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RESEARCH PAPER

Nematode infection and reproduction in transgenic and mutant *Arabidopsis* and tobacco with an altered phenylpropanoid metabolism

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Abstract

Transgenic and mutant *Arabidopsis* and tobacco plants with altered phenylpropanoid metabolism were infected with the plant parasitic root knot nematode *Meloidogyne incognita* to assess the effect of the transgene or mutation on nematode infection and reproduction. Modifications in the lignin biosynthetic pathway which alter lignin composition in roots affected reproduction. In *Arabidopsis* with increased levels of syringyl lignin, reproduction was lower than in wild-type plants, while in tobacco with reduced levels of syringyl lignin, life cycle progression was stimulated. Overexpression of a MYB transcription factor of phenylpropanoid metabolism in tobacco significantly stimulated reproduction of *M. incognita*, while overexpression of L-phenylalanine ammonia-lyase had no effect. *Arabidopsis transparent testa* mutants with deficiencies in flavonoid pathway enzymes did not affect reproduction of *M. incognita* in the present infection tests.

Key words: Chlorogenic acid, flavonoid, guaiacyl lignin, *Meloidogyne incognita*, syringyl lignin, *transparent testa* mutants.

Introduction

The phenylpropanoid pathway of secondary metabolism has been altered by metabolic engineering and induced mutations mainly in *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.] and tobacco (*Nicotiana tabacum* L.), with the purpose of increasing knowledge on pathway structure, regulation, and flux control. Engineering provides options for increased lignin degradation (paper industry) and forage digestibility, for the enhancement of the antioxidant capacity of food for health reasons, and for increased disease resistance. Pathway regulation and flux control have been investigated in tobacco by sense and antisense expression of L-phenylalanine ammonia-lyase (PAL) and cinnamic acid 4-hydroxylase (C4H), two enzymes of the 'core' phenylpropanoid pathway from which lignin and flavonoid pathways are derived (Howles *et al.*, 1996; Blount *et al.*, 2000). Jung *et al.* (2000) and Yu *et al.* (2000) transferred the gene coding for isoflavone synthase from soybean to non-leguminous species—*Arabidopsis*, tobacco, and maize—and obtained the synthesis of legume-specific isoflavonoids in these plants. With respect to alterations in lignin metabolism, different endogenous and heterologous enzyme-coding genes were expressed in sense and antisense direction mainly in tobacco but also in *Arabidopsis* and tree species (reviewed by Boerjan *et al.*, 2003). Efforts have been made to increase the flavonoid and chlorogenic acid content of tomatoes to create 'functional food' with higher antioxidant levels (Muir *et al.*, 2001; Niggeweg *et al.*, 2004). Metabolic engineering for disease resistance has been focused so far on fungal pathogens (Punja, 2001). The most successful and widespread applied approach is the expression of the stilbene synthase gene from grapevine or peanut in heterologous plant species. Increased fungal resistance has been obtained in tobacco,

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Abbreviations: C4H, cinnamic acid 4-hydroxylase; F5H, ferulic acid 5-hydroxylase; OMT, *O*-methyltransferase; PAL, L-phenylalanine ammonia-lyase; *tt*, *transparent testa*.

tomato, rice, barley, wheat, alfalfa, papaya, and aspen (reviewed by Punja, 2001; Zhu *et al.*, 2004). The overexpression of the gene coding for isoflavone *O*-methyltransferase (OMT) in alfalfa increases the levels of the phytoalexin medicarpin and increases resistance against fungal infection (He and Dixon, 2000). Finally, it was shown that *PAL*-overexpressing tobacco plants have improved resistance against fungal infection (Howles *et al.*, 1996; Way *et al.*, 2002; Shadle *et al.*, 2003).

The role of the phenylpropanoid pathway in plant defence mechanisms includes the synthesis of preformed or inducible physical and chemical barriers against infection and the synthesis of signal molecules involved in local and systemic signalling for defence gene induction (Nicholson and Hammerschmidt, 1992; Dixon *et al.*, 2002). Research on phenylpropanoids active in plant defence against nematode infection is limited, compared with fungal infection, although phenolic compounds were already mentioned by Rohde (1972) and Giebel (1982). Levels of phenylpropanoid compounds are higher in nematode-resistant cultivars—chlorogenic acid in tomato and pepper roots and rice (Hung and Rohde, 1973; Plowright *et al.*, 1996; Pegard *et al.*, 2005), isoflavonoids and medicarpin in alfalfa roots (Edwards *et al.*, 1995; Balbridge *et al.*, 1998)—or increase as a result of nematode infection (Mahmood and Saxena, 1986; Huang and Barker, 1991; Cook *et al.*, 1995; Kennedy *et al.*, 1999). It has also been shown that enzymes of the phenylpropanoid biosynthetic pathway are induced in plants after nematode infection (Edens *et al.*, 1995; Balbridge *et al.*, 1998). Bioassays involving natural plant products, including phenylpropanoids, have shown the existence of potential chemical barriers to nematode infection among these compounds (Chitwood, 2002; Wuyts *et al.*, 2006). Lignin is a potential physical barrier to nematode infection as it is known that the endodermis of the vascular cylinder in plant roots is not crossed by most endoparasitic nematodes, except cyst nematodes, because of lignification.

Engineering for resistance against nematodes in plants has, so far, not involved phenylpropanoid metabolism (Atkinson *et al.*, 2003). As such, potential phytoanticipins or phytoalexins have not been tested *in vivo* by (over)-production of these compounds in plants. Therefore, mutants and transgenics with an altered phenylpropanoid metabolism are interesting candidates for nematode infection and reproduction tests. They include plants with alterations in the lignin biosynthetic pathway (Atanassova *et al.*, 1995; Meyer *et al.*, 1998), in the flavonoid pathway (Goldsbrough *et al.*, 1996; He and Dixon, 2000; Muir *et al.*, 2001; Peer *et al.*, 2001), and in the 'core' part of the phenylpropanoid pathway with effects on lignin and flavonoid metabolism (Howles *et al.*, 1996; Blount *et al.*, 2000; Borevitz *et al.*, 2000). As such, the perspectives of metabolic engineering for resistance against plant parasitic nematodes can be explored. Moreover, it could become clear which part of the phenylpropanoid pathway is utilized in defence or which part is required by nematodes for successful infection and establishment in plants.

The specific objective of the study was to evaluate infection and reproduction of plant parasitic nematodes in mutant and transgenic *Arabidopsis* and tobacco plants in comparison with their wild types. Before the actual infection with nematodes, roots of these plants were histochemically stained or root extracts were analysed by liquid chromatography to confirm the altered profile of metabolites as described for leaf and stem tissue. Infection tests were performed with the root knot nematode *Meloidogyne incognita*, which is a sedentary endoparasite. Juvenile nematodes infect roots in the elongation zone just behind the root tip and migrate intercellularly through the cortex towards the root apex. Once they have arrived, they turn round towards the vascular cylinder, where they induce cell (re)differentiation processes to establish a permanent feeding site, which functions as a nutrient sink in host roots (Wyss *et al.*, 1992; Gravato Nobre *et al.*, 1995). Nematodes are serious threats to agricultural production worldwide. Current control measures still rely mostly on a few expensive, unsafe, and environmentally hazardous chemical pesticides. Natural or transgenic nematode-resistant cultivars are promising alternatives as part of an integrated pest management strategy for large-scale as well as small-scale cultivation.

Materials and methods

Transgenic and mutant plants

In Table 1 a summarized description is provided of the mutant and transgenic plants that were included in the nematode infection tests. Seeds of the different mutant and transgenic plant lines were obtained as follows: *Arabidopsis* C4H-F5H and control plants from Dr Clint Chapple (Department of Biochemistry, Purdue University, West Lafayette, IN, USA); tobacco OMT antisense line B_{10} from Dr Michel Legrand (Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, Strasbourg, France); tobacco PAP2 and control plants from Dr Justin Borevitz (Plant Biology, Salk Institute, La Jolla, CA, USA); tobacco PAL-sense line 10-6-OX434 plants and control line C17 plants from Dr Richard Dixon (Plant Biology, The Samuel Roberts Noble Foundation, Inc., Ardmore, OK, USA); *Arabidopsis tt3* (line 84), *tt4* (line UV118a), *tt5* (line 86), and *tt7* (line 88) and L*er* from Dr Wendy Peer (Department of Horticulture, Purdue University) and Dr Maarten Koornneef (Plant Sciences, Wageningen University, Wageningen, The Netherlands). The *tt4* (line UV118a) was originally a gift of Dr Brenda Winkel (Fralin Center for Biotechnology, Virginia Tech, Blacksburg, VA, USA) to Dr Wendy Peer.

Plant growth

Nematode infection tests were performed *in vitro*. Tobacco and *Arabidopsis* seeds were surface-sterilized by a 1 min soak in 70% ethanol, followed by 2 min in 0.8% NaOCl with 0.1% Tween-20, and excessive rinsing in water. Seeds were plated on MS-medium (Murashige and Skoog, 1962) (Duchefa Biochemie, Haarlem, The Netherlands) containing 1% sucrose and 0.8% agar for germination.

Table 1. *Transgenic and mutant plants selected because of alterations in their secondary metabolism and submitted to infection and reproduction tests with plant parasitic nematodes*

C4H, cinnamic acid 4-hydroxylase; CHI, chalcone isomerase; CHS, chalcone synthase; DFR, dihydroflavonol 4-reductase; F3′H, flavonoid 3′-hydroxylase; F5H, ferulic acid 5-hydroxylase; OMT, *O*-methyltransferase; PAL, L-phenylalanine ammonia-lyase.

Secondary metabolism	Species	Wild type	Mutant/transgenic	Mutated enzyme	Inserted protein	Described phenylpropanoid profile	Origin
Lignin metabolism	*Arabidopsis thaliana*	Columbia (Col)	C4H-F5H	–	F5H	Almost entirely syringyl lignin	Meyer *et al.* (1998)
	Nicotiana tabacum	Samsun NN	OMT antisense	–	OMT	Decreased levels of syringyl lignin	Atanassova *et al.* (1995)
Lignin+flavonoid metabolism	*Nicotiana tabacum*	Xanthi	PAP2	–	MYB transcription factor	Purple plants due to increased levels of anthocyanidins	Borevitz *et al.* (2000)
	Nicotiana tabacum	Xanthi	PAL sense	–	PAL	Increased levels of chlorogenic acid	Elkind *et al.* (1990); Howles *et al.* (1996)
Flavonoid metabolism	*Arabidopsis thaliana*	Landsberg *erecta* (*Ler*)	*tt3*	DFR	–	Increased levels of kaempferol and quercetin	Koornneef (1981, 1990); Koornneef *et al.* (1982); Shirley *et al.* (1995)
			tt4	CHS	–	No flavonoids	
			tt5	CHI	–	Only naringenin chalcone, no other flavonoids	
			tt7	F3′H	–	Increased levels of kaempferol	

Seven-day-old seedlings of transformed, mutant, and control plants were randomly transferred to modified KNOP medium (Sijmons *et al.*, 1991) in six-well tissue culture plates (Greiner Bio-One, Wemmel, Belgium), with each plate containing one or two plants of each type. Twelve seedlings per plant type were included in the infection tests and at least three independent tests were done. Seedlings were grown at 23 °C with a 16 h light/8 h dark cycle. When plants had grown a sufficient root system (1 week on KNOP medium), they were inoculated with nematodes.

Confirmation of alterations in phenylpropanoid metabolism in roots

Only for the *tt*-mutants has the phenylpropanoid profile been analysed in roots (Peer *et al.*, 2001). For the other plants, analysis has been performed on leaves or stem tissue only. Also only *in-vivo* grown (growth chamber/greenhouse) plants have been analysed for phenylpropanoids, while the nematode infection tests were performed *in vitro*. Therefore, roots of *in vitro*-grown transformed, mutant, and wild-type plants of tobacco and *Arabidopsis* were analysed either by HPLC or histochemical staining at the time of nematode inoculation.

HPLC analysis was performed on methanol extracts of fresh tissues of *in vitro*-grown tobacco. Approximately 500 mg of stems and leaves or 50 mg of roots of PAL-sense and control plants were ground with a small pestle in 1 ml methanol and extracted for 5 min at room temperature under constant shaking. For the PAP2 and control plants ~200 mg of stems and leaves or 30 mg of roots were extracted in 1 ml methanol for 15 min. Samples were passed through 0.45 µm PTE filters (Machery-Nagel, Düren, Germany). HPLC analysis was carried out on a HP1100 system (Hewlett Packard, Waldbronn, Germany) using an Inertsil ODS2 (250×3 mm, particle size 5 µm) RP18 analytical column (Chrompack, Middelburg, The Netherlands). Extracts (15 µl) were separated at 25 °C at a flow rate of 0.4 ml min^{-1} using a gradient of acetonitrile in water (MilliQ water at pH 3.0 with phosphoric acid): 12% for 10 min, 18% for 5 min, 45% for 20 min, 100% for 7 min, and a step back to 12% for 10 min. A diode array detector (Hewlett Packard) was used to record the online spectra (254, 280, 330, 350, and 366 nm) of compounds eluting from the column. Peak identification and integration were carried out on HP ChemStations software using phenylpropanoid standards (Sigma-Aldrich, Inc., Bornem, Belgium). Peaks were identified by comparing their retention times and UV absorbance spectra with those of standards. Standard calibration curves were determined for quantification.

Lignin was detected in the roots of *Arabidopsis* C4H-F5H and Col wild-type plants and in tobacco OMT antisense and Samsun NN wild-type plants by histochemical staining. As fresh sections could not be made of the very thin and transparent *in vitro* roots of *Arabidopsis* and tobacco, whole roots were stained in solutions to which detergent (Triton X-100) was added. Plants were removed from agar, gently washed, and submerged in the staining solutions. Both Mäule and Wiesner staining procedures were applied according to standard protocols (Chapple *et al.*, 1992; Atanassova *et al.*, 1995). Stained roots were examined by bright-field microscopy at 40- and 100-fold magnification. Digital photographs were taken with a SPOT RT camera and SPOT software version 3.3 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA).

For the detection of phenylpropanoid compounds, *Arabidopsis* roots were stained with saturated (0.25%, w/v) diphenylboric acid 2-aminoethyl ester (DPBA) (Sigma-Aldrich, Inc., Bornem, Belgium) with 0.02% (v/v) Triton X-100, and observed under an epifluorescence microscope equipped with a DAPI filter (excitation 340–380 nm, suppression LP 430 nm) and a FITC filter (excitation 450–490 nm, suppression LP 520 nm) (Peer *et al.*, 2001). Photodocumentation of roots was achieved with a SPOT RT camera and SPOT software version 3.3.

Nematode inoculum and infection

Meloidogyne incognita was cultured *in vitro* on Ri T-DNA-transformed tomato roots (hairy roots) (Verdejo *et al.*, 1988). Egg masses were isolated from roots and allowed to hatch in sterile distilled water for 5–7 d to obtain juvenile nematodes. Depending on the availability, *M. incognita* populations isolated from tomato (Belgium), banana (Morocco), or fig tree (Spain) were used. Per plant, i.e. in one well of a six-well plate, 10 or 20 juvenile nematodes were inoculated on the culture medium in a small drop of water. The plates were sealed with Parafilm and incubated at 23 °C or 26 °C depending on the nematode population (indicated in Table 2).

Analysis of infection and reproduction

The infection of plants by nematodes was monitored up to 3 weeks after inoculation by light microscopy. Nematode reproduction was evaluated 10 weeks after inoculation. The number of sedentary females, egg masses, and juveniles in roots and medium were counted by direct observations of the six-well plates on a reverse-contrast light microscope. This is possible because of the transparency of the culture medium and plates and the transparency and thin diameter of the *in vitro*-cultured roots (Sijmons *et al.*, 1991). Afterwards, the egg masses were hand-picked from the roots and transferred per root system to a 10% commercial bleach solution (0.4% NaOCl) to dissolve the gelatinous matrix (Hussey and Barker, 1973). The number of eggs per egg mass and per root system was then determined.

Statistical analysis

Data on nematode reproduction were analysed using ANOVA (Statistica® Release 6, Statsoft, Inc., Tulsa, OK, USA). For multiple comparisons of group means the Tukey test was applied. Data were $\log(x+1)$ transformed prior to analysis to reduce the natural variance in nematode reproduction data. When the conditions for ANOVA—normal distribution and homogeneity of variances—were not met, the non-parametric alternative Kruskal–Wallis analysis of variance by ranks was applied (Statistica®). When the Kruskal–Wallis statistic was significant, multiple comparisons between treatments were calculated as described by Siegel and Castellan (1988).

Results

Nematode infection and reproduction in in vitro*-grown* Arabidopsis *and tobacco*

Meloidogyne incognita infected and reproduced in both *Arabidopsis* and tobacco, but reproduction ratios (final number of nematodes and eggs/inoculated number of nematodes) varied much between repetitions. This was mainly due to the source (or pathotype) of the nematode population, with *M. incognita* isolated from tomato plants giving the lowest reproduction (Table 2). At 1 d after inoculation, juveniles accumulated in tobacco root tips and after 5 d root knots were visible. In *Arabidopsis* roots, at 5 d after inoculation, swollen bodies of developing females were noted. Male nematodes occurred rarely. Overall, *M. incognita* significantly infected and reproduced in both *Arabidopsis* and tobacco under the test conditions.

Table 2. *Reproduction ratio*[a] *range of* Meloidogyne incognita *populations on* Arabidopsis *ecotypes Landsberg* erecta (*L*er) *and Columbia (Col) and tobacco cultivars Xanthi and Samsun NN under* in vitro *conditions*

Reproduction ratios were determined 10 weeks after inoculation. –, Not tested.

Species	Ecotype or cultivar	Source of population Incubation temperature		
		Tomato 23 °C	Fig tree 26 °C	Banana 23 °C
Arabidopsis	Col	<1	30–78	–
	L*er*	<1–7	32–130	–
Tobacco	Xanthi	6–32	18–63	7–46
	Samsun NN	12–22	31–54	12–44

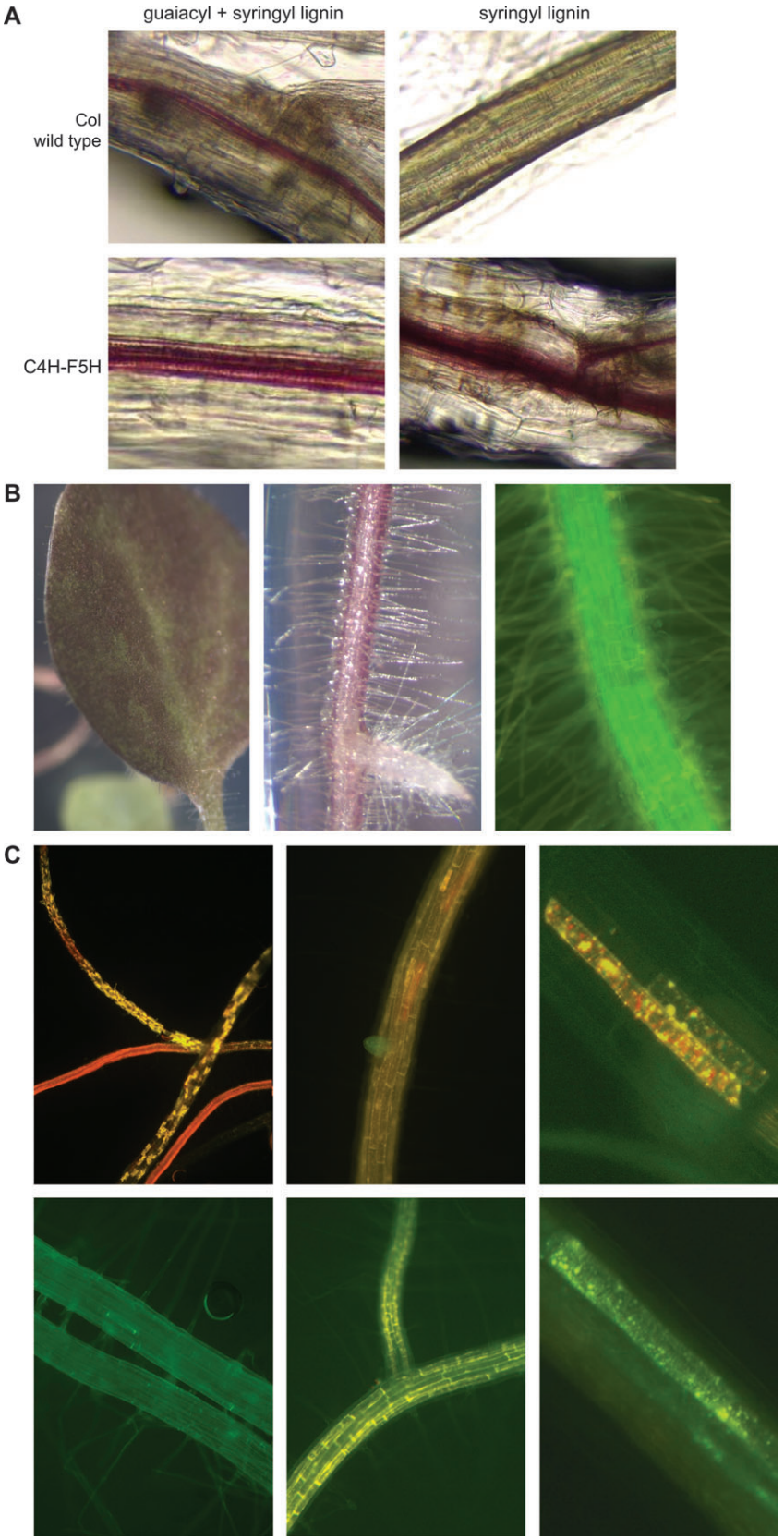
[a] Final number of nematodes and eggs/inoculated number of nematodes.

Alterations which affect lignin metabolism

The *fah1-2* mutant of *Arabidopsis thaliana* is deficient in ferulic acid 5-hydroxylase (F5H). This enzyme catalyses the irreversible hydroxylation of precursors for guaiacyl lignin towards precursors for syringyl lignin biosynthesis. As such, the mutant only accumulates guaiacyl lignin (Chapple *et al.*, 1992). The gene encoding F5H in *Arabidopsis* (Meyer *et al.*, 1996) has been used in a transcriptional fusion construct with the promoter of C4H, an early enzyme in the phenylpropanoid pathway, for transformation of the *fah1-2* mutant. The resulting C4H-F5H plants contain a lignin that is highly enriched in syringyl monomer units: an average of 76 mol% compared with 20 mol% in wild-type *Arabidopsis* (Meyer *et al.*, 1998; Marita *et al.*, 1999). Moreover, in wild-type plants, guaiacyl and syringyl lignin monomers are restricted to cells of the vascular bundles and to cells of the sclerified parenchyma that flank the vascular bundles, respectively. In the C4H-F5H plants, syringyl units also replace guaiacyl units in the cells of the vascular bundles (Meyer *et al.*, 1998).

Using the Mäule and Wiesner staining procedures, the result of alterations in the lignin pathway in roots of transgenics was checked. The Mäule reaction is specific for free syringyl units, while the Wiesner stain reflects the total lignin content (syringyl and guaiacyl lignin) as it reacts with cinnamaldehyde residues in lignin (Monties, 1989). The overall changes in the syringyl/guaiacyl ratio that have been described for stem and leaf tissues of C4H-F5H plants were confirmed in roots of these plants when grown under the conditions of the infection tests. The vascular cylinder of roots of the C4H-F5H plants stained very bright red (more than Col wild-type roots), indicative of syringyl lignin, in the treatment with the Mäule reagent. Total lignin was detected in roots of all plants (Fig. 1A).

Infection of lignin-altered *Arabidopsis* with *M. incognita* was repeated four times, including three experiments with the tomato population and one with the fig-tree population. Only in one experiment with the tomato population, was significant reproduction obtained. The numbers of egg masses, juveniles, and the resulting reproduction ratio were significantly lower in the C4H-F5H plants than in the wild-type plants at 10 weeks after inoculation (P <0.01, 0.001)



(Table 3). In the experiment with the fig-tree population, much higher reproduction ratios were obtained. Reproduction was again significantly lower in the C4H-F5H plants than in the wild type (P <0.05) (Table 3). The number of egg masses did not differ between plants at 10 weeks after inoculation. The difference lay in the number of juveniles (Table 3) and the number of eggs produced per egg mass (Table 4). The percentage of juveniles in the population was used as an indicator of the time required for the transition between life cycles (egg→mobile juvenile). This was again significantly lower in the C4H-F5H plants (P <0.05). Also the number of developing females, i.e. the nematodes that have become sedentary but have not yet produced an egg mass (sedentary→reproductive), was lower in the C4H-F5H plants (P <0.05) (Table 4).

The selected tobacco plants are altered in another enzyme of the lignin biosynthesis pathway, OMT, which catalyses the conversions of hydroxycinnamic CoA esters to syringyl lignin units (Guo *et al.*, 2001; Zhong *et al.*, 1998). In the transgenic line with antisense expression, OMT activity in stem tissue is reduced to 8% of the wild-type level, together with a decrease in the syringyl monomer content to 10% of the wild-type level and a syringyl/guaiacyl ratio of 0.07 compared with 0.9–1.1 in the wild type (Atanassova *et al.*, 1995).

Stained lignin was detected in the epi- and endodermis of roots of the wild-type Samsun NN plants. In the endodermis, the lignin polymer contained syringyl units. Staining of OMT antisense roots resulted in no syringyl units and little total lignin in the endodermis compared with the wild type.

Infection of lignin-altered tobacco with *M. incognita* was repeated three times (Table 5). In one out of three experiments, the average reproduction ratio obtained for the OMT antisense plants was significantly higher than for wild-type plants (P <0.001). Although not significantly different, the same trend was observed in all experiments (Table 5). The number of egg masses and the number of eggs per egg mass were equal among the transgenic and wild-type plants (Table 4). The difference in reproduction lay in the number of juveniles, which was significantly higher in OMT antisense plants (P <0.05, 0.001) in two out of three experiments. As the percentage of juveniles in the final nematode population was higher than in the wild-type plants, the life cycle of *M. incognita* appeared to be altered in roots of OMT-transgenic plants.

Alterations which affect lignin and/or flavonoid metabolism

During the evaluation of activation-tagged *Arabidopsis*, a mutant was observed with intense purple pigmentation in vegetative organs, including roots, throughout development (*pap1-D*). In this plant a massive activation of the phenylpropanoid pathway occurs due to the constitutive expression of a MYB transcription factor. Enhanced activity of key enzymes in the pathway leads to increased accumulation of hydroxycinnamic acids, syringyl and guaiacyl lignin, and flavonoids, including anthocyanidins. The purple pigmentation has been transferred to tobacco plants by transformation with a CaMV 35S promoter:: cDNA fusion construct, containing coding sequences of an expressed sequence tag (*PAP2*) with high homology to the coding sequence of PAP1, which is a MYB transcription factor (Borevitz *et al.*, 2000). No quantitative studies of enzyme activities or phenylpropanoid levels have been performed before on PAP2 tobacco plants.

PAP2 tobacco plants, grown under *in vitro* conditions, showed the intense purple pigmentation, indicative for anthocyanidins, as has been described for *in vivo*-grown plants. Roots were purple as well (Fig. 1B). In methanol extracts of root tissue, mainly chlorogenic acid and a glycoside of 4-coumaric acid were detected by HPLC analysis. In stem and leaf tissue, two other phenylpropanoids, caffeic acid and rutin (a glycoside of the flavonol quercetin), were present. Levels of chlorogenic acid were higher in root tissue than in stem and leaf tissues. PAP2 roots contained 3.5-fold more chlorogenic acid than roots of wild-type Xanthi, while 4-coumaric acid was not detected any more. Levels of caffeic acid and rutin were also higher in PAP2 stem and leaf tissue (Table 6).

Meloidogyne incognita infected PAP2 and Xanthi wild-type tobacco plants under *in vitro* conditions (Table 7). In three independent experiments, reproduction was significantly higher in the PAP2 plants than in the wild-type control plants (P <0.01, 0.001). The number of egg masses did not differ, but the number of eggs per egg mass and the number of juveniles was significantly higher in the PAP2 plants (P <0.05, 0.001) (Tables 4, 7). The percentage of

Fig. 1. (A) Histochemical staining for lignin in roots of *Arabidopsis* C4H-F5H and wild-type Col plants. Roots on the left were stained with the Wiesner reagent to detect total lignin (guaiacyl and syringyl monomers), while the roots on the right were treated with the Mäule reagent to visualize syringyl monomers. (B) PAP2 tobacco plants show strong purple pigmentation in leaves (left) and roots (middle). Treatment of roots with DPBA and observation under an epifluorescence microscope reveals the presence of chlorogenic acid (green fluorescence) along the axis of a PAP2 root (right). (C) DPBA-treated *Arabidopsis* L*er* and *tt* roots viewed under an epifluorescence microscope (FITC filter) for the detection of flavonoids. Upper left and middle images: in roots of L*er* wild-type plants the fluorescence of naringenin chalcone (yellow), quercetin (and its glycosides) (orange), and kaempferol (and its glycosides) (green) is observed along the root axis and in root tips. Upper right image: in *tt3* mutants, root cells contain granules with a high concentration of naringenin chalcone, quercetin, and to a lesser extent kaempferol. Lower left image: *tt4* mutants are deficient in the first enzyme of the flavonoid pathway (chalcone synthase) and, as such, only the autofluorescence of sinapic acid esters in cell walls is seen. Lower middle image: in *tt5* mutants, only naringenin chalcone is synthesized. Lower right image: in *tt7* mutants, only the kaempferol part of the flavonoid pathway remains, which results in kaempferol accumulation.

juveniles in the population was identical in the control and transgenic plants, which means that the life cycle of *M. incognita* was not affected.

The PAL-sense plants are first generation (T_1) overexpressor plants from a primary transformant (T_0) harbouring a heterologous (bean) *phenylalanine ammonia-lyase* (*PAL*) gene with CaMV 35S enhancer sequences in its promoter. PAL is the enzyme which catalyses the first step in the phenylpropanoid pathway: the conversion of L-phenylalanine into cinnamic acid. The T_0 showed homology-dependent silencing of the endogenous tobacco *PAL* genes, which resulted in reduced PAL activity and lower levels of phenylpropanoids, including chlorogenic acid and rutin (Elkind *et al.*, 1990). In the T_1 plants PAL-activity is up to 5- and 2-fold greater in leaf and stem tissue, respectively, compared with wild-type levels, due to the transgene-encoded PAL. In leaf tissue, this results in increased levels of chlorogenic acid and a 4-coumaric acid glycoside. Levels of rutin are not increased which suggests that additional flux control points exist in the flavonoid pathway (Howles *et al.*, 1996). *PAL* overexpression has little effect on the total lignin content or on lignin composition in stem tissue (Sewalt *et al.*, 1997).

Table 3. *Reproduction of* Meloidogyne incognita *in* Arabidopsis *C4H-F5H and wild-type Col plants under* in-vitro *conditions 10 weeks after inoculation of 20 (tomato) or 10 (fig tree) juveniles*

Data were log(x+1) transformed prior to ANOVA. *, **, *** Averages within a column and experiment followed by different letters are different at P <0.05, 0.01, 0.001, respectively, according to the Tukey test. *n*, Number of plants; EM, number of egg masses; Juveniles, number of juveniles; R_r, reproduction ratio (=final number of nematodes and eggs/inoculated number of nematodes).

Population	Plant	*n*	EM	Juveniles	R_r
Tomato	Control (Col)	10	4.5 a**	73.7 a***	3.7 a***
	C4H-F5H	10	1.2 b	4.7 b	0.2 b
Fig tree	Control (Col)	8	2.3 a	326 a*	78.7 a*
	C4H-F5H	8	2.9 a	143 b	36.3 b

PAL sense tobacco plants showed higher levels of all four phenylpropanoids in stem and leaf tissue compared with control plants. In root tissue the level of chlorogenic acid was twice as high. The 4-coumaric acid glycoside was still detected and levels were similar to control roots (Table 6). Control plants for the PAL sense plants were the first-generation progeny line that had lost the bean *PAL* transgene through segregation (Sewalt *et al.*, 1997).

Unlike for the PAP2 plants, reproduction in the PAL sense plants was not different from the control in three independent experiments (data not shown).

Alterations which affect flavonoid metabolism

The flavonoid accumulation patterns of *Arabidopsis transparent testa* (*tt*) mutants are well described and result from defects in different intermediate steps in the flavonoid biosynthetic pathway (Koornneef, 1981, 1990; Koornneef *et al.*, 1982; Shirley *et al.*, 1995). A deficiency in a pathway enzyme results in the accumulation of precursors.

The flavonoid profiles in the roots of *tt*-mutants and L*er* plants that have been described by Peer *et al.* (2001) were confirmed for plants grown under *in vitro* conditions. In 2–7-d-old seedlings flavonoids accumulate at specific sites along the root axis, i.e. at sites of organ transition and at the root tip (Peer *et al.*, 2001). In the roots of the plants in the present study, which were 14-d-old, flavonoids were also detected more randomly along the root axis (Fig. 1C).

Infection tests with *M. incognita* (fig tree) were repeated in four independent experiments. Nematodes reproduced well in all plants (average reproduction ratio of 59), but no significant differences (P <0.05) in reproduction between *tt*-mutants or between mutants and L*er* occurred (data not shown).

Discussion

Based on nematode feeding behaviour, it is possible that infection and reproduction by root knot nematodes is

Table 4. *Indicators of life cycle alteration and fecundity of* Meloidogyne incognita *(fig tree) on transgenic, mutant and wild-type* Arabidopsis *and tobacco under* in vitro *conditions 10 weeks after inoculation of 10 juveniles*

Data were log(x+1) transformed prior to ANOVA. *, ** Averages within a column and plant group followed by different letters are different at P <0.05, 0.01, respectively, according to the Tukey test. *n*, Number of plants; DF, number of developing females; TF, total number of females (EM+DF); eggs, number of eggs; EM, number of egg masses; %juv, percentage juveniles in final population.

Plant	*n*	Life cycle alteration			Fecundity	
		DF	TF	% juv	Eggs	Eggs/EM
Control (Col)[a]	8	4.9 a*	7.1 a	43 a*	461 a*	209 a**
C4H-F5H	8	1.1 b	4.0 a	24 b	221 b	78 b
Control (Samsun NN)[b]	12	0 a	3.3 a	35 a**	181 a	60 a
OMT antisense	11	0 a	4.4 a	51 b	262 a	69 a
Control (Xanthi)[b]	12	0 a	4.9 a	43 a	93 a*	34 a*
PAP2	6	0 a	4.3 a	49 a	361 b	92 b

[a,b] Results of fourth and third experiments, respectively.

Table 5. *Reproduction of* Meloidogyne incognita *in tobacco OMT antisense and wild-type Samsun NN plants under* in vitro *conditions 10 weeks after inoculation of 20 (tomato and banana) or 10 (fig tree) juveniles*

Data were log(x+1) transformed prior to ANOVA. *, *** Averages within a column and experiment followed by different letters are different at P <0.05, 0.001, respectively, according to the Tukey test. n, Number of plants; EM, number of egg masses; Juveniles, number of juveniles; R_r, reproduction ratio (=final number of nematodes and eggs/inoculated number of nematodes).

Population	Plant	n	EM	Juveniles	R_r
Tomato	Control (Samsun NN)	12	7.3 a	239 a	12 a
	OMT antisense	12	7.8 a	438 a	22 a
Banana	Control (Samsun NN)	12	8.2 a	229 a***	12 a***
	OMT antisense	12	7.1 a	882 b	44 b
Fig tree	Control (Samsun NN)	12	3.3 a	127 a*	31 a
	OMT antisense	11	4.4 a	277 b	54 a

Table 6. *Indicative values of the phenylpropanoid content of transgenic and control tobacco plants as determined by HPLC analysis*

nd, Not detected.

	Stem and leaf tissue (μg g^{-1} FW)	Root tissue (μg g^{-1} FW)
Chlorogenic acid		
Xanthi	80	137
PAP2	187	490
Control[a]	60	316
PAL sense	237	645
Caffeic acid		
Xanthi	29	nd
PAP2	62	nd
Control[a]	21	nd
PAL sense	64	nd
4-Coumaric acid glycoside[b]		
Xanthi	3.2	3.0
PAP2	3.4	nd
Control[a]	5.6	5.8
PAL sense	12.1	4.8
Rutin		
Xanthi	0.9	nd
PAP2	13.1	nd
Control[a]	0.9	nd
PAL sense	1.1	nd

[a] Transgenic control which has lost its transgene.
[b] Expressed as 4-coumaric acid equivalents (μg) g^{-1} fresh weight.

Table 7. *Reproduction of* Meloidogyne incognita *in tobacco PAP2 and wild-type Xanthi plants under* in vitro *conditions 10 weeks after inoculation of 20 (tomato and banana) or 10 (fig tree) juveniles*

Kruskal–Wallis ANOVA by ranks. *, **, *** Averages within a column and experiment followed by different letters are different at P <0.05, 0.01, 0.001, respectively, according to the multiple comparisons procedure as described by Siegel and Castellan (1988). n, Number of plants; EM, number of egg masses; Juveniles, number of juveniles; R_r, reproduction ratio (=final number of nematodes and eggs/inoculated number of nematodes).

Population	Plant	n	EM	Juveniles	R_r
Tomato	Control (Xanthi)	12	6 a	55 a***	3 a***
	PAP2	12	7 a	298 b	15 b
Banana	Control (Xanthi)	12	6 a	194 a***	10 a***
	PAP2	12	7 a	915 b	46 b
Fig tree	Control (Xanthi)	12	5 a	84 a*	18 a**
	PAP2	6	4 a	272 b	63 b

affected by changes in lignin content or composition. These nematodes establish their permanent feeding sites inside the vascular cylinder, which is the main site of secondary wall formation and lignification in roots. In C4H-F5H *Arabidopsis* plants, infection by *M. incognita* was not affected by the transgenic phenylpropanoid profile as the number of egg masses was equal to wild-type Col plants. Fecundity and development, however, were significantly reduced compared with the wild type: fewer eggs were produced per egg mass, fewer eggs hatched, and fewer juveniles developed into sedentary females. *Meloidogyne incognita* juveniles select procambial cells in the differentiation zone at the entry of the vascular cylinder, just above the root tip, to initiate the development of giant cells (permanent feeding site). As the root matures, cells of the vascular cylinder undergo secondary wall thickening. At this point, juveniles are already feeding from the initiated giant cells (Gravato Nobre *et al.*, 1995). In wild-type *Arabidopsis*, the sclerified parenchyma of the vascular cylinder contains mainly syringyl lignin, while the xylem bundles only contain guaiacyl lignin. In the vascular cylinder of C4H-F5H plants, lignified tissue contains significantly more syringyl units, and this syringyl lignin also replaces guaiacyl lignin in the xylem bundles (Meyer *et al.*, 1998). The increased levels of syringyl lignin in the vascular bundles possibly impede the flow of nutrients to the nematode's giant cells or hamper the nematode in feeding from its giant cells, which leads to reduced reproduction, a reduced 'quality' of eggs and overall a change in the development rate. Bell (1981) stated that the welfare of sedentary nematodes depends on the establishment and maintenance of the giant cells. In naturally resistant cultivars, nematode numbers often do not decline rapidly after infection. Instead, the nematode's development rate is slowed as fewer females develop egg masses and each mass contains fewer eggs. The transgenic C4H-F5H *Arabidopsis* plants seemed to affect *M. incognita* reproduction accordingly. In *in vitro* bioassays, sinapic acid and syringic acid inhibit motility of *M. incognita* juveniles, but the effect is limited and only temporary. Neither of these compounds affects hatching (Wuyts *et al.*, 2006).

Arabidopsis and tobacco have been altered with enzymes of the lignin biosynthetic pathway to increase knowledge on control points for lignin content and lignin composition with respect to improved paper pulping and forage digestibility. Guaiacyl units in the lignin polymer are monomethoxylated, which means that the C-5 aromatic position is available for carbon–carbon linkage with, for example, another guaiacyl unit during the polymerization

process. As syringyl units are dimethoxylated, syringyl-containing lignin has fewer 5-5′ linkages and is therefore less condensed. For the paper industry, this means that wood containing this type of lignin (angiosperm 'hard' wood) is easier to delignify (Boudet *et al.*, 1995). On the other hand, syringyl-rich lignins are more linear and penetrate a larger fraction of the cell wall. This limits the digestibility of polysaccharides by hydrolytic enzymes and decreases the nutritional value of forages (Jung and Deetz, 1993). In relation to plant pathogens, it has been shown that induced lignification in response to fungal infection is accompanied by an increased syringyl content in wheat leaves (Ride, 1975) and increased levels of sinapyl alcohol dehydrogenase activity in banana roots (de Ascensao and Dubery, 2000). Cellulases and pectinases have been detected in secretions of nematodes, including *Meloidogyne* spp. (Williamson and Gleason, 2003), but a relationship between lignin composition in cell walls and susceptibility to degradation by nematode enzymes has not been reported. Also no correlation has been established between the level of lignification in the vascular cylinder and the metabolic sink capacity of nematode giant cells nor the capacity of nematodes to feed on their giant cells in mature roots.

In roots of OMT antisense tobacco plants with a reduced syringyl lignin content, the life cycle of *M. incognita* progressed faster than in wild-type plants, as significantly more eggs had developed into juvenile nematodes. This contrasts nicely with the effect of increased levels of syringyl lignin in *Arabidopsis* plants. The number of egg masses was not higher in OMT-suppressed plants, probably because the decrease in syringyl lignin did not affect giant cell induction or because roots of *in vitro* plants simply do not sustain higher infection levels.

Roots of both PAP2 and PAL sense plants contained higher levels of chlorogenic acid than those of control plants. The 4-coumaric acid glycoside was not detected in roots of PAP2 plants, while PAL sense roots contained similar levels as control roots. Only in PAP2 roots was reproduction of *M. incognita* stimulated, which means that the 4-coumaric acid glycoside negatively affected reproduction or that metabolites other than chlorogenic acid in PAP2 affected reproduction in a positive sense. The purple colour of the PAP2 roots is an indication of the accumulation of other compounds, including anthocyanidins, as in stem tissue (Borevitz *et al.*, 2000). The analysis of anthocyanidins and related compounds would have required other extraction and detection methods. In PAL sense plants the effects of increased PAL activity are believed to be limited to increased levels of chlorogenic acid and 4-coumaric acid in stem tissue (Howles *et al.*, 1996).

Infection tests with *Arabidopsis tt*-mutants have the potential of providing information on the involvement of flavonoids in infection and reproduction of nematodes. Flavonoids affect nematode behaviour in *in vitro* bioassays, including chemotaxis and motility (Wuyts *et al.*, 2006). Indirect effects may arise from auxin transport inhibition in plants by flavonoids which results in local auxin accumulation. Flavonoids act on auxin accumulation by inhibiting or stimulating auxin turnover (Mathesius, 2001) or by modulating auxin transport (Peer *et al.*, 2004). Feeding-site induction by sedentary (cyst and root knot) nematodes requires cell cycle activation, which itself is regulated by the phytohormones auxin and cytokinin (Goverse *et al.*, 2000). Flavonoids are candidate modulators of local auxin manipulation in nematode infection mechanisms.

No significant differences in infection and reproduction of *M. incognita* were observed between *tt* mutants and wild-type plants or between the different *tt* mutants. However, between the six-well plates of each experiment, results varied considerably. Other test methods, including the growth and infection of individual plants in soil, may still be considered to test effects of altered flavonoid profiles.

Metabolically engineered and mutant *Arabidopsis* and tobacco with altered phenylpropanoid pathways were infected with the sedentary root knot nematode *M. incognita* to assess the effect of the transgene or mutation on the infection and reproduction of nematodes. Besides the ones that were used in this study, a large number of other plants have been designed with different or complementing alterations in the phenylpropanoid pathway of secondary metabolism, which can be tested and contribute to the knowledge on plant–nematode interactions.

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