia developing in the roots of *PYK10:CKX3* line compared with those in Col-0 and *ipt3/5/7* plants (Fig. 3*E* and Dataset S1). These data confirmed the results of our infection assays, which suggested that the decrease in susceptibility to nematodes was much more pronounced in *PYK10:CKX3* compared with *ipt3/5/7* plants (Figs. 2 *D–F* and 3*E*). Noteworthy, the concentrations

AGRICULTURAL SCIENCES

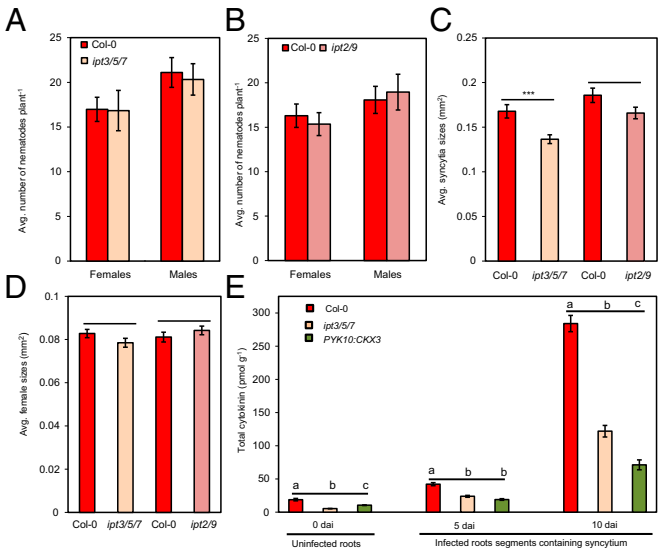

Fig. 3. Nematode infection assays in *ipt* mutant lines and cytokinin quantification in root segments. (*A* and *B*) Average number of nematodes present in Col-0, *ipt3/5/7* (*A*) and *ipt2/9* (*B*) plants at 14 dai. (*C* and *D*) Average sizes of plant syncytia (*C*) and female (*D*) at 14 dai. (*A*–*D*) Bars represent mean ± SE for 3 independent biological replicates. Data were analyzed using single-factor ANOVA ($P < 0.05$) and Dunnett post hoc test. Asterisks represent statistically significant difference to corresponding Col-0. ***$P < 0.001$. (*E*) Total cytokinin contents in uninfected roots, root segments containing syncytia at 5 or 10 dai in Col-0, *ipt3/5/7*, and *PYK10:CKX3* lines. Data were analyzed using single-factor ANOVA ($P < 0.05$) and Fisher's LSD post hoc test. Bars represent mean of three independent experiments ± SE. Values that do not share same letter are significantly different from each other.

of free bases (*tZ* and *iP*) were strongly decreased in *ipt3/5/7* plants compared with Col-0 in uninfected roots and at 10 dai. This decrease was not observed at 5 dai when the levels of one of the free bases (*iP*) were even significantly increased in *ipt3/5/7* (*SI Appendix 1*, Fig. S10). These observations support the findings of previous studies suggesting that cytokinin may be produced and released by nematodes into the host plants especially during the early stages of infection (13, 18). To confirm these observations, we measured the cytokinin content of nematodes and found that cytokinin was present in the preinfective J2 of *H. schachtii* (*SI Appendix 1*, Fig. S11 and Dataset S1). The total cytokinin in *H. schachtii* consists of ∼90% of *iP*-type cytokinin, which is consistent with a functional role in parasitism (*SI Appendix 1*, Fig. S11).

Silencing *HsIPT* Disrupts the Infection Process. To determine whether nematodes contribute to the levels of cytokinin detected at the infection site, we used the *tRNA-IPT* sequence from *Caenorhabditis elegans* to identify an ortholog in the recently annotated *H. schachtii* transcriptome. We identified a single gene in *H. schachtii* (*HsIPT*) encoding a tRNA-IPT (*SI Appendix 1*, Fig. S12). We were able to access the *Heterodera glycines* draft genome for further analysis and identified one putative full-length and two partial IPT protein sequences suggesting that *HgIPT* belongs to a small gene family (*SI Appendix, Experimental Procedures*). An alignment between *HgIPT* on scaffold 40 and the *HsIPT* sequence identified a high percentage similarity between the sequences (*SI Appendix 1*, Fig. S13*A*).

A blast against the Affymetrix Soybean GeneChip Consensus Probe database (www.affymetrix.com) identified two probesets for *HgIPT*: HgAffx.20363.1.S1_at and HgAffx.23529.1.S1_at. We then searched the microarray dataset generated previously to determine the expression profile of *HgIPT* throughout *H. glycines* development (26). These two probesets fell into developmental expression clusters 1 and 4 (*SI Appendix 1*, Fig. S13*B*), indicating that expression of this gene is highest during the early stages of infection and declines as the nematode proceeds through its life cycle, which is consistent with a role for nematode-secreted cytokinin during the initial establishment of feeding sites.

Further, we experimentally tested the potential of *HsIPT* as a source of cytokinin during the plant-nematode interaction. To this end, we performed in vitro RNAi targeting of *HsIPT*. Our results showed that RNAi caused a significant decrease in transcript abundance of the *HsIPT* gene in J2 of *H. schachtii* (*SI Appendix 1*, Fig. S14*A*). We then asked whether targeting of *HsIPT* by RNAi influenced the survival and infectivity of J2 nematodes. To test viability, we treated J2 nematodes with dsRNA targeted against *GFP* or *IPT*. These nematodes were incubated in water and monitored for survival over the course of 30 d. The survival of the nematodes measured by the percentage of live nematodes did not vary significantly between dsRNA-*IPT*– and dsRNA-*GFP*–treated nematodes. To test infectivity, we assessed whether there were defects in syncytium establishment by monitoring the cessation of stylet movements for nematodes that invaded the roots 4 h after inoculation. We did not see any difference in the syncytial cells establishment between dsRNA-*GFP*– and dsRNA-*IPT*–treated J2 nematodes (*SI Appendix 1*, Fig. S14 *B* and *C*).

We infected the *ARR5:GUS* and *CycB1;1:GUS* transgenic *Arabidopsis* plants with J2s soaked in dsRNA targeting *GFP* or *IPT* and performed GUS staining at 1, 3, 5, and 10 dai to analyze whether silencing of the *IPT* gene interferes with the expression of *ARR5:GUS* and *CycB1;1:GUS* on nematode infection. The results showed that the intensity, as well as the number of GUS-positive infection sites, decreased at various time points in plants infected with J2 nematodes soaked in dsRNA targeting *HsIPT* compared with *GFP*-treated nematodes (*SI Appendix 1*, Figs. S15 and S16). These results strongly suggest that *HsIPT* is involved in producing cytokinin that is secreted into host plants to activate cytokinin signaling and, as a consequence, the cell cycle required for successful parasitism.

Next, we used in situ hybridization to localize *HsIPT* transcripts in J2 of *H. schachtii*. The antisense riboprobe of *HsIPT* labeled with digoxigenin hybridized in the entire nematode body; however, the signal was particularly strong in the dorsal esophageal gland cells of *H. schachtii* (Fig. 4*A*). We infected *Arabidopsis* Col-0 with nematodes soaked in dsRNA directed against *GFP* or *HsIPT* and counted the number of developed nematodes at 14 dai. There was a significant decrease in the number of female nematodes found on plants infected with J2 nematodes soaked in dsRNA targeting *IPT* compared with *GFP* (Fig. 4 *B* and *C*). Similarly, the average size of the females and the size of the syncytia were significantly reduced (Fig. 4 *D* and *E*). We evaluated whether the silencing of *HsIPT* also had an effect on sugar beet infection. Infection assays showed that silencing of *HsIPT* caused no change in the size of female nematodes but the number of females and the size of the syncytium were reduced in a pattern resembling that of *Arabidopsis* (*SI Appendix 1*, Fig. S16 *A*–*C*).

To analyze whether silencing of *HsIPT* reduces the cytokinin levels in nematodes, we treated J2 of *H. schachtii* with dsRNA against *GFP* or *IPT* and measured the level of cytokinin. A very strong and significant decrease (60%) in the concentration of free base *iP* (one of the major active forms of cytokinin in the infection zone) occurred in nematodes treated with dsRNA against *IPT* compared with *GFP*. However, there was no significant decrease in the total cytokinin content of J2 nematodes irrespective of the treatment (Fig. 4*F* and Dataset S2). These data support our idea that the *HsIPT* gene is involved in the production of cytokinin in nematodes.

Discussion

The role of cytokinin in the formation of galls induced by RKN on plant roots has been demonstrated previously (15). It was found that *Lotus japonicus* plants expressing *Arabidopsis ARR5:GUS* showed little or no activation of cytokinin signaling during penetration and migration of J2s of RKN inside roots. However, a high level of *GUS* expression was observed once the nematode reached the vascular cylinder. Moreover, overexpression of *CKX* genes rendered hairy roots resistant to nematode infection (15).

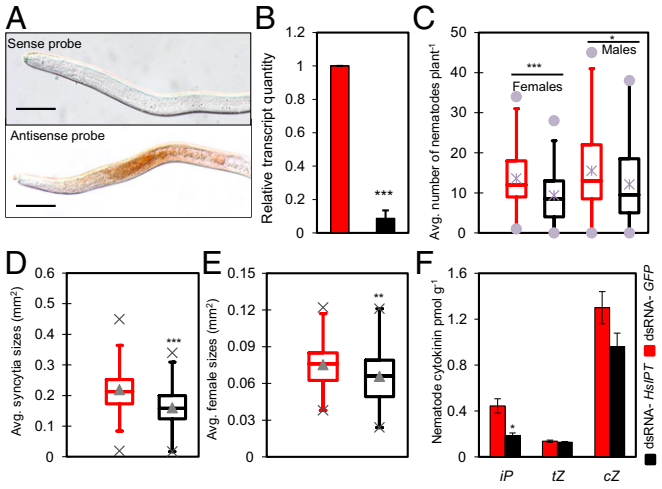

Fig. 4. Effect of *HsIPT* knockdown on nematode infection. (*A*) In situ hybridization of the *HsIPT* gene in J2 of *H. schachtii*. (Scale bar, 40 μm.) (*B*) Change in transcript abundance of *HsIPT* gene in J2 nematodes soaked in dsRNA targeting *HsIPT* or GFP. Data were analyzed using a *t* test ($P < 0.05$). (*C*) Average number of nematodes per plant in Col-0 infected with J2 soaked in dsRNA targeting *HsIPT* or *GFP* at 14 dai. (*D* and *E*) Average sizes of plant syncytia (*D*) or female nematodes (*E*) in Col-0 infected with J2 soaked in dsRNA targeting *HsIPT* or *GFP* at 14 dai. (*C–E*) Data are represented from five independent biological replicates and was analyzed using a *t* test (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). (*F*) Average amount of free bases for cytokinin in *H. schachtii* treated with dsRNA targeting *GFP* or *IPT*. Bars represent mean ± SE for four independent biological replicates. Data were analyzed using a *t* test ($P < 0.05$). Asterisks represent statistically significant difference to control. Red color bars represent dsRNA-*GFP* and black color bars represent dsRNA-*IPT*.

Nevertheless, mechanistic details for a role of cytokinin in the plant-nematode interaction remained elusive. In this study we establish a molecular framework for cytokinin function during the cyst nematode-plant interaction. We showed that activation of cytokinin signaling on cyst nematode infection is mediated mainly by the AHK3 and CRE1/AHK4 cytokinin receptors and that this positively regulates the establishment and development of nematode feeding sites. The detection of a strong signal of cytokinin reporter genes (*ARR5:GUS* and *TCSn:GFP*) in and around the expanding syncytium is in line with previous observations and strongly suggests the involvement of cytokinin in cell cycle reactivation after nematode infection (27). Indeed, analysis with a *CycB1;1:GUS* reporter showed a strong reduction in cell cycle reactivation in cytokinin receptor double mutants (*ahk3/4*) after nematode infection, which supported our observations of decreased susceptibility of *ahk3/4* double mutants to nematodes.

We also propose that nematodes produce cytokinin that is released into host cells at the site of infection. This cytokinin is subsequently involved in the activation of the cell cycle in cells adjacent to the feeding site, thereby playing an important role in the expansion of the syncytium. In support of this hypothesis, we found that silencing of the *HsIPT* gene in nematodes strongly reduced the levels of the free base *iP* and diminished cytokinin signaling, which in turn reduced activation of the cell cycle and caused a reduction in the infectivity of nematodes to plants. However, we also found that the size of syncytia in *ipt3/5/7* plants was reduced significantly compared with Col-0, suggesting that the cytokinin secreted by nematodes is not sufficient for optimal development and expansion of the syncytium. Based on these results, we postulate that nematode-derived cytokinin is particularly important during the early stages of feeding site initiation and that host-derived cytokinin plays a more dominant role during later stages of syncytium expansion and maintenance. This hypothesis is supported by cytokinin measurements of host roots segments, which showed that *ipt3/5/7*-derived cytokinin contributes more to the total cytokinin in control roots (72%), as well as in infected tissues at 10 dai (65%) compared with 5 dai (44%) (Fig. 3*E*). Interestingly, the same trend was observed in levels of *iP*-type cytokinin, especially in levels of the free base *iP*. The cytokinin receptor CRE1/AHK4 has been shown to have high affinity to *iP* (11). Therefore, the observation that *ahk4* mutants are particularly impaired in their response to nematode infection supports a role for nematode-derived cytokinin, in particular for *iP*, in nematode parasitism.

Our results also raise the question of whether the amount of cytokinin secreted by the nematode is physiologically relevant to play a significant role in feeding site development. Previous analysis showed that *H. schachtii* produces cytokinin in the amount of $\sim 1.8 \times 10^{-18}$ mol/J2^{-1}h^{-1}, whereas the average concentration of cytokinin in roots is $\sim 10^{-18}$ mol/nL (18). With an average volume of a root cell ranging between 0.0005 and 0.5 nL, additional cytokinin produced by the nematode may therefore be sufficient to activate the cell cycle at the feeding site during the initial stages of infection. This hypothesis is supported by the observation that expression of the *HgIPT* gene is highest during the early stages of infection and that it declines after the nematodes become sedentary. However, it is also plausible that once the nematode becomes sedentary and starts feeding, the profile and secretion of cytokinin may change through changes in expression of the nematode *IPT* gene in specific tissues, such as gland cells. Because *H. glycines* microarray analysis was performed using RNA from entire nematodes, changes in gene expression in local tissues might not be reflected.

The discovery that nematodes produce and release cytokinin raises the question of how a nematode tRNA-IPT produces *iP*-type cytokinin. In *Arabidopsis*, two different pathways that are responsible for the formation of distinct types of cytokinin have been described. Whereas the tRNA-IPT pathway was shown to be mainly responsible for the bulk of *cZ*-type cytokinin, the ATP/ADP-IPT pathway is responsible for the bulk of cytokinin including the *tZ* and *iP* types (8). In contrast to *Arabidopsis*, the bryophyte *Physcomitrella patens* possesses only tRNA-IPTs and therefore predominantly produces *cZ*-type cytokinin. Nevertheless, the *iP*-type and *tZ*-type cytokinins are still present and are also the most active forms in this moss (28). In a recent study, it was also suggested that a tRNA-independent pathway exists in case of *P. patens* (29). Therefore, it may be that the nematode tRNA-IPT enzyme catalyzes the production of a broader range of cytokinins.

Thus far, the best-characterized microbial system for the production of cytokinin is the crown gall-forming bacterium *Agrobacterium tumefaciens*. It contains a cytokinin synthetic *IPT* gene in its Ti-plasmid, which is integrated into plant genomes during infection. Similar *IPT* genes have been found in other bacteria of the same genus (30). In addition to bacteria, phytopathogenic and mutualistic fungi have also been shown to produce and secrete cytokinin into their host plants (31). However, evidence that an animal can produce cytokinin and use it to manipulate the plant hormone system to establish a parasitic interaction is not yet known.

Our findings are reminiscent of those in plant-bacterial and plant-fungal interactions. We show the ability of a nematode to produce and secrete its own cytokinin and not just to manipulate the host's cytokinin production system. In sum, this work provides, to our knowledge, first genetic evidence of cytokinin synthesis by an animal to induce and maintain long-term parasitic interactions with their host plants. Clarifying further details of the nematode cytokinin synthesis pathway, including details of the activity of HsIPT, and studying its action on host cell development will provide exciting information on cyst nematode parasitism.

Experimental Procedures

Plant Material and Growth Conditions. *A. thaliana* plants were grown in Petri dishes containing agar medium supplemented with modified Knop's nutrient medium under conditions described previously (32). The *CKX* lines and *ARR5:GUS*, *AHK:GUS*, *TCSn:GFP*, *CycB1;1:GUS*, *ahk*, and *ipt* mutant lines have been described before (8–11, 19–24). The *CycB1;1:GUS* reporter line (24) was introgressed into the cytokinin double receptor mutants *ahk2-5 ahk3-7*, *ahk2-5 cre1-2*, and *ahk3-7 cre1-2* (10), and lines homozygous for the reporter

gene and the receptor mutations were selected in subsequent generations using selection marker genes and genotyping.

Nematode Infection Assays. The nematode infection assays on *Arabidopsis* plants were performed as previously described (32). Sugar beet seeds were surface sterilized in 6% (vol/vol) sodium hypochlorite (NaClO) for 30 min and were grown in 0.2 Knop medium and infected with nematodes under the same conditions as described for *Arabidopsis*.

***IPT* Gene Identification and RNAi.** We used the amino acid sequences of *tRNA-IPT* from *C. elegans* (GenBank accession no. T27538) and *Arabidopsis* (*IPT2*) to identify their ortholog in *H. schachtii*. This analysis resulted in a single gene coding for 449 amino acids (*HsIPT*). A further sequence analysis showed that *HsIPT* retains the consensus pattern of *tRNA-IPTs* as suggested previously (30). The silencing of *HsIPT* in *H. schachtii* was performed as described previously (33), including some modifications (34). Transcript abundance of *HsIPT* after silencing was analyzed using StepOne Plus Real-Time PCR System (Applied Biosystems). Samples were analyzed in three technical replicates. An *Actin* gene was used as an internal control. Relative expression was calculated by the Pfaffl's method (35), where the expression of the *HsIPT* gene was normalized to the actin gene to calculate fold change. The reliability of *Actin* as a reference gene in response to dsRNA-*GFP* and dsRNA-IPT treatment was confirmed by using a *60S* acidic ribosomal protein-encoding gene. The same primers for 60S gene were used as described previously for cyst nematode *Globodera rostochiensis* (36). All primer sequences are listed in *SI Appendix 1*, Fig. S18.

Cytokinin Measurement. *Arabidopsis* plants were grown and infected with *H. schachtii,* as described above. The root segments containing infection sites were cut at 5 or 10 dai. Seventeen-day-old uninfected root segments were used as a control. For the cytokinin measurement of *H. schachtii*, preinfective J2s were collected from cysts under sterile conditions. Extraction, purification, and quantification of endogenous cytokinin was performed as described previously (37) with modifications. The concentrations of the various cytokinins were calculated using a standard isotope dilution method (38).

ACKNOWLEDGMENTS. We thank Jeremy Schmutz, Matt Hudson, Kris Lambert, and the Department of Energy Joint Genome Institute for providing prepublication access to the draft genome of soybean cyst nematode *H. glycines*. We also thank Stephan Neumann, Gisela Sichtermann, and Robert Heinz for nematode maintenance; Bernhard Fuss, Andreas Meyer, Arpine Mikayelyan, and Aleksandr Jurkevic for assistance with microscopy work; Barbara Klinzer for assistance in preparation of the microscopic figures; Bruno Müller for providing the *TCSn:GFP* seed; and Eva Hirnerová and Thomas Gerhardt for helpful technical assistance. This work was supported by grants from Ministry of Education, Youth and Sports, Czech Republic (Grant LO1204 to M. Strnad), National Science Centre, Poland (Grant 2013/09/B/NZ9/02381 to M. Sobczak), and National Science Foundation (NSF) (Grant IOS-1456047 to M.G.M.).

1. Sasser JN, Freckman DW (1986) A world perspective on nematology: The role of the society. *J Nematol* 18(4):596–597.
2. de Almeida-Engler J, et al. (1999) Molecular markers and cell cycle inhibitors show the importance of cell cycle progression in nematode-induced galls and syncytia. *Plant Cell* 11(5):793–807.
3. Vieira P, et al. (2014) The cyclin-dependent kinase inhibitor KRP6 induces mitosis and impairs cytokinesis in giant cells induced by plant-parasitic nematodes in Arabidopsis. *Plant Cell* 26(6):2633–2647.
4. de Almeida-Engler J, Engler G, Gheysen G (2011) Unravelling the plant cell cycle in nematode induced feeding sites. *Genomics and Molecular Genetics of Plant-Nematode Interactions*, eds Jones J, Gheysen G, Fenoll C (Springer, Dordrecht, The Netherlands), pp 349–368.
5. Goverse A, et al. (2000) Cell cycle activation by plant parasitic nematodes. *Plant Mol Biol* 43(5-6):747–761.
6. Ha S, Vankova R, Yamaguchi-Shinozaki K, Shinozaki K, Tran LS (2012) Cytokinins: Metabolism and function in plant adaptation to environmental stresses. *Trends Plant Sci* 17(3):172–179.
7. Kieber JJ, Schaller GE (2014) Cytokinins. *Arabidopsis Book* 12:e0168.
8. Miyawaki K, et al. (2006) Roles of Arabidopsis ATP/ADP isopentenyltransferases and tRNA isopentenyltransferases in cytokinin biosynthesis. *Proc Natl Acad Sci USA* 103(44):16598–16603.
9. Inoue T, et al. (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. *Nature* 409(6823):1060–1063.
10. Riefler M, Novak O, Strnad M, Schmülling T (2006) Arabidopsis cytokinin receptor mutants reveal functions in shoot growth, leaf senescence, seed size, germination, root development, and cytokinin metabolism. *Plant Cell* 18(1):40–54.
11. Higuchi M, et al. (2004) *In planta* functions of the Arabidopsis cytokinin receptor family. *Proc Natl Acad Sci USA* 101(23):8821–8826.
12. Nishimura C, et al. (2004) Histidine kinase homologs that act as cytokinin receptors possess overlapping functions in the regulation of shoot and root growth in Arabidopsis. *Plant Cell* 16(6):1365–1377.
13. Dimalla GG, Vanstaden J (1977) Cytokinins in root-knot nematode, *Meloidogyne incognita*. *Plant Sci Lett* 10(1):25–29.
14. Vanstaden J, Dimalla GG (1977) Comparison of endogenous cytokinins in roots and xylem exudate of nematode resistant and susceptible tomato cultivars. *J Exp Bot* 28(107):1351–1356.
15. Lohar DP, et al. (2004) Cytokinins play opposite roles in lateral root formation, and nematode and rhizobial symbioses. *Plant J* 38(2):203–214.
16. Walters DR, McRoberts N (2006) Plants and biotrophs: A pivotal role for cytokinins? *Trends Plant Sci* 11(12):581–586.
17. Abelenda JA, Prat S (2013) Cytokinins: Determinants of sink storage ability. *Curr Biol* 23(13):R561–R563.
18. De Meutter J, et al. (2003) Identification of cytokinins produced by the plant parasitic nematodes *Heterodera schachtii* and *Meloidogyne incognita*. *Mol Plant Pathol* 4(4):271–277.
19. Zürcher E, et al. (2013) A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network *in planta*. *Plant Physiol* 161(3):1066–1075.
20. D'Agostino IB, et al. (2000) Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiol* 124(4):1706–1717.
21. Stolz A, et al. (2011) The specificity of cytokinin signalling in *Arabidopsis thaliana* is mediated by differing ligand affinities and expression profiles of the receptors. *Plant J* 67(1):157–168.
22. Werner T, et al. (2010) Root-specific reduction of cytokinin causes enhanced root growth, drought tolerance, and leaf mineral enrichment in Arabidopsis and tobacco. *Plant Cell* 22(12):3905–3920.
23. Werner T, et al. (2003) Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* 15(11):2532–2550.
24. Colon-Carmona A, You R, Haimovitch-Gal T, Doerner P (1999) Spatio-temporal analysis of mitotic activity with a labile cyclin-GUS fusion protein. *Plant J* 20(4):503–508.
25. Golinowski W, Grundler FMW, Sobczak M (1996) Changes in the structure of *Arabidopsis thaliana* during female development of the plant-parasitic nematode *Heterodera schachtii*. *Protoplasma* 194(1):103–116.
26. Elling AA, et al. (2007) Divergent evolution of arrested development in the dauer stage *of Caenorhabditis elegans* and the infective stage of *Heterodera glycines*. *Genome Biol* 8(10):R211.
27. Absmanner B, Stadler R, Hammes UZ (2013) Phloem development in nematode-induced feeding sites: The implications of auxin and cytokinin. *Front Plant Sci* 4:241.
28. von Schwartzenberg K, et al. (2007) Cytokinins in the bryophyte *Physcomitrella patens*: Analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. *Plant Physiol* 145(3):786–800.
29. Lindner A, et al. (2014) Isopentenyltransferase-1 (*IPT1)* knockout in *Physcomitrella* together with phylogenetic analyses of IPTs provide insights into evolution of plant cytokinin biosynthesis. *J Exp Bot* 65(9):2533–2543.
30. Kakimoto T (2001) Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate: ATP/ADP isopentenyltransferases. *Plant Cell Physiol* 42(7):677–685.
31. Giron D, Frago E, Glevarec G, Pieterse CMJ, Dicke M (2013) Cytokinins as key regulators in plant-microbe-insect interactions: Connecting plant growth and defence. *Funct Ecol* 27(3):599–609.
32. Siddique S, et al. (2014) Parasitic worms stimulate host NADPH oxidases to produce reactive oxygen species that limit plant cell death and promote infection. *Sci Signal* 7(320):ra33.
33. Urwin PE, Lilley CJ, Atkinson HJ (2002) Ingestion of double-stranded RNA by preparasitic juvenile cyst nematodes leads to RNA interference. *Mol Plant Microbe Interact* 15(8):747–752.
34. Sukno SA, et al. (2007) Quantitative detection of double-stranded RNA-mediated gene silencing of parasitism genes in *Heterodera glycines*. *J Nematol* 39(2):145–152.
35. Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* 29(9):e45.
36. Lozano-Torres JL, et al. (2014) Apoplastic venom allergen-like proteins of cyst nematodes modulate the activation of basal plant innate immunity by cell surface receptors. *PLoS Pathog* 10(12):e1004569.
37. Svacinova J, et al. (2012) A new approach for cytokinin isolation from Arabidopsis tissues using miniaturized purification: Pipette tip solid-phase extraction. *Plant Methods* 8(1):17.
38. Rittenberg D, Foster GL (1940) A new procedure for quantitative analysis by isotope dilution, with application to the determination of amino acids and fatty acids. *J Biol Chem* 133(3):737–774.