Poplar–Root Knot Nematode Interaction: A Model for Perennial Woody Species

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Plant root-knot nematode (RKN) interaction studies are performed on several host plant models. Though RKN interact with trees, no perennial woody model has been explored so far. Here, we show that poplar (*Populus tremula* × *P. alba*) grown in vitro is susceptible to *Meloidogyne incognita*, allowing this nematode to penetrate, to induce feeding sites, and to successfully complete its life cycle. Quantitative reverse transcription-polymerase chain reaction analysis was performed to study changes in poplar gene expression in galls compared with noninfected roots. Three genes (*expansin A*, *histone 3.1*, and *asparagine synthase*), selected as gall development marker genes, followed, during poplar-nematode interaction, a similar expression pattern to what was described for other plant hosts. Downregulation of four genes implicated in the monolignol biosynthesis pathway was evidenced in galls, suggesting a shift in the phenolic profile within galls developed on poplar roots. Raman microspectroscopy demonstrated that cell walls of giant cells were not lignified but mainly composed of pectin and cellulose. The data presented here suggest that RKN exercise conserved strategies to reproduce and to invade perennial plant species and that poplar is a suitable model host to study specific traits of tree-nematode interactions.

Root-knot nematodes (RKN) (*Meloidogyne* spp.) are sedentary obligate biotrophic plant parasites with a broad host range including more than 6,500 host plants. They are responsible for major economic damages caused to both annual and perennial plant species worldwide (Koenning et al. 1999; Trudgill and Blok 2001).

The life cycle of *Meloidogyne incognita* lasts from 4 to 8 weeks, depending on the host and the environmental conditions, and consists of four molts (Moen and Perry 2009). The first molt, from juvenile J1 to infective juvenile J2 stage, occurs within the egg. During the plant-RKN interaction, newly hatched J2s move to

the root elongation zone, migrating intercellularly toward the root apical meristem and turning upwards to the vascular cylinder (Caillaud et al. 2008). Then, J2s pierce five to seven vascular parenchymatic cells with their stylet and inject secretions containing effectors that contribute to the transdifferentiation of parenchymatic host cells into giant feeding cells (GCs) (Bellafiore and Briggs 2010; Bellafiore et al. 2008; Gheysen and Mitchum 2011; Jaubert et al. 2002). Subsequently, J2s become sedentary, feed from GCs, and then, molt into J3 and J4 stages to finally reach the female adult stage. *M. incognita* reproduces via parthenogenesis and males are only formed in adverse conditions (McClure and Viglierchio 1966; Triantaphyllou 1960). Males are vermiform and leave the root, whereas females are pear-shaped and lay eggs in a protective gelatinous matrix called an egg mass onto the host root surface or within the host root (Fernandez et al. 2015).

GC differentiation involves multiple mitotic events without cytokinesis, followed by several endoreduplication cycles causing nuclear and cellular enlargement (de Almeida Engler et al. 1999, 2004; Vieira et al. 2013). GCs are also characterized by cytoskeleton rearrangements, a fragmented vacuolar system, and an increased cytoplasm density with a large number of organelles, such as mitochondria, plastids, endoplasmic reticulum, Golgi stacks, and ribosomes (Banora et al. 2011; de Almeida Engler et al. 2004; Rodiuc et al. 2014). Typical patchy thickenings and ingrowths are seen in GC walls, as encountered in transfer cell walls (Cabrera et al. 2014; Rodiuc et al. 2014).

Plant-RKN interaction studies at the cellular and the molecular levels have been performed on several host plant models, such as *Solanum lycopersicum*, *Arabidopsis thaliana*, *Lotus japonicus*, *Glycine max*, and *Medicago truncatula* for dicots (Bartlem et al. 2014; Escobar et al. 2015; Holbein et al. 2016; Mantelin et al. 2015; Wiczorek 2015) and *Oryza sativa* for monocots (Kyndt et al. 2014). Tree-RKN interactions have been scarcely studied. Previous reports have shown that some tree species, like *Salix alba* and *S. babylonica* (Santamour and Batzli 1990) as well as *Catalpa bignonioides* (Sasanelli and Pierangeli 1994), are susceptible to RKN. The most documented impact of nematodes on tree species is on olive trees, leading to a reduction of growth mainly in nurseries (Ali et al. 2014).

In view of the economic and ecological importance of forest trees, research related to tree-RKN interaction deserves investigation. Poplars (*Populus* spp.) are trees of major

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agro-economic interest with high biomass productivity, used for pulping, veneer, timber, and biofuel production (Aylott et al. 2008; Polle et al. 2013). Poplar has been developed as a model to study specific traits of woody perennial plants, such as wood formation and seasonal dormancy (Jansson and Douglas 2007). Poplar-microorganism interactions have been studied, for instance, with *Melampsora larici-populina* (Azaiez et al. 2009) and mycorrhiza, such as *Laccaria bicolor* (Felten et al. 2009).

Here, we show that poplar (*P. tremula* × *P. alba*) can be considered as a model tree for interaction studies with the RKN *M. incognita*. Morphological and histological analyses of in vitro infection of poplar by *M. incognita* and its life cycle are described. We have shown that, at the molecular level, RKN gall development was accompanied by changes in gene expression, including *expansin A (EXPA)*, *histone 3.1 (H3.1)*, and

asparagine synthase (ASNS), as well as genes associated to the monolignol biosynthesis pathway.

RESULTS

RKN galls are induced on poplar roots and allow nematodes to complete their life cycle.

To assess poplar susceptibility to *M. incognita* infection, around 100 J2 larvae were applied per 3-week-old in vitro plant. Typical root swellings caused by gall formation were observed in infected poplar roots at 7, 14, and 21 days after inoculation (dai) (Fig. 1A to C, respectively) but not in the corresponding 21-day-old uninfected roots (Fig. 1D). During gall expansion, a reddish coloration accumulated at its surface (Fig. 1A to C), probably due to the accumulation of anthocyanins in cortical gall cells (Hipskind et al. 1996). This coloration was not

Fig. 1. Histological features of root-knot nematode gall development on in vitro poplar. **A to C**, Galls at 7, 14, and 21 days after inoculation (dai). **D**, Uninfected root corresponding to infected root at 21 dai. **E to H**, Longitudinal sections of an uninfected root and of 7-, 14-, and 21-dai galls, respectively, stained with toluidine blue. The white arrows indicate nuclei. **I to K**, Longitudinal sections of 7-, 14-, and 21-dai galls, respectively, stained with 4,6-diamidino-2-phenylindole, showing enlarged nuclei in giant feeding cells (GC). C = cortex, G = gall, N = nematode, NC = neighboring cells, VT = vascular tissue, X = xylem. Asterisks (*) indicate GC. Bars = 1 mm (A to D), 150 (E to H) and 100 µm (I to K).

observed on uninfected or in gall-free infected root segments. Longitudinal sections of uninfected poplar roots (Fig. 1E) and galls, made at the three different developmental stages, were stained with toluidine blue and were observed by light microscopy

Fig. 2. *Meloidogyne incognita* female, gall, and egg mass observation and infection tests on in vitro poplar. **A**, Female of *M. incognita*, at 35 days after inoculation (dai) in a partially dissected gall, showing a typical pear-shaped morphology, as observed by scanning electron microscope. **F** = female. **B**, Uninfected and **C**, infected root at 21 dai (arrows show individual galls). **D**, Egg mass linked to a gall at 42 dai. **E**, Number of galls and egg masses per plant at 21 and 42 dai, respectively. Values are expressed as mean values $\pm$ standard error for three independent experiments, $n \geq 15$ plants per experiment. EM = egg mass. Bars = 100 μ m (A), 1 cm (B and C), and 1 mm (D).

(Fig. 1F to H). Developing galls, at 7 dai (Fig. 1F), contained GCs harboring several distinct nuclei (Fig. 1I), multiple small vacuoles, a dense cytoplasm, and were surrounded by dividing neighboring cells (NCs) within the vascular system, as typically observed on other plant hosts. At 14 and 21 dai (Fig. 1G and H, respectively), galls further developed within the root vascular tissue. Within GCs, the nuclei were numerous, enlarged, and often, clustered, showing irregular contours (Fig. 1J and 1K).

Typical pear-shaped female nematodes were observed by scanning electron microscopy (SEM) in partly dissected mature galls at 35 dai (Fig. 2A). Control and infected roots with multiple galls at 21 dai are illustrated in Figure 2B and C, respectively. Galls were recorded on infected roots at 21 dai (Fig. 2C) and egg masses at 42 dai (Fig. 2D). A mean of approximately 13 galls and five egg masses were counted per plant, demonstrating RKN infection and reproduction on in vitro grown poplar (Fig. 2E). Numerous galls at different developmental stages were observed 14 weeks after inoculation, corresponding to about two successive life cycles (Fig. 3A), indicating that eggs were able to hatch and to reinfect the plants. At this stage, suckers appeared on infected roots above and under the gall area (Fig. 3A and B), which was not observed on uninfected plants at the same age (Fig. 3C).

GCs in poplar galls show cell-wall structures typical of transfer cells.

Light and transmission electron microscopy (TEM) were used to visualize GC walls (Fig. 4). Fragments of aborted cell walls indicative of incomplete cytokinesis were observed (Fig. 4A). All GCs contained a dense cytoplasm filled with organelles (Fig. 4B), and their walls formed patchy thickenings and branched ingrowths not oriented to NCs (Fig. 4C to E). TEM images revealed that the GC walls displayed different staining intensities, probably representing different layers within the wall (Fig. 4F).

Drastic xylem proliferation in poplar galls.

Confocal microscopy was used to detect cell-wall autofluorescence under UV excitation in gall sections at 21 dai. Cell walls containing lignin usually show prominent autofluorescence, as observed in xylem elements in plant tissues (Donaldson 2013). Proliferation of xylem around GCs was conspicuous in poplar galls (Fig. 5A), compared with uninfected root (Fig. 5C). A close-up of the feeding site zone (Fig. 5B) shows a low autofluorescence of GC walls compared with xylem cell wall, suggesting the absence of lignin in the thickened GC walls. Xylem proliferation was also obvious in galls stained with toluidine blue (Fig. 5E), as compared with uninfected root (Fig. 5D).

Raman analysis reveals chemical composition of the GC wall.

Chemical composition of the GC wall was characterized by confocal Raman microspectroscopy, using galls at 21 dai. As a preliminary experiment and to validate the methodology on this material, a focus was made on the identification of cellulose, pectin, and lignin, which are the three main cell-wall components that are well-characterized with Raman microspectroscopy (Agarwal et al. 2013; Gierlinger and Schwanninger 2007; Gierlinger et al. 2012; Petry et al. 2003). Two main spectra from 464 to 1,853 cm^{-1} were identified in an 80- μ m gall cross-section comprising the whole feeding site surrounded by xylem and NCs (Supplementary Fig. S1A and B). The first spectrum presented a main peak at 1,600 cm^{-1} corresponding to lignin, which is mainly present in xylem cells, as seen during confocal microscopy observations. A second spectrum showed several major peaks, including the peak of pectin at 854 cm^{-1} and the characteristic double peak (1,098 and 1,120 cm^{-1}) corresponding to cellulose (Agarwal et al. 2013; Gierlinger et al. 2012). In

parallel, Raman analysis was performed on an uninfected root (Supplementary Fig. S2). Further emphasis on the Raman analysis of the GC wall (Fig. 6A) illustrated a similar localization pattern for pectin and cellulose (Fig. 6B and D). These compounds presented a heterogeneous distribution within the GC wall. Spectra for pectin and cellulose are shown in Figure 6C and E, respectively. No spectrum corresponding to lignin could be identified in the GC wall, confirming the lack of autofluorescence observed by confocal microscopy (Fig. 5).

RKN-associated marker genes are expressed during poplar gall formation.

To conduct expression analysis associated with poplar-RKN interaction, preliminary experiments were performed in order to select reference genes that can be used for expression normalization. A set of housekeeping genes for poplar (Pettengill et al. 2012) or for RKN-plant interaction in *A. thaliana* and *Medicago truncatula* (Baldacci-Cresp et al. 2012; Clément et al. 2009) was evaluated. Table 1 shows the expression stability of the different genes tested, using BestKeeper and NormFinder programs in uninfected roots and in galls at 7, 14, and 21 dai. As regards expression stability and considering all analyzed samples, including roots and galls, at all time points, NormFinder ranked *PTI*, *CDC2*, and *UBP22* and BestKeeper ranked *Cyc063*, *PTI*, *CDC2*, and *UBP22* as suitable marker genes (Table 1). In addition, Table 1 displays data related to expression stability with respect to comparison of galls versus roots at different stages of gall development. According to the obtained results using the two algorithms, *PTI*, *CYC063*, and *CDC2* were selected as suitable genes for expression normalization.

Gall marker genes have been reported for several plant hosts but, as far as we know, not yet for poplar or any other known host tree. These genes include defense genes usually down-regulated during early stage of gall formation, as well as genes linked to gall development, and genes involved in primary metabolism generally described as upregulated in both developing and mature galls as compared with uninfected roots. Based on previous transcriptomic analysis (Baldacci-Cresp et al. 2012; Barcala et al. 2010; Grunewald et al. 2008; Hamamouch et al. 2011; Ibrahim et al. 2011; Jammes et al. 2005; Portillo et al. 2013), we selected *pathogenesis-related protein 1* (*PR1*), *WRKY23*, and *phenylalanine ammonia-lyase 3* (*PAL3*) as defense genes, *EXPA*, *H3.1*, and *cell cycle switch protein 52A* (*CCS52A*) for gall development, and *cytosolic malate dehydrogenase* (*cMDH*), *ASNS*, and *nitrate reductase 2* (*NR2*) for carbon and nitrogen metabolism. Supplementary Table S1 gives details on the selected marker genes, their probable function, the RKN-plant interaction model, and their expression profiles upon infection.

The expression of these genes was analyzed at different stages of gall development in whole galls and was compared with their expression in corresponding uninfected poplar root (Fig. 7). Considering the defense genes, *PR1* was up-regulated by 6.7-fold in galls at 7 dai, *PAL3* was down-regulated by 1.9-fold in galls at 14 dai, whereas *WRKY23* was not differentially expressed in galls at 7, 14, or 21 dai (Fig. 7A). The genes associated with gall development, including *EXPA* and *H3.1*, were up-regulated at 7 dai (by 23.9- and 8.1-fold, respectively) and at both 14 and 21 dai (by about 9- and 2.5-fold, respectively), whereas *CCS52A* expression was not affected (Fig. 7B). For the genes involved in primary metabolism, *NR2* expression was not changed, *cMDH* was down-regulated by 1.8-fold at 7 dai but not differentially expressed at 14 and 21 dai, and *ASNS* was down-regulated by 2.3-fold at 7 dai and by 1.6-fold at both 14 and 21 dai (Fig. 7C). As a result, the expression profile of three genes can be selected as a marker for poplar-RKN interaction: the upregulation of *EXPA* and *H3.1* for gall development and the downregulation of *ASNS* for gall metabolism, similarly to *A. thaliana*, *Glycine max*, and *Medicago truncatula*.

Since xylem development is induced in galls of poplar roots (Fig. 5), the expression of eight genes involved in the monolignol biosynthetic pathway was investigated during gall development (Fig. 8). These genes, including *hydroxycinnamoyl-CoA shikimate/quinic acid hydroxycinnamoyl transferase 1* (*HCT1*), *4-coumarate 3-hydroxylase 3* (*C3H3*), *caffeoyl CoA 3-O-methyltransferase 1/2* (*CCoAOMT1/2*), *cinnamoyl-CoA reductase 2* (*CCR2*), *ferulate 5-hydroxylase 1 and 2* (*F5H1* and *F5H2*), *caffeic acid 3-O-methyltransferase* (*COMT2*), and *cinnamyl alcohol dehydrogenase 1* (*CAD1*), have been reported to be expressed in xylem (Shi et al. 2010). Their expression was analyzed in galls at 7, 14, and 21 dai and was compared with uninfected roots. As shown in Figure 8, *HCT1* was down-regulated by 1.8- and 1.4-fold in galls at 7 and 14 dai, respectively, *COMT2* was down-regulated by 1.4-fold in galls at 14 dai, and both *F5H1* and *F5H2* were down-regulated by two- to fourfold, depending on the stage of gall development analyzed, suggesting changes in the profile of monolignol and related compounds in RKN-infected poplar roots as compared with noninfected ones. The expression of the four other genes (*C3H3*, *CCoAOMT1/2*, *CCR2*, and *CAD1*) was not significantly different in infected and uninfected roots.

DISCUSSION

Poplar: a tree model host for RKN interaction.

Herein, we present poplar as a host of potential interest to study tree-RKN interaction. We show the successful

Fig. 3. De novo infection from a first inoculation and sucker production on in vitro poplar root. **A**, In vitro poplars 14 weeks after inoculation. **B**, Sucker produced close to a gall on the poplar root system. **C**, Uninfected in vitro poplars after 14 weeks. Bars = 2.1 cm (A and C) and 2 mm (B). The presence of suckers was observed on 15 plants spread on three biological replicates.

completion of the life cycle of *M. incognita* on *P. tremula* × *P. alba* roots demonstrating the induction of functional feeding sites typically called ‘galls.’ Histological analysis revealed that feeding sites in poplar roots characteristically consisted of five

to seven GCs with all distinctive features of RKN-induced galls. GCs presented a dense cytoplasm rich in organelles, multiple small vacuoles, cell-wall thickenings holding in-growths, and an increased number of oversized nuclei with

Fig. 4. Light and electron micrographs showing giant feeding cell (GC) wall thickenings at 21 days after inoculation. **A**, Section of a feeding site stained with toluidine blue. White arrows show aborted cell walls and black arrows show cell-wall thickening zones. **B**, An overview of a GC showing dense cytoplasm filled with organelles and **C** and **D**, wall ingrowths. **E**, Cell-wall ingrowths between two GCs stained by toluidine blue. **F**, An electron micrograph of a GC with a thickened cell wall (CW) in contact with neighboring cells (NC) (with large vacuoles [V]). X = xylem. **A**, Bars = 50 (A), 5 (B), 1.4 (C), 2.3 (D), 17 (E), and 2 μ m (F).

irregular shapes (Fig. 1). Characteristic cell-wall stubs resulting from an incomplete cytokinesis as well as cell-wall thickenings with profuse cell-wall ingrowths were also observed in GCs (Fig. 4). During feeding site development, nematodes matured into fertile females that laid viable egg masses capable of de novo infection (Fig. 3). Nematode life cycle was completed in about 6 weeks on in vitro-infected poplars.

The observed GC wall structures are similar to transfer cell walls and are considered to increase plasma membrane area, allowing enhanced nutrient exchange between cells (Rodiuc et al. 2014). As suggested by Baldacci-Cresp et al. (2015a), these cell-wall thickenings could also provide mechanical support to maintain cell shape in response to the high osmotic pressure present in galls. As far as we know, there is no report on the chemical composition of GC wall structures induced by RKN. Therefore, we performed Raman microspectroscopy to analyze cell-wall compounds in galls. Raman microspectroscopy is a nondestructive imaging technique, suitable for living organisms for the characterization of the molecular composition of a liquid or the outer part of a solid material (Brauchle and Schenke-Layland 2013; Gierlinger and Schwanninger 2007; Roman et al. 2011; Uragami et al. 2014). Based on this methodology, we were able to show that, in 21-dai galls, GC walls were composed of pectin and cellulose deposited heterogeneously. Besides, previous transcriptomic studies of whole galls or microdissected GCs indicate that the primary cell-wall metabolism is modified during plant-RKN interaction, as shown by the differential expression of genes coding for expansins, pectate lyases, pectin methylesterases, and glucanases (Damiani et al.

2012; Ibrahim et al. 2011; Jammes et al. 2005; Portillo et al. 2013). Raman analysis did not reveal lignin occurrence in GC walls. Similarly, by Wiesner staining, Ji et al. (2015) did not detect lignin in feeding sites in developing galls at 3, 5, and 7 dai in rice. In poplar galls, histological analysis (Fig. 5) revealed a strong natural autofluorescence indicative of drastic xylem development in galls around GCs. Xylem proliferation within RKN gall tissue was first described by Jones and Northcote (1972) in *Coleus blumei*, and this cytological feature was hypothesized to maintain vascular system continuity to compensate for anatomical disruption due to GC development and nematode presence inside the root structure (Goto et al. 2013). The overall observed features following poplar-RKN interaction are also encountered in other plant hosts, suggesting that RKN share conserved strategies to reproduce and to invade perennial plant species.

A peculiar observation was the induction of sucker formation at the poplar root–gall interface. Suckers were never observed in uninfected poplar roots during our in vitro experiments (Fig. 3). However, in vivo root suckering may occur under stress conditions as a vegetative reproduction mode of poplars from the *Populus* (*Leuce* Duby) section, of which *P. tremula* and *P. alba* are part (Slavov and Zhelev 2010). Wan et al. (2006) suggested that sucker development is possibly inhibited by the auxin produced in aerial parts and promoted by cytokinin, produced by the root apex, that accumulates in roots following disconnection from the stem. Indeed, Karczmarek et al. (2004) and Lohar et al. (2004) showed that hormonal balance is modified within gall tissue. One can hypothesize that the

Fig. 5. Xylem development in galls 21 days after inoculation and corresponding uninfected root. **A** and **B**, Autofluorescence of cell walls in a gall section and **C**, in a cross section of an uninfected root. **D** and **E**, Longitudinal sections of an uninfected root and of a gall, respectively, stained with toluidine blue. Images in **A** through **C** are represented in false color with an artificial 255 color intensity scale. FS = feeding site, X = xylem. Asterisks (*) indicate GC. Bars = 500 (**A**), 200 (**B**), 20 (**C**), 100 (**D**), and 250 μ m (**E**).

Fig. 6. Chemical characterization of giant feeding cell (GC) walls, with Raman microspectroscopy. **A**, A 80- μm section in a feeding site 21 days after inoculation, observed by light microscopy. **B**, Pectin localization in the feeding site, with a dark (no pectin) to light (maximum pectin intensity) shade range. **C**, Specific pectin-isolated spectra on the feeding site, analyzed between 700 and 1,300 cm^{-1} . **D**, Cellulose localization in the feeding site with a dark (no cellulose) to light (maximum cellulose intensity) shade range. **E**, Specific cellulose-isolated spectra on the feeding site, analyzed between 700 cm^{-1} and 1,300 cm^{-1} . Asterisks (*) indicate GC. Bars = 50 μm (A, B, and D).

formation of suckers upon nematode infection is the result of changes in the hormonal balance within the root system following RKN infection. Profiling changes in auxin and cytokinin metabolism during poplar gall development may help to decipher the physiological processes associated to sucker development in elderly RKN-infected poplar roots cultured in vitro.

Poplar RKN galls undergo conserved changes in gene expression.

Three potential marker genes, showing similar altered expression levels in poplar galls as in other nematode hosts, were selected for analysis during poplar-RKN interaction (Fig. 7). The first gene, Potri.008G088300, encoding an expansin homologous to the *Arabidopsis* *EXPA1*, was up-regulated during poplar gall development stages at 7, 14, and 21 dai. *EXPA1* was also up-regulated in *A. thaliana* galls at 7, 14, and 21 dai, together with six other *EXPA* genes and two *EXPB* genes (Jammes et al. 2005). Expansins are cell wall-loosening enzymes involved in the extension of the plant cell wall (Sampredo and Cosgrove 2005) and have been suggested to play a role in the establishment of RKN parasitism in tomato (Gal et al. 2006). A second gene, Potri.014G096900, encodes a histone H3.1 upregulated during all poplar gall development stages, similarly to its *Arabidopsis* homolog (Jammes et al. 2005). The histone H3.1 is part of the chromatin structure and the upregulation of its corresponding gene is most likely linked to transcription regulation occurring in galls (Ji et al. 2013). The third gene investigated, Potri.009G072900, coding for an ASNS (EC 6.3.5.4, an enzyme that generates asparagine from aspartate), was down-regulated during all analyzed gall developmental stages in poplar. Potri.009G072900 is homologous to *Medicago truncatula* *ASNS2-1* (MTR_5g071360) also found

Table 1. Analysis of expression stability of reference genes for normalization using NormFinder and BestKeeper softwares

NormFinder ^a				
Stability value				
Gene	All samples	R7 vs. G7	R14 vs. G14	R21 vs. G21
<i>CYC063</i>	0.02	0.018	0.027	0.018
<i>PT1</i>	0.006	0.015	0.001	0.002
<i>UBQ7</i>	0.015	0.005	0.028	0
<i>18S</i>	0.028	0.023	0.053	0.031
<i>CDC2</i>	0.009	0.017	0.009	0
<i>40S</i>	0.017	0.023	0.013	0.023
<i>UBP22</i>	0.013	0.007	0.002	0.018
BestKeeper ^b				
Correlation coefficient				
Gene	All samples	R7 vs. G7	R14 vs. G14	R21 vs. G21
<i>CYC063</i>	0.971	1.005	0.982	1.009
<i>PT1</i>	0.835	1.001	1.019	-0.898
<i>UBQ7</i>	0.786	-0.993	1.021	0.898
<i>18S</i>	-0.64	1.007	-0.961	0.995
<i>CDC2</i>	0.914	1.003	-1.033	0.984
<i>40S</i>	-0.466	-1	-1.014	-0.99
<i>UBP22</i>	0.835	1.003	1.006	0.993

^a The expression of each candidate reference gene was evaluated by the model-based and pairwise comparison approaches. A stability value close to 0 is indicative of high expression stability. The three best reference genes and values are shown in bold.

^b Each candidate reference gene was compared with the BestKeeper Index to calculate a Pearson's coefficient of correlation. Correlation coefficients near to 1 indicate greater correlation with the index and, therefore, a high expression stability. The best reference genes and values are shown in bold. G7, G14, and G21, galls at 7, 14, and 21 dai, respectively.

to be downregulated during *Medicago*-RKN interaction (Baldacci-Cresp et al. 2012).

The analysis of eight genes coding for enzymes involved in the monolignol biosynthesis pathway was investigated and revealed alteration in this biosynthesis pathway during RKN infection (Fig. 8). The biosynthesis pathway of the three main monolignols (*p*-coumaroyl, coniferyl, and sinapyl alcohols) and their precursors comprises numerous enzymatic steps. This pathway starts with the general phenylpropanoid pathway, responsible for the production of *p*-coumaric acid from phenylalanine, which is common to a variety of molecules including hydroxycinnamic acids, flavonoids, stilbenes, coumarins, and monolignols (Vanholme et al. 2012a). Following their biosynthesis, the monolignols can be polymerized and incorporated into the lignin polymer and are then called the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units (Fig. 8A) (Boerjan et al. 2003). Alternatively, the monolignol biosynthesis pathway has been involved in the defense response of plants. Actually, enzymes of this pathway are induced upon defense (Naoumkina et al. 2010) and intermediates of the monolignol biosynthesis pathway have been reported to have nematocidal properties (Ohri and Pannu 2010). In this report, we show that *HCT1*, *COMT2*, *F5H1*, and *F5H2* are downregulated in whole gall tissues in poplar. Similarly, a global downregulation of the phenylpropanoid pathway was reported in rice galls (Kyndt et al. 2012). In poplar and with respect to

lignin composition, a natural mutation of *HCT* has been shown to lead to an increase in the amount of H units (Vanholme et al. 2013) and the downregulation of *COMT*, decreasing the S unit content (Van Doorselaere et al. 1995). *F5H* has been demonstrated to be a limiting enzyme for the synthesis of S units, as the *F5H*-deficient *fah1 Arabidopsis* mutant is devoid of S units (Meyer et al. 1998), whereas the *C4H:F5H* transgenic *Arabidopsis* (Meyer et al. 1998) or poplar (Franke et al. 2000) overexpressing *F5H* have a lignin with an increased proportion of S units. Therefore, the downregulation of these four genes might suggest a modified lignin composition in poplar gall tissue.

However, it has been demonstrated that alteration of individual genes of the monolignol pathway may not only impact the lignin content and composition but have a wider impact at the metabolic level, resulting in a shift in the phenolic profile (Vanholme et al. 2012b). For instance, the *comt* and *f5h1 Arabidopsis* mutant showed a downregulation in a gene involved in both shikimate and phenylpropanoid pathways albeit having normal lignin content (Vanholme et al. 2012b), suggesting changes in phenol-related secondary metabolites that might affect physiological and developmental processes. In that context, Wuyts et al. (2006) observed a lower fecundity (fewer eggs, juveniles, and mature females) for overexpressing *C4H:F5H Arabidopsis* transgenic plants compared with the wild type. Besides, the transcriptomic analysis of transgenic tomato

Fig. 7. Identification of marker genes for poplar root-knot nematode gall development. **A**, The relative expression of genes involved in plant defense (*PR1*, *WRKY23*, and *PAL3*), **B**, gall development (*EXPA*, *H3.1*, and *CCS52A*), and **C**, gall metabolism (*NR2*, *cMDH*, and *ASNS*) was analyzed by quantitative reverse transcription-polymerase chain reaction in galls at 7, 14, and 21 days after inoculation (dai) and was compared with their expression in corresponding uninfected roots. Expression in uninfected root was normalized to 1 for each development stage and is represented by a dashed line. Values are expressed as mean values $\pm$ standard error for three independent experiments. $n \geq 15$ plants per experiment. Statistical analysis was performed using Student's *t* test. nd = not determined. * indicates $P \leq 0.05$.

Fig. 8. Relative expression of genes coding for enzymes involved in the lignin biosynthesis pathway during root-knot nematode gall development in poplar root. **A**, Schematic view of the lignin biosynthesis pathway. **B**, Relative expression evaluated by quantitative reverse transcription-polymerase chain reaction in galls compared with corresponding uninfected roots at 7, 14, and 21 days after inoculation (dai). Values are expressed as mean values $\pm$ standard error for three independent experiments. $n \geq 15$ plants per condition per experiment. Statistical analysis was performed using Student's *t* test. * indicates $P \leq 0.05$.

hairy roots overexpressing the *M. javanica* gene, encoding fatty acid- and retinol-binding protein (Mj-FAR-1), involved in nematode development and reproduction, has been shown to lead to downregulation of genes implicated in the phenylpropanoid pathway, such as *PAL2* and *4CL3* in noninfected roots (Iberkleid et al. 2015). As suggested by these authors, the nematode may repress the defense mechanisms of the plant to facilitate infection. Altogether, there is seemingly a link between RKN infection and the phenylpropanoid biosynthesis pathway. Further investigations on the occurrence and the role of phenylpropanoid-derived secondary metabolites during RKN interaction is required to better characterize RKN gall development in poplar. However, the expression analysis was made in whole poplar gall tissue including GCs, neighboring cells, and vascular tissues, therefore reflecting the global change during RKN-poplar interaction. The specific expression profile of particular cell types may be studied on microdissected tissues. In agreement with our general observation, a downregulation of the phenylpropanoid pathway was reported on microdissected GCs in both tomato and *Arabidopsis* (Portillo et al. 2013).

In conclusion, we demonstrate that poplar is a suitable host for RKN and we propose that the poplar-*M. incognita* interaction represents an attractive model to study this interface in woody perennial species. In addition, we have shown that several genes coding for enzymes of the phenylpropanoid biosynthesis pathway have an altered expression during RKN gall development, suggesting modifications in phenylpropanoid metabolism in poplar gall. As the proportion of lignified tissue, especially the xylem, is largely increased in polar galls and lignin is absent in GC walls, it is worthwhile to further investigate the impact of the modification of the phenylpropanoid pathway on poplar-RKN interaction by using specific poplar mutants in this pathway.

MATERIALS AND METHODS

Plant material, growth conditions, and nematode infection.

P. tremula × *P. alba* clone INRA 717 1-B4 was used in all experiments. Micropropagated poplars were cultured 15 days in sterile boxes (Duchefa, Haarlem, The Netherlands) containing WPM medium (Duchefa) supplemented with 0.7% agar under a photoperiod of 16 h of light and 8 h of darkness at 24°C (1,800 to 2,200 lux, five fluorescent tubes; Fluora T8 Osram agro, München, Germany). Plants were transferred to squared plates (12 × 12 cm) on WPM medium containing 1% agar. Before transfer, the medium was removed from 3 to 4 cm at the top of each plate to prevent a contact between the leaves and the medium. Plates were positioned horizontally for 2 days and were then inclined (around 45 degrees) for the rest of the experiment. To keep the roots in dark conditions, the plates were covered by aluminum foil at the level of the roots.

For nematode infection, approximately 100 surface-sterilized (Baldacci-Cresp et al. 2012) freshly hatched *M. incognita* J2 larvae were inoculated to each plant. After infection, plates were placed horizontally for 24 h and were then inclined (around 45 degrees) for the rest of the experiment. Galls (from 61 plants, four independent biological replicates) were counted at 21 dai and egg masses (from 56 plants, four independent biological replicates) were counted at 42 dai. For egg mass counting, infected roots were stained with a solution of Eosin Y (0.5 g/liter, staining of egg mass matrix) (Sigma-Aldrich, St. Louis) for 30 min, were rinsed with water, and were observed with an Olympus SZX9 stereomicroscope (Olympus, Tokyo).

For Raman hyperspectral imaging analyses, inoculated plants were cultivated in the same conditions as above, but the plates were totally covered by aluminum foil, except for a small window (2 × 12 cm) at the level of the aerial part, to limit chlorophyll synthesis due to the high autofluorescence background in Raman microspectroscopy analyses.

Morphological analysis.

Galls were collected at 7, 14, and 21 dai. Samples were fixed with 2.5% glutaraldehyde in 50 mM phosphate buffered saline (PBS), pH 7.2, for 30 min under vacuum and were incubated under rotation at 4°C for a minimum of 48 h (galls up to 14 dai) to 7 days (galls older than 14 dai). Galls were then dehydrated in increasing ethanol series (15 to 100% ethanol for 30 min each, followed by three times for 30 min in 100% ethanol). Samples were embedded in Technovit resin following manufacturer instructions (Technovit, Heraeus Kulzer, Hanau, Germany). Sections of 5 µm were stained with 0.05% toluidine blue in distilled water, and images were taken using an Olympus BX60 microscope coupled with a Color View II camera (Olympus), using standard bright field optics. For nuclei observations, 5-µm sections were stained for 15 min with 1 µg of 4',6-diamidino-2-phenylindole per milliliter, which binds specifically to DNA.

Confocal microscopy analysis.

Sections of 80 to 150 µm, formed using a vibroslicer HM 650 V (Microm, Francheville, France), were imaged with a LSM710 confocal laser-scanning microscope (Zeiss, Jena, Germany) equipped with a 20× objective, using the proprietary ZEN software. Images show the autofluorescence of the sample, under 405 nm excitation.

SEM and TEM analysis.

For SEM, samples were fixed with 4% paraformaldehyde and 2.5% glutaraldehyde in 50 mM PBS buffer, pH 7.2. Galls were then sectioned (150 µm) with a vibroslicer HM 650 V. Observations were made on a FEI Quanta 200 FEG electron microscope (FEI, Hillsboro, OR, U.S.A.) and were processed with AnalySIS and Adobe Photoshop softwares.

For TEM, the complete applied procedure was previously described by Baucher et al. (2011).

Gene expression analysis by quantitative reverse transcription-polymerase chain reaction (RT-qPCR).

For gene expression analysis, three independent biological replicates were analyzed. Each replicate was a pool of galls harvested from a minimum of 15 infected plants or a pool of roots harvested from a minimum of 25 uninfected plants. Total RNA was extracted using the Plant RNA isolation mini kit (Agilent Technology, Santa Clara, CA, U.S.A.), according to the manufacturer protocol with the following modifications. The first centrifugation was performed at 16,000 × g for 10 min before the prefiltration column step and the second centrifugation during the wash step was performed at 20,000 × g. One microgram of RNA from whole gall and uninfected roots was reverse-transcribed using the reverse transcription system kit (Promega, Fitchburg, WI, U.S.A.). qPCR reactions were performed using a Light Cycler 480 II (Roche, Basel, Switzerland) and a qPCR MasterMix Light Cycler SYBR green I (Roche). In each reaction, 5 µl of 50-fold diluted cDNA and 0.3 µM primer (primer sequences are given in Supplementary Table S2) were used. PCR was performed at 95°C for 2 min, followed by 40 to 45 cycles at 95°C for 10 s, 60°C for 45 s. Results are the mean of three independent biological experiments with technical duplicates. The specificity of the amplification was confirmed by a single peak in the dissociation curve at the end of the PCR reaction. Data were quantified using a Light Cycler II (Roche).

and were normalized with the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). The endogenous controls were validated by BestKeeper and NormFinder application (Andersen et al. 2004) as presented above. The absence of genomic DNA contamination in the RNA samples was assessed according to Baldacci-Cresp et al. (2015b).

Database, sequence, and statistical analysis.

DNA sequences were analyzed using BLAST against the databases of the National Center for Biotechnology Information (<http://blast.ncbi.nlm.nih.gov/>) and Phytozome 11 (<http://phytozome.jgi.doe.gov/pz/portal.html>).

All data represent, at minimum, three independent biological repetitions. Statistical analysis was performed using Student's *t* test ($P \leq 0.05$).

Raman hyperspectral imaging analyses.

Confocal Raman hyperspectral images of the GC walls were performed with a Labram HR Evolution (Horiba Scientific, Villeneuve-d'Ascq, France) equipped with a two-dimensional EMCCD detector ($1,600 \times 200$ pixels sensor) (Andor Technology Ltd., Belfast, Ireland), a Leica 50 $\times$ Fluotar LWD objective and a 785-nm laser (XTRA II, Toptica Photonics AG, Gräfelfing, Germany) with a power of 45 mW at the sample (five galls from three independent biological replicates). A neutral-density filter reducing the laser power of 75% was used to preserve the sample from burning. The spectral range of 464 to 1,853 cm^{-1} was used to perform the mappings. Seven accumulations of 2-s exposures were used. The spectra were collected with the LabSpec 6 (Horiba Scientific) software. The feeding site image was mapped with a step resolution of 3 μm and an image size of 94×130 pixels ($282 \times 390 \mu\text{m}$).

For data processing, once acquired, the hyperspectral images were unfolded and the spectra were smoothed (Savitzky-Golay filter with a window width of 5) (Savitzky and Golay 1964) and baseline-corrected using the asymmetric least squares (Eilers 2004) algorithm. Pure spectra of the general map were resolved using multivariate curve resolution-alternating least squares (MCR-ALS) (Felten et al. 2015) with a nonnegativity constraint. The obtained spectra were compared with those presented in the literature (Chylińska et al. 2014; Gierlinger et al. 2012; Mansfield et al. 2013). Once the spectra of lignin, cellulose, and pectin were resolved, they were used to perform classical least squares analysis on the full map. The feeding site map was analyzed by MCR-ALS with nonnegativity constraint. All computations were realized with Matlab R2014b (version 8.4; The Mathworks, Natick, MA, U.S.A.) and the PLS Toolbox 7.9.5 (Eigenvector Research, Wenatchee, WA, U.S.A.).

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