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ORIGINAL ARTICLE

# Overexpression of the *GmEXPA1* gene reduces plant susceptibility to *Meloidogyne incognita*

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## Abstract

***Key message*** **The overexpression of the soybean *GmEXPA1* gene reduces plant susceptibility to *M. incognita* by the increase of root lignification.**

**Abstract** Plant expansins are enzymes that act in a pH-dependent manner in the plant cell wall loosening and are associated with improved tolerance or resistance to abiotic or biotic stresses. Plant-parasitic nematodes (PPN) can alter the expression profile of several expansin genes in infected root cells. Studies have shown that overexpression or downregulation of particular expansin genes can reduce plant susceptibility to PPNs. Root-knot nematodes (RKN) are obligate sedentary endoparasites of the genus *Meloidogyne* spp. of which *M. incognita* is one of the most reported species. Herein, using a transcriptome dataset and real-time PCR assays were identified an expansin A gene (*GmEXPA1*; *Glyma.02G109100*) that is upregulated in the soybean nematode-resistant genotype PI595099 compared to the susceptible cultivar BRS133 during plant parasitism by *M. incognita*. To understand the role of the *GmEXPA1* gene during the interaction between soybean plant and *M. incognita* were generated stable *A. thaliana* and *N. tabacum* transgenic lines. Remarkably, both *A. thaliana* and *N. tabacum* transgenic lines overexpressing the *GmEXPA1* gene showed reduced susceptibility to *M. incognita*. Furthermore, plant growth, biomass accumulation, and seed yield were not affected in these transgenic lines. Interestingly, significant upregulation of the *NtACC oxidase* and *NtEFE26* genes, involved in ethylene biosynthesis, and *NtCCR* and *Nt4CL* genes, involved in lignin biosynthesis, was observed in roots of the *N. tabacum* transgenic lines, which also showed higher lignin content. These data suggested a possible link between *GmEXPA1* gene expression and increased lignification of the root cell wall. Therefore, these data support that engineering of the *GmEXPA1* gene in soybean offers a powerful biotechnology tool to assist in RKN management.

**Keywords** *Glycine max* · Plant-nematode interaction · Root-knot nematodes · Root lignification · Root cell wall · RKN management

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## Introduction

Expansins (EXPs) are extracellular enzymes that induce in a pH-dependent manner the plant cell wall loosening and stiffness relaxation leading to cell extension, as well as affecting several other developmental processes without causing the rupture of the hemicellulose polymers (Cosgrove 1998; Zhu et al. 2014). Conceptually, the plant expansin superfamily is classified into $\alpha$-expansin (EXPA), $\beta$-expansin (EXPB), expansin-like A, and expansin-like B subfamilies based on phylogenetic relationship (Sampedro and Cosgrove 2005). Plant expansins are typically 250–300 amino acids long and contain up to two catalytic domains (DPBB and CBM) preceded by a signal peptide (Sampedro and Cosgrove 2005; Zhu et al. 2014). The N-terminal domain (DPBB) is a six-stranded double-psi beta-barrel composed of the His-Phe-Asp motif and some conserved polar residues structurally similar to the family 45 glycosyl hydrolases (Yennawar et al. 2006; Cosgrove 2015). In contrast, the C-terminal domain (CBM) is composed of conserved aromatic amino acids required for binding to polysaccharides and organized on the surface of a $\beta$-sandwich fold similar to the motifs found in the family-63 carbohydrate-binding module (CBM63) (Georgelis et al. 2012; Cosgrove 2015). EXPAs are described as major players of acid-induced cell wall loosening, whereas EXPBs are enzymes belonging to grass pollen allergens subgroup-1 whose functions are not yet well defined (Cosgrove 2015; Sampedro et al. 2015).

Plant expansins are associated with tolerance or resistance to biotic or abiotic stresses once gene expression levels are often altered in plants under stress (Marowa et al. 2016; Chen et al. 2018). The downregulation of some expansin genes expression inhibits cell expansion and increases plant resistance to pathogens, through increased cell wall hardiness and recalcitrance (Gal et al. 2006; Ding et al. 2008). In contrast, high expression levels of other expansin genes improve tolerance to oxidative stress (Han et al. 2015), salt (Han et al. 2012; Chen et al. 2017), water deficit (Li et al. 2013), low temperature (Peng et al. 2019), and pathogens (Guimaraes et al. 2017; Perini et al. 2017). It has also been shown that plant infection by plant-parasitic nematodes (PPNs) can alter expression profiles of several expansin genes, as well as other genes also related to root cell wall (Wieczorek et al. 2006; Ithal et al. 2007; Klink et al. 2007; Fudali et al. 2008). Interestingly, stable overexpression of some expansin genes, such as the expansins-like B *AdEXLB8* gene, reduced plant susceptibility to *M. incognita* (Guimaraes et al. 2017; Brasileiro et al. 2021). So, the specific and individual role of these plant expansin genes in reducing plant susceptibility to PPNs is not yet fully understood.

Root-knot nematodes (RKNs) are obligate sedentary endoparasites belonging to the genus *Meloidogyne* spp. (Trudgill and Blok 2001). *Meloidogyne incognita* is one of the most commonly reported species inducing gall formation, consequently causing damage to several plant species of economic importance worldwide (Abad et al. 2008; Moreira et al. 2022; Mendes et al. 2022c). The RKN life cycle typically comprises five stages: eggs that will hatch into pre-parasitic second-stage juveniles (ppJ2), which upon root infection will become parasitic pJ2, and then molt to non-feeding J3 and J4 juveniles, becoming finally parasitic feeding females (Abad et al. 2008). However, the restricted availability and use of chemical control agents due to the toxicity and the reduced number of tolerant or resistant cultivars have limited the RKN control and management (Seo and Kim 2014; Bernard et al. 2017). Given this, the current agriculture has great demand for new elite traits or cultivars that show a good resistance or tolerance level to these pathogens.

Herein, we identified the *GmEXPA1* gene (*Glyma.02G109100*) as being upregulated in response to *M. incognita* in the soybean nematode-resistant genotype PI595099 compared to the nematode-susceptible cultivar BRS133. Then, we characterized the *GmEXPA1* gene by overexpressing in *Arabidopsis thaliana* and *Nicotiana tabacum* transgenic plants. The *A. thaliana* and *N. tabacum* transgenic lines showed reduced susceptibility to *M. incognita*, without penalty in plant growth, biomass accumulation, and seed yield. Two remarkable genes involved in the ethylene biosynthesis pathway and two other genes involved in the lignin biosynthesis pathway were upregulated in the transgenic roots. The biochemical analysis showed higher lignin content in transgenic roots compared with wild-type (non-transgenic) roots. Before that, the link between *GmEXPA1* gene expression and increased root lignification in transgenic lines and soybean plants, as well as the biotechnological potential of this gene in commercial soybean cultivars are discussed here.

## Material and methods

### *GmEXPA1* gene expression in soybean cultivars during plant infection by *M. incognita*

The *GmEXPA1* gene expression was considered based on RNAseq data as differentially regulated in the nematode-resistant genotype PI595099 compared with the nematode-susceptible cultivar BRS133 during soybean infection by *M. incognita* race 1 (Arraes et al. 2022; Suppl. Table S1). Then, the *GmEXPA1* gene expression level was again evaluated by real-time PCR in soybean PI595099 genotype and BRS133 cultivar inoculated with 1500 M. *incognita* ppJ2 race 1. Infected soybean plants were harvested at 0, 4, 8, 12, and 30 days after inoculation (DAI), while RNA total was

isolated using TRIzol Reagent (Invitrogen, Waltham, Massachusetts, USA). The RNA concentration was estimated using a spectrophotometer (NanoDrop 2000, Thermo Scientific, Massachusetts, USA), and integrity was evaluated by agarose gel electrophoresis. RNA samples were treated with RNase-free RQ1 DNase I (Promega, Madison, Wisconsin, USA). Then, 2 µg DNase-treated RNA was used as a template for cDNA synthesis using oligo-$(dT)_{20}$ primer and SuperScript III RT kit (Life Technologies, Carlsbad-CA, USA). The cDNA samples were diluted 1:10 (v/v) with nuclease-free water. The real-time PCR assays were performed in an Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, Massachusetts, USA) using 0.2 µM primer (Suppl. Table S3) and GoTaq qPCR Master Mix (Promega, Madison, Wisconsin, USA). The gene expression level was represented as values calculated with the 2^-ΔCT formula using the *GmCYP18* as an endogenous reference gene (Suppl. Table S3). Three biological replicates were used for each independent line and five plants for each biological replicate, while all samples were carried out in technical triplicates. Primer efficiencies and target-specific amplification were confirmed by a single and distinct peak in melting curve analysis.

### *GmEXPA1* gene sequence analysis

The *GmEXPA1* gene sequence was retrieved from Williams 82 cultivar (*Glycine max* Wm82.a4.v1 dataset) and used as a reference to find the corresponding gene sequences in the PI595099 genotype and BRS133 cultivar (Suppl. Information S1). For this, were used genome sequencing datasets generated *in-house* (unpublished data). The raw genome sequences were mapped using the STAR program (Dobin et al. 2012) and *bam* files were assembled using the Trinity genome guided v2.0.6 program (Grabherr et al. 2011). The *GmEXPA1* gene sequences were searched against the assembled genomes by the Blastn program (Altschul et al. 1990). Sequences and features from all soybean expansin A genes were retrieved from the *Glycine max* Wm82.a4.v1 dataset (Schmutz et al. 2010) by the Phytozome database v.13 (Goodstein et al. 2012). Phylogenetic analyses were performed using the Phylogeny.fr web service (Dereeper et al. 2008). For this analysis, sequences were aligned using MUSCLE software (Edgar 2004), curated by the Gblocks model, and phylogenetic trees were performed using the maximum likelihood estimation method by PhyML software with approximate likelihood ratio test (aLRT) SH-like branch support and GTR and WAG substitution model for nucleotide and amino acid sequences, respectively. Pairwise identity matrices from nucleotide and amino acid sequences were generated using the Sequence Demarcation Tool version 1.2 software (Muhire et al. 2014). Positional conservation of the Expansin A domain (Cdd:PLN00050) domain was generated from multiple sequence alignments by Color Align Conservation software (Stothard 2000). In addition, conserved domains in gene or protein sequences were checked using the Conserved Domain Database (CDD) (Marchler-Bauer et al. 2017). The organ and tissue-specific expression of the soybean expansin A genes were presented by a heatmap plot created by the PhytoMine tool using all gene expression data in the tissue- and organ-specific expression database.

### Plant genetic transformation mediated by *Agrobacterium tumefaciens*

The binary vector was synthesized and assembled by Epoch Life Science (Sugar Land, TX, EUA). The *GmEXPA1* gene (CDS sequence) was cloned under control of the CaMV35S promoter and *in tandem* fused with *GFP* reporter protein gene. The *bar/PAT* gene was used as a selection marker gene also under control of the CaMV35S promoter (Fig. 1a). *A. thaliana* Col-0 was transformed by the floral dip method (Clough and Bent 1998), while *N. tabacum* var. SR1 was transformed as described by Park et al. (1998). The *N. tabacum* lines were selected in vitro using 5 mg/L glufosinate-ammonium (Finale, Liberty Link, Bayer). Both *A. thaliana* and *N. tabacum* transgenic plants were screened in vivo by glufosinate-ammonium spray (64 mg/L glufosinate-ammonium). The surviving transgenic plants were genotyped by PCR using specific primers (Suppl. Table S3). In addition, QuickStix™ Kit (Envirologix, Inc., USA) was used for PAT/bar protein detection in transgenic plants according to the manufacturer's instructions. The plant generations were advanced with all transgenic lines (10 from *A. thaliana* and 26 from *N. tabacum*), while several homozygous lines (*A. thaliana*: AtEv1 to AtEv3; *N. tabacum*: NtEv1 to NtEv6) were chosen for the growth room or greenhouse bioassays.

### Plant growth, biomass, and seed yield

*A. thaliana* and *N. tabacum* transgenic lines were evaluated for growth, biomass (leaf and root), and seed yield under growth room or greenhouse conditions. For this, *A. thaliana* seeds were placed on plates containing half-strength Murashige and Skoog (Murashige and Skoog 1962) supplemented with 0.8% agar and 1% sucrose (w/v), and kept in the dark for three days at 4 °C for stratification. Two-week-old seedlings were transferred to 50 mL cups containing commercial substrate (Plantmax) and maintained in a growth room at 22 °C with 100 µmol photons $m^{-2}$ $s^{-1}$ light intensity, and 16/8 h photoperiod. For *A. thaliana* root architecture analysis, newly germinated seeds were transferred to Petri plates containing MS ½ supplemented with 7% Phytagel (Sigma-Aldrich, San Luis, Missouri, USA) and 1% sucrose (w/v). Then, plates were maintained inverted in

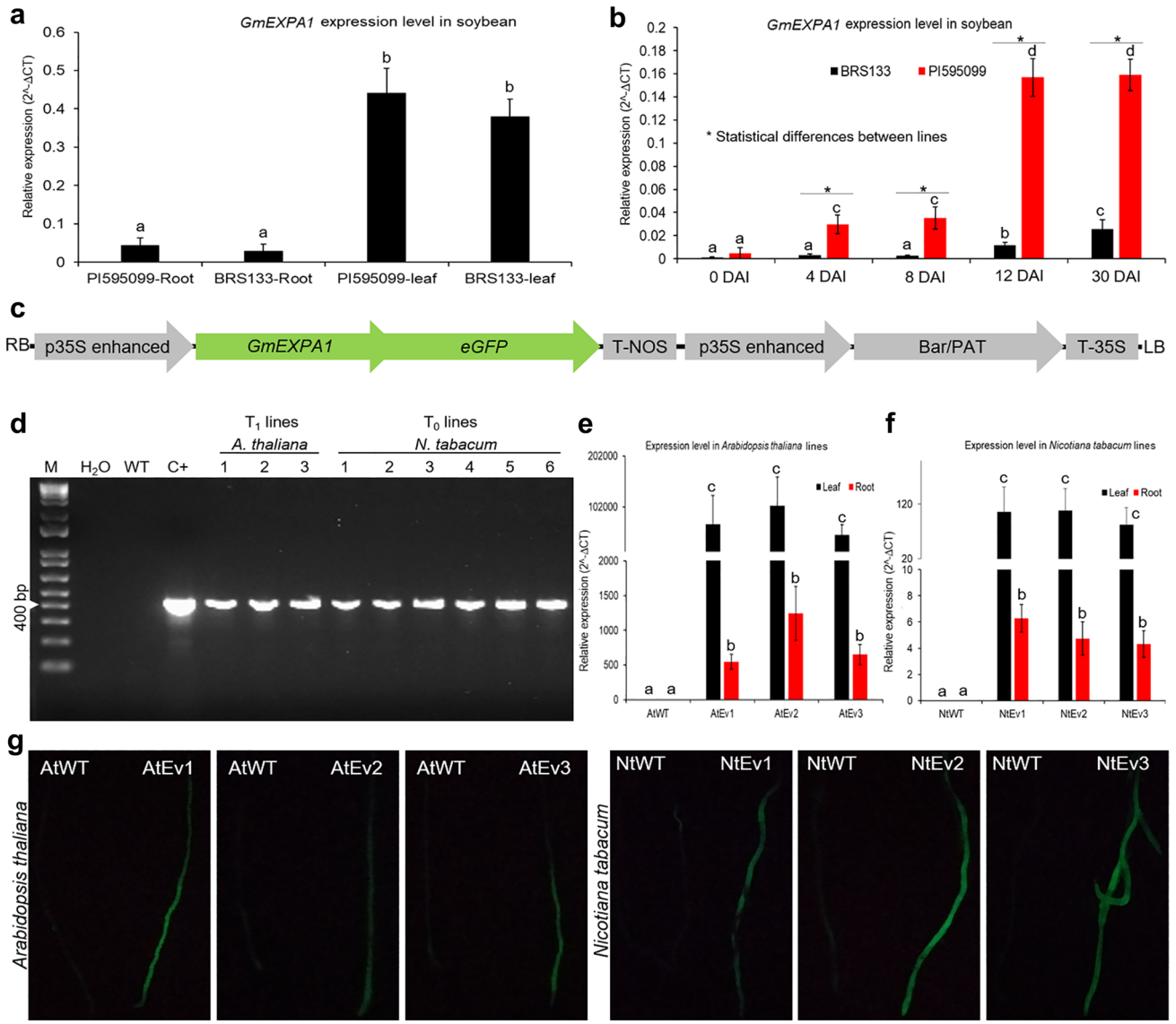


**Fig. 1** Expression level of *GmEXPA1* (*Glyma.02G109100*) gene in soybean, and genetic transformation of *A. thaliana* and *N. tabacum*. **a** *GmEXPA1* gene expression in soybean leaves and roots of nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133. Error bars represent the standard deviation of three biological replicates composed of four plants each. Plants were evaluated at the second trifolium opening stage. **b** *GmEXPA1* gene expression in nematode-resistant genotype and nematode-susceptible cultivar soybean roots at 0, 4, 8, 12, and 30 days after inoculation (DAI) of *M. incognita* ppJ2 race 1. The relative gene expression was represented by values calculated with the 2^-ΔCT formula using *GmCYP18* as an endogenous reference gene (Suppl. Table S3). Error bars represent confidence intervals for three biological replicates, while each biological consisted of five plants. Different letters indicate significant differences between nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133 according to Tukey's test at a 5% significance level. The hashtag indicates significant differences between treatment at 4, 8, 12, and 30 DAI compared to 0 DAI according to Tukey's test at a 5% significance level. **c** T-DNA overview for constitutive overexpression of *GmEXPA1* (*Glyma.02G109100*) gene, used in plant genetic transformation mediated by *A. tumefaciens*. **d** PCR detection of the transgene inserted in *A. thaliana* $T_1$ and *N. tabacum* $T_0$ transgenic lines using specific primers to the *GFP* gene. Marker: 1.0-kb DNA ladder (Invitrogen, Waltham, Massachusetts, USA, Cat. 10,787,018); WT: wild-type plant used as a negative control to PCR analyses and bioassays performed under growth room or greenhouse conditions; C+: DNA plasmid used as a positive control for PCR analyses. *GmEXPA1* gene expression profile in leaves and roots of the **e** *A. thaliana* and **f** *N. tabacum* transgenic lines using real-time PCR. The gene expression levels were represented by values calculated with the 2^-ΔCT formula. The *AtActin 2* and *NtActin 4* were used as endogenous reference genes in *A. thaliana* and *N. tabacum*, respectively (Suppl. Table S3). Error bars represent confidence intervals for three biological replicates, and each biological replicate consisted of four plants. **g** Visualization of the GmEXPA1 protein fused to GFP protein in transgenic roots of *A. thaliana* and *N. tabacum* lines was conducted under a fluorescence stereomicroscope (Leica M205 FA) with GFP Long Pass filter (excitation 395–455 and emission 480 nm)

the same growth room as described above. For biomass and seed yield, 10 to 15 plants for each *A. thaliana* line were evaluated, while root architecture analysis was performed for at least 20 plants for each transgenic line. In contrast, *N. tabacum* transgenic plants were germinated in a commercial substrate, transplanted into pots containing sand/soil (1:1 w/w), maintained under greenhouse conditions, and evaluated after 30 days.

### *GmEXPA1* gene expression in transgenic lines

Leaves and roots from *A. thaliana* and *N. tabacum* plants of the three transgenic lines were harvested and total RNA was isolated using TRIzol Reagent (Invitrogen, Waltham, Massachusetts, USA). High-purity RNA samples were used for cDNA synthesis as described above. The cDNA samples were diluted 1:10 (v/v) with nuclease-free water and used in real-time PCR assays as described above. The *AtActin 2* and *NtActin 4* were used as reference genes in *A. thaliana* and *N. tabacum*, respectively (Suppl. Table S3). Three biological replicates for each transgenic line and four plants for each biological replicate were used, while all cDNA samples were carried out in technical triplicates. Primer efficiencies and target-specific amplification were confirmed by a single distinct peak in the melting curve analysis. The relative expression level was calculated using the 2^-ΔCT formula (Schmittgen and Livak 2008). In addition, the GmEXPA1 fused to the GFP protein was detected in roots of *A. thaliana* and *N. tabacum* transgenic lines using fluorescence stereomicroscope (Leica M205 FA) with GFP Long Pass filter (excitation 395–455 and emission 480 nm).

### Susceptibility level to *M. incognita* of transgenic lines

The *M. incognita* ppJ2 race 3 inoculum was obtained from tomato plants cv. Santa clara kept under greenhouse conditions. Infected roots were washed and macerated, while eggs were harvested using 100 to 550 µm sieves (Hussey and Barker 1973). Subsequently, eggs were hatched in the dark under aerobic conditions at 28 °C, and ppJ2s were harvested every two days, decanted, and counted under a binocular microscope using counting chambers. *A. thaliana* were transplanted into pots containing 45 g autoclaved sand/substrate mixture (1:1; w/w) and kept in a growth room as described above. Two-week after transplanting, plants were inoculated with 500 M. *incognita* ppJ2. Fifteen plants per transgenic line were used while wild-type plants were used as a negative control. After 60 DAI, plants were evaluated for the number of eggs per gram of root, number of hatched ppJ2 per gram of root, number of gall per plant and, then, the nematode reproduction factor was calculated. The *M. incognita* reproduction factor was determined using Oostenbrink's formula as follows: nematode reproduction factor = the final number of ppJ2/initial number of ppJ2 or nematode final population/initial population (Oostenbrink 1966; Windham and Williams 1987). In contrast, *N. tabacum* plants were transplanted to pots containing 125 g sterile sand/soil mixture (1:1; w/w) and kept under greenhouse conditions. Fifteen days after transplanting, plants were inoculated with 1,000 freshly hatched *M. incognita* ppJ2. Sixteen plants per transgenic line were used while wild-type plants were used as a negative control. After 60 DAI, plants were evaluated for the number of eggs per gram of root, number of hatched ppJ2 per gram of root, number of gall per gram of root and, then, the nematode reproduction factor was calculated as described above. For root morphological analysis, galls were collected from nematode-infected roots of *A. thaliana* wild-type and transgenic lines at 10 and 45 DAI. Galls were then fixed in 2% glutaraldehyde in 50 mM PIPES buffer pH 6.9, dehydrated, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). Galls were sectioned at 3 µm, stained in 0.05% toluidine blue, and mounted in Depex (Sigma-Aldrich, San Luis, Missouri, USA). The microscopy analysis was performed using bright-field optics and images were acquired with a digital camera (Axiocam, Carl Zeiss, Berlin, Germany).

### Marker gene expression profiles in uninfected *N. tabacum* transgenic lines

From two *N. tabacum* transgenic lines (NtEv1 and NtEV2) with about 30 days old maintained under greenhouse conditions, roots were collected and total RNA was isolated using TRIzol Reagent (Invitrogen, Waltham, Massachusetts, USA) as described above. DNA-free RNA samples were used for cDNA synthesis and the gene expression level was evaluated by real-time PCR. The following defense marker genes *NtGOLS2*, *NtP5CS*, *NtPR2*, *NtHSR201*, *NtACC oxidase*, *NtGST1*, *NtEFE26*, and *NtEDS1* (Suppl. Table S3) were evaluated in uninfected transgenic lines. Then, the expression profile of the *NtCCR* and *Nt4CL* genes, which are directly involved in the lignin biosynthesis pathway (Song et al. 2021), was evaluated in these same transgenic lines and wild-type plants. The *NtActin 4* was used as an endogenous reference gene (Suppl. Table S3). All cDNA samples were carried out in technical triplicates, while each treatment was composed of three biological replicates composed of four plants.

### Lignin content in roots of *N. tabacum* transgenic lines

Lignin quantification was performed in roots of two *N. tabacum* transgenic lines (NtEv1 and NtEv2) after total protein removal according described to Moreira-Vilar et al. (2014).

After that, 20 mg root dry mass was homogenized in 50 mM potassium phosphate buffer and washed successively with 1% Triton X-100, 1 M sodium chloride, and twice with pure acetone, a protein-free cell wall was obtained. The cell wall samples were washed with water and dried at 45 ℃, and 1 mg of each sample was suspended in 25% acetyl bromide in acetic acid and incubated at 70 ℃ for 30 min. Then, 200 µL of a solution containing 1–5 M sodium hydroxide, 0–5 M hydroxylamine hydrochloride, and glacial acetic acid were added to the mixture. After 1400 *g* centrifugation for 5 min, the absorbance of the supernatant was measured at 280 nm.

## Results

### *GmEXPA1* gene expression in nontransgenic soybean cultivars

The real-time PCR data showed a higher *GmEXPA1* gene expression in soybean leaves than roots, both for the nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133 (Fig. 1a). This *GmEXPA1* gene expression profile in different soybean organs was consistent with information retrieved from the Phytozome database (using the PhytoMine tool), indicating that *GmEXPA1* gene is indeed highly expressed in different soybean organs, most prominently in leaf, stem, and pod, and including young roots (Suppl. Fig. S1e). The 49 soybean expansin A genes were previously organized into five groups by Zhu et al. (2014), with the *GmEXPA1* gene belonging to group I (Suppl. Fig. S1e). In addition, the *GmEXPA1* gene showed an expression profile with a highly Pearson correlation for the following expansin A genes: *Glyma.01G050100*, *Glyma.02G240900*, *Glyma.04G222210*, *Glyma.06G143300*, *Glyma.09G236200*, and *Glyma.011G160000* (Suppl. Fig. S1f). In our previous study using a RNAseq approach, the *GmEXPA1* gene was identified as upregulated in both genotypes (PI595099 and BRS133) during plant infection by *M. incognita* race 1 (Suppl. Table S1 and Suppl. File S1). Among these 49 expansin A genes in these two soybean genotypes, the *GmEXPA1* gene showed the higher upregulation throughout the plant infection cycle by the nematode, while *Glyma.01G050100* gene showed the expression profile closest to the *GmEXPA1* gene (Suppl. Fig. S2a and S2b). In this way, to confirm this *GmEXPA1* gene expression modulation in response to plant infection by *M. incognita*, soybean genotype PI595099 and cultivar BRS133 were inoculated with *M. incognita* ppJ2 race 1, and the gene expression profile was monitored by real-time PCR at 0 (previous to inoculation), 4, 8, 12, and 30 DAI. The *GmEXPA1* gene expression was increasingly upregulated at 4 to 30 DAI in both soybean genotype and cultivar, but higher gene upregulation was observed in the nematode-resistant genotype PI595099 (Fig. 1b). So, the *GmEXPA1* gene expression profile observed by real-time PCR validated the RNAseq data (Suppl. Table S1; Suppl. File S1). Therefore, these data showed that the *GmEXPA1* gene was significantly upregulated in the nematode-resistant genotype PI595099 compared to the susceptible cultivar BRS133 during plant infection by *M. incognita*. These data provided new evidence of a link between the *GmEXPA1* gene upregulation with reduced plant susceptibility to *M. incognita*.

### Sequence analysis of the soybean expansin A genes

The *GmEXPA1* gene sequences were retrieved from both genomes of the genotype PI595099 and cultivar BRS133 using the cultivar Willians 82 as a reference genome (Suppl. Fig. S1a to S1d; Suppl. Information S1). The two *GmEXPA1* gene copies were identified in the genome of cultivar PI595099 and cultivar BRS133, and at least one of these *GmEXPA1* copies in each soybean genotype or cultivar showed high identities (100%) for the transcript, CDS, and amino acid sequences when compared with *GmEXPA1* (*Glyma.02G109100*) gene from reference cultivar Willians 82 (Suppl. Fig. S1a to S1d; Suppl. Information S1). In a previous study, Zhu et al. (2014) identified in the soybean (*Glycine max*) genome and grouped the 49 expansin A genes. Herein, in this study was summarized the sequence characteristics of these 49 (*GmEXPA1* to *GmEXPA49*) genes (Suppl. Table S2). Interestingly, all these 49 genes showed high sequence conservation in the typical Cdd:PLN00050 conserved domain (Suppl. Fig. S3). Additional sequence (nucleotide and amino acid) and phylogenetic analysis suggested that the *GmEXPA1* gene has a higher identity and evolutionary linkage to *Glyma.07G229000* and *Glyma.20G033900* genes (Suppl. Fig. S4a to S4b; Suppl. Fig. S5a to S5b). Interestingly, among these 49 expansin genes, the *GmEXPA1* gene was the most upregulated gene in both genotype PI595099 and cultivar BRS133 during plant infection by *M. incognita* (Suppl. File S1). Finally, sequence characteristics confirmed that the *GmEXPA1* gene is highly conserved in the three soybean cultivars studied here, and gene expression analysis confirmed that the *GmEXPA1* gene was indeed the major expansin A gene modulated during soybean parasitism by *M. incognita*.

### *GmEXPA1* gene overexpression reduces plant susceptibility to *M. incognita*

Ten transgenic lines of *A. thaliana* and 26 transgenic lines of *N. tabacum* overexpressing the *GmEXPA1* gene were successfully generated (Fig. 1c). The real-time PCR data from three *A. thaliana* transgenic lines and other three *N. tabacum* transgenic lines showed that *GmEXPA1* gene expression was

higher in leaves than in roots of both *A. thaliana* (Fig. 1e) and *N. tabacum* (Fig. 1f) transgenic lines. Also, the *GmEXPA1* gene fused to the *GFP* gene allowed to confirm the presence of these proteins in transgenic roots (Fig. 1g). Three and six *A. thaliana* and *N. tabacum* transgenic lines, respectively, were selected for further studies according to the higher *GmEXPA1* gene expression level (Suppl. Fig. S6a and S6b). Subsequently, these transgenic lines were challenged by *M. incognita* ppJ2 race 3, and the plant susceptibility level was determined after 60 DAI by counting the number of eggs and number of hatched ppJ2, the number of gall and, then, the nematode reproduction factor was estimated (Fig. 2a–h). In all *A. thaliana* transgenic lines, a significant reduction in the number of eggs and hatched ppJ2 per gram of roots (Fig. 2a) was recorded, the number of galls (Fig. 2b), and the nematode reproduction factor (Fig. 2c) ranged from 60 to 90%, compared with wild-type plants. Similarly, reduced plant susceptibility was also observed in *A. thaliana* transgenic lines infected with *M. incognita* pJ2 strain Morelos, when a reduced number of galls and number of egg masses per plant were observed (Fig. 2d). In addition, the gall morphology of these *A. thaliana* transgenic lines infected with *M. incognita* pJ2 strain Morelos was evaluated after 10 and 45 DAI. Interestingly, galls and giant cells overexpressing the *GmEXPA1* gene were smaller; the feeding cells were more vacuolated and apparently contained fewer neighboring cells compared with wild-type plants (Fig. 2i–l). These data suggested that this gall phenotype probably resulted in delayed nematode development. The *GmEXPA1* gene overexpression in *N. tabacum* also hampered nematode development and resulted in reduced nematode reproduction (Fig. 2e). A reduction in the number of galls and hatched ppJ2 was also observed, while the nematode reproduction factor decreased from 30 to 90% in transgenic lines compared with wild-type plants (Fig. 2f–h). Therefore, these collective data showed that higher *GmEXPA1* gene expression can be associated with decreased susceptibility of *A. thaliana* and *N. tabacum* to *M. incognita*.

### *GmEXPA1* gene overexpression did not result in yield penalty in transgenic lines

Uninfected plants from *A. thaliana* and *N. tabacum* transgenic lines were evaluated under growth room or greenhouse conditions for plant growth, biomass, and seed yield. *A. thaliana* transgenic lines showed equivalent vegetative biomass (Fig. 3a), root biomass and seed yield (Fig. 3b), and primary root length, but a slightly higher number of lateral roots (Fig. 3c, d) compared with wild-type plants. Similarly, plants from *N. tabacum* transgenic lines also showed equivalent vegetative and root biomass yield (Fig. 3e). Therefore, these data indicated that *GmEXPA1* gene overexpression did not result in an apparent yield penalty in transgenic lines, but small changes in the root architecture were observed.

### Marker genes expression profile in *N. tabacum* transgenic lines

The *NtGOLS2*, *NtP5CS*, *NtPR2*, *NtHSR201*, *NtGST1*, and *NtEDS1* genes did not show significant differences in the expression profile between transgenic lines compared with wild-type plants (data not shown). However, significant upregulation of *NtACC oxidase* (Fig. 4a) and *NtEFE26* (Fig. 4b) genes was observed in roots of uninfected *N. tabacum* transgenic lines overexpressing the *GmEXPA1* gene. Interestingly, these two upregulated genes in the transgenic lines are related to the ethylene biosynthesis pathway, which might be linked to the lignification of the root cell wall. In accordance, the lignin biosynthesis pathway genes *NtCCR* and *Nt4CL* were also upregulated in at least one of the transgenic lines evaluated compared with wild-type control plants (Fig. 4c, d). These gene expression data suggested that the *GmEXPA1* gene can act by increasing the lignification of the root cell wall of transgenic lines or in soybean root tissues infected by *M. incognita*.

### Lignin content in roots of *N. tabacum* transgenic lines

Significant increase was observed in the lignin content in roots of uninfected *N. tabacum* transgenic lines compared with wild-type plants (Fig. 4e). These data supported that changes in the root cell wall occurred under higher *GmEXPA1* gene expression levels and may be associated with reduced plant susceptibility to *M. incognita*.

## Discussion

Soybean is one of the major agricultural commodities worldwide and important raw material for many essential products or by-products (Hartman et al. 2011; Hamawaki et al. 2019). The improvement of crop systems and/or development of new traits or soybean cultivars more productive and less susceptible to biotic and abiotic stresses are primary measures currently focusing on making soybean cultivation profitable and increasingly sustainable (Fragoso et al. 2022). Among the main constraints in soybean crops, yield losses caused by the high RKN and cyst nematode incidence have increased worldwide (Bernard et al. 2017; Gillet et al. 2017). The annual crop rotation (for example between soybeans, corn, and cotton) is not effective in reducing plant infection by nematodes. Thus, the development of new traits and soybean cultivars with reduced susceptibility to RKN are extremely important

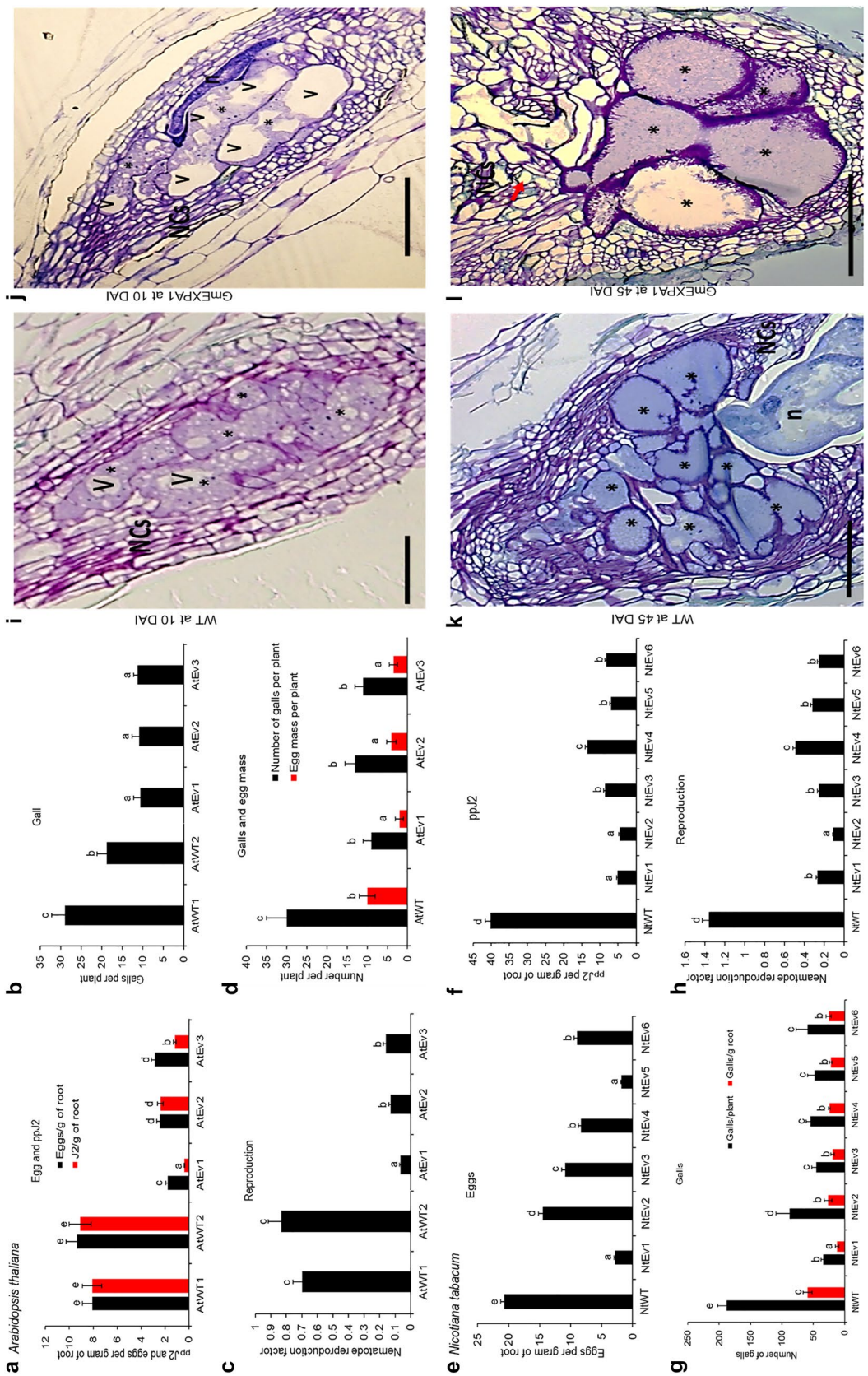


**Fig. 2** Susceptibility level to *M. incognita* of the *A. thaliana* and *N. tabacum* transgenic lines overexpressing the *GmEXPA1* (*Glyma.02G109100*) gene. **a** Number of eggs per gram of root and number of ppJ2 per gram of root, **b** number of galls per plant or per gram of root, and **c** nematode reproduction factor in *A. thaliana* transgenic lines after plant infection by *M. incognita* race 3. **d** Number of galls per plant and number of egg masses per plant measured at 45 DAI with *M. incognita* ppJ2 strain Morelos. Error bars represent confidence interval for 12 biological replicates ($n = 12$ plants). Different letters indicate significant differences according to Tukey's test at a 5% significance level. **e** Number of eggs per gram of root, **f** number of ppJ2 per gram of root, **g** number of galls per plant or per gram of root, and **h** nematode reproduction factor in *N. tabacum* transgenic lines after plant infection by *M. incognita* race 3. Error bars represent confidence intervals corresponding to three technical replicates composed of 15 plants ($n = 15$ plants). **i** Bright-field images of toluidine blue-stained gall sections illustrated gall morphology of wild-type plants after 10 DAI; **j** galls from transgenic lines after 45 DAI; and **l** galls from transgenic lines after 45 DAI. The red arrow indicates a group of neighboring cells with abnormally dividing neighboring cells or aborted giant cells. *, giant cell; *NCs* neighboring cells, *n* nematode, *v* vacuoles. Bars = 50 µm

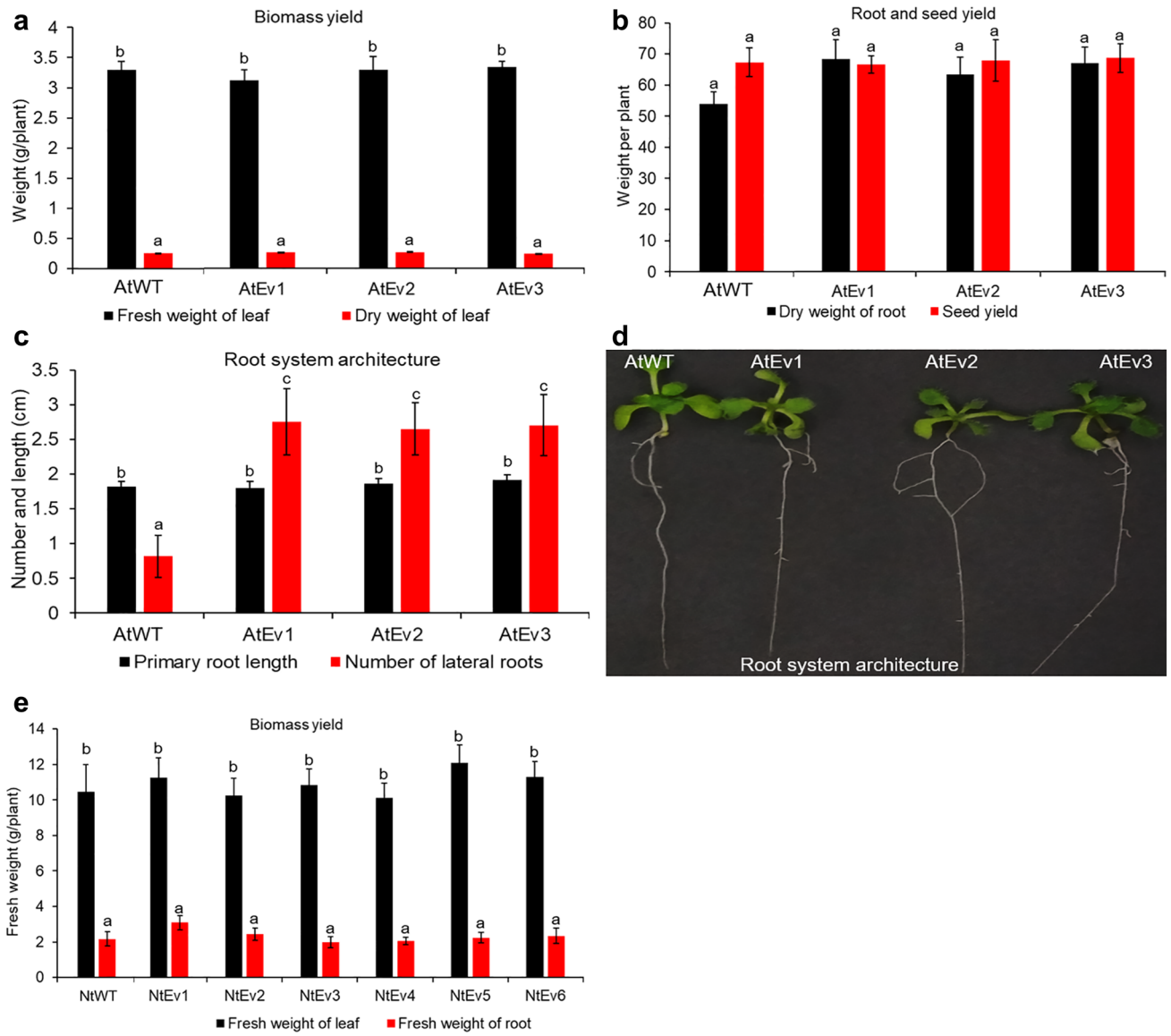


**Fig. 3** Plant growth, vegetative and root biomass, and seed yield of uninfected *A. thaliana* and *N. tabacum* transgenic lines under growth room or greenhouse conditions. **a** Fresh and dry leaves biomass of three *A. thaliana* transgenic lines. Error bars represent confidence interval for 15 biological replicates ($n = 15$ plants). **b** Root dry weight and seed yield of three *A. thaliana* transgenic lines compared with wild-type (WT) plants. Error bars represent confidence interval for 15 biological replicates ($n = 15$ plants). **c** and **d** Root system architecture (primary root length and the number of lateral roots) of three *A. thaliana* transgenic lines compared with wild-type plants kept in vitro under growth room conditions. Error bars represent confidence interval for 20 biological replicates ($n = 20$ plants). **e** Leaf fresh weight and root fresh weight of six *N. tabacum* transgenic lines compared with wild-type plants. Error bars represent confidence interval for 16 biological replicates ($n = 16$ plants). Different letters indicate significant differences according to Tukey's test at a 5% significance level

to overcome these drawbacks (Kim et al. 2011; Jiao et al. 2015; Ali et al. 2017). The prospection of new powerful genes associated with reduced plant susceptibility is essential for the generation of these new cultivars, leading to more efficient management of these pathogens (Bellafiore et al. 2008; Basso et al. 2019, 2020a; Mejias et al. 2019; Mendes et al. 2021b). The *M. incognita*, *Rotylenchulus reniformis*, and *Heterodera glycines* are the major species responsible for high annual losses in soybean yield worldwide (Wilkes et al. 2020; Basso et al. 2020b; Tylka and Marett 2021). In this present work, through RNAseq and real-time PCR data generated from two contrasting genotypes: nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133, were identified an expansin A gene as differentially expressed and associated with reduced soybean susceptibility to *M. incognita*. The soybean genotype PI595099 is consistently considered resistant to almost all economically important nematode

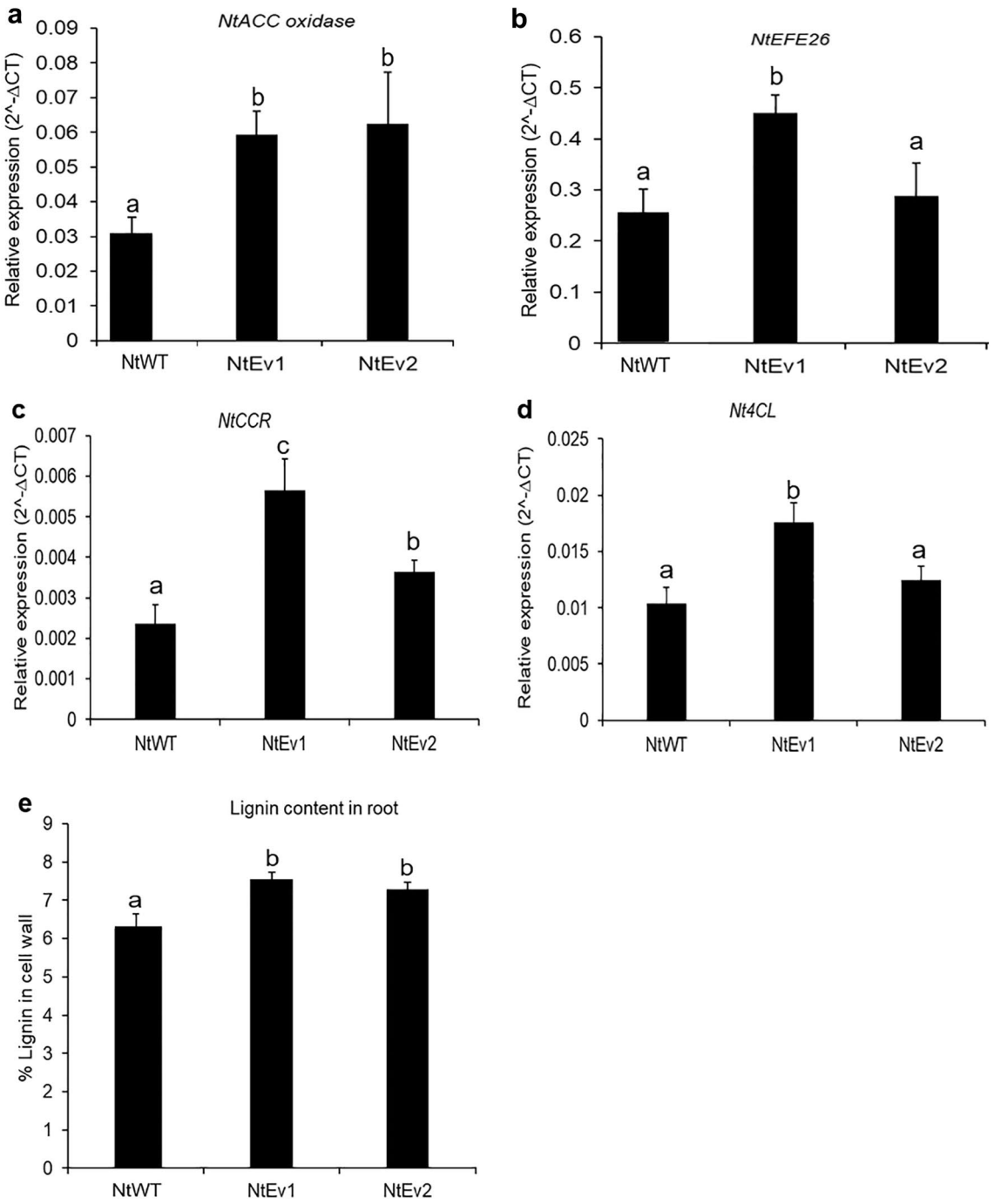


**Fig. 4** Gene expression profile and lignin content in roots of uninfected *N. tabacum* transgenic lines. The expression level of ethylene biosynthesis pathway-associated genes, **a** *NtACC oxidase* and **b** *NtEFE26.* The gene expression level of lignin biosynthesis pathway, **c** *NtCCR* and **d** *Nt4CL.* The *NtActin 4* was used as a reference gene (Suppl. Table S3). Error bars represent confidence intervals for three biological replicates composed of four plants each. **e** Lignin content in roots harvested from uninfected *N. tabacum* transgenic lines compared with wild-type (WT) plants. Error bars represent confidence intervals for five biological replicates composed of 30 plants each. Different letters indicate statistical significance according to Tukey's test at a 5% significance level

species, while the soybean cultivar BRS133 is considered highly susceptible (Mendes et al. 2021a, b; Basso et al. 2022).

This previous RNAseq study using these contrasting soybean genotype and cultivar, allowed us to identify several genes as being significantly modulated during soybean parasitism by *M. incognita*. Among them, the *GmEXPA1*

gene attracted our special attention since it was upregulated in both soybean genotype and cultivar in response to plant infection by *M. incognita*. The real-time PCR data showed that the *GmEXPA1* gene is significantly expressed in both roots and leaves of uninfected soybean plants, but up to 10 times more expressed in leaves compared with roots. In agreement, in silico expression data showed that this gene has strong expression also in soybean pods and stems. In contrast, the lowest expression levels were found only in soybean roots under high concentrations of nitrogen or when in symbiotic interactions. This higher expression of the *GmEXPA1* gene in uninfected soybean leaves can indicate that this expansin A protein may act in some way by conferring greater rigidity or relaxation of the cell wall as a natural mechanism for adapting plant tissues to adverse or natural conditions (Cosgrove 2015). At the same time, the expression of the *GmEXPA1* gene in uninfected soybean roots must also meet the same objective, and the lower expression level of this gene in root tissues compared with soybean leaves may be dependent on the quantitative demand of this expansin A protein for tissue-specific adaptation of the cell wall when under certain conditions. Regarding its expression in infected plants, the *GmEXPA1* gene was strongly more upregulated in roots of nematode-resistant genotype PI595099 than in the nematode-susceptible cultivar BRS133 during the early stages of plant infection by *M. incognita*. Curiously, in general, the expansin genes act on the cell wall, but can indirectly act on different plant developmental processes not fully understood ranging from plant growth to plant defense during various biotic and abiotic stresses (Chen et al. 2016, 2020; Muthusamy et al. 2020; Narváez-Barragán et al. 2020). Although different soybean expansin A proteins can play different roles, they are almost all localized in the plant cell wall (Guo et al. 2011; Li et al. 2014, 2015; Lo et al. 2015; Nawaz et al. 2017; Montechiarini et al. 2021; Yang et al. 2021). At this moment, better understanding the role of the *GmEXPA1* gene that results in reduced plant susceptibility to *M. incognita* is of great importance. In order to address this point, were generated several *A. thaliana* and *N. tabacum* transgenic lines constitutively overexpressing the *GmEXPA1* gene. The bioassay data, in which we evaluated the reduction in the number of ppJ2, eggs, galls, and the nematode reproduction factor, showed that both *A. thaliana* and *N. tabacum* transgenic lines were less susceptible to *M. incognita* compared with wild-type plants. Meanwhile, the gall morphology data revealed that nematodes that parasitized transgenic roots developed anomalies, with evident delay in the progress of plant infection, development of feeding sites, and gall formation. These phenotypic characteristics and the previous information based on RNAseq and real-time PCR data supported our initial hypothesis that *GmEXPA1* gene expression can be likely associated with reduced soybean susceptibility to *M. incognita*. Curiously, among the 49 expansin A genes exploited in our RNAseq data, only the *GmEXPA1* gene and, with less intensity, the *Glyma.01G050100* gene showed consistent upregulation in soybean galls during plant parasitism by *M. incognita*. Similar to this, Wieczorek et al. (2006) showed that several *AtEXPA* and *AtEXPB* genes were upregulated specifically in syncytia caused by *Heterodera schachtii* but not in surrounding *A. thaliana* root tissue, indicating that these expansin genes can act in cell growth and division, as well as in the cell wall disassembly and expansion during syncytium formation. Collaborating with this observation, Zhang et al. (2017) showed that certain soybean expansin genes were also up- or downregulated in response to *H. glycines*. Curiously, in our study, some expansin A genes that were previously considered upregulated during the plant-pathogen interaction, mainly nematodes, here it was found that their ortholog genes in soybean were not differentially modulated during plant infection by *M. incognita*.

On the other hand, the plant phenotype data showed that *GmEXPA1* gene overexpression did not significantly affect plant growth, vegetative or root biomass accumulation, and seed yield in *A. thaliana* and *N. tabacum* transgenic lines when grown under growth room or greenhouse conditions. However, significant changes were observed in root architecture of the *A. thaliana* transgenic lines, where were verified a significant increase in the number of lateral roots, although these were apparently thinner compared with wild-type plants. These remarkable phenotypic changes in transgenic roots can be related initially to the role of expansins in acting on cell wall loosening, likely causing cell extension and shrinkage (Lee et al. 2003; Ma et al. 2013; Kong et al. 2019). Similarly, Liu et al. (2021) showed that *AtEXPA4* gene overexpression in *A. thaliana* improved primary root elongation. Also, Abbasi et al. (2021) showed that *AtEXPA18* gene overexpression in *N. tabacum* resulted in improved drought stress tolerance and higher biomass yield in transgenic plants under drought stress conditions. Similarly, Calderini et al. (2021) showed that ectopic overexpression of the *TaEXPA6* gene to the wheat endosperm, aleurone, and pericarp layers leads to a significant increase in grain size without reducing the number of grains. Also, Wu et al. (2022) showed that *AcEXPA23* gene overexpression in kiwifruit increased the number of kiwifruit lateral roots.

At the same time, previous studies have shown a close relationship between the expression of some expansin genes, ethylene hormone, and increased root lignification. It is worth noting that, Huang et al. (2013) showed that the ethylene-regulated inhibition of primary root elongation was caused by an increase in cell wall lignification and the suppression of lateral root formation was linked mainly to expansin A. In this same context, Fujimoto et al. (2015) showed that the sclareol, a natural diterpene known as an antimicrobial and defense-related molecule, reduced *A.*

*thaliana* susceptibility to RKN by mediating the accumulation of ethylene-dependent lignin in roots. In this present study, we verified the upregulation of *NtACC oxidase* and *NtEFE26* genes in roots of uninfected *N. tabacum* transgenic lines, both genes involved in the ethylene biosynthesis pathway (Chen et al. 2003). In addition, the *NtCCR* and *Nt4CL* genes involved in the lignin biosynthesis pathway were also upregulated in transgenic lines. Similar to what we observed, Sato et al. (2021) showed that infection of *Solanum torvum* by the avirulent pathotype A2-O of *M. arenaria* rapidly induced (at 3 DAI) the expression of genes encoding the lignin biosynthesis, while biochemical analysis confirmed high lignin accumulation at the root tip, suggesting that physical reinforcement of cell walls with lignin is an important defense mechanism against RKN. In this same sense, Kumar et al. (2019) showed that several lignin biosynthesis pathway genes were upregulated in an RKN-resistant cotton genotype compared with a susceptible genotype.

Subsequently, our biochemical data revealed higher lignin accumulation in the root cell wall of *N. tabacum* transgenic lines. These data suggested that the GmEXPA1 protein can indirectly act by modulating the cell wall of transgenic roots, and that the increased root lignification may explain the reduced plant susceptibility to *M. incognita*. Consistent with these data, it is well known that the ethylene hormone increases lignin content by regulating the activities of key enzymes of the lignin biosynthesis pathway, and the root morphological changes can be correlated with higher lignin content (Tanimoto et al. 1995; Huang et al. 2013; Feng et al. 2017). It is worth noting that the lignin polymer is present in the cell wall of vascular plants, where it rigidifies and strengthens the cell wall structure through covalent cross-linkages to cell wall polysaccharides (Sattler and Funnell-Harris 2013; Liu et al. 2018). Also, lignin content is associated with the modulation of root and plant growth and directly related to plant defense against pathogens (Franke et al. 2002; Xie et al. 2018). Thus, our collective data provided evidence that allows associates the *GmEXPA1* gene upregulation with reduced plant susceptibility to *M. incognita*, which leads to changes in the cell wall and lignin content. A little similar to this study, Basso et al. (2022) identified that the *GmGlb1-1* gene was also upregulated in the nematode-resistant cultivar PI595099 during plant infection by *M. incognita* and showed that the constitutive overexpression of the *GmGlb1-1* gene reduced *A. thaliana* and *N. tabacum* susceptibility to *M. incognita*, possibly by interfering in the dynamic of reactive oxygen species production and nitric oxide scavenging, enhancing the acquired systemic acclimation to biotic and abiotic stresses, and improving the cellular homeostasis.

In conclusion, RNAseq and real-time PCR data allowed us to identify the *GmEXPA1* gene as being upregulated in the soybean nematode-resistant genotype PI595099 in response to *M. incognita*. The genome sequence analysis revealed that the *GmEXPA1* gene was present and highly conserved in both soybean nematode-resistant genotype and susceptible cultivar. The *A. thaliana* and *N. tabacum* transgenic lines overexpressing the *GmEXPA1* gene allowed us to validate the association of *GmEXPA1* gene expression with reduced plant susceptibility to *M. incognita*. The upregulation of two marker genes involved in the ethylene biosynthesis pathway and two other genes involved in the lignin biosynthesis pathway, supported by the increased accumulation of lignin in the transgenic roots, provided strong evidence that the *GmEXPA1* gene can act in cell wall modification, increasing root lignification. Therefore, the *GmEXPA1* gene represents a powerful biotechnological tool to be applied in soybean genetic engineering to reduce soybean susceptibility to *M. incognita* and, possibly, other economically important PPNs.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00299-022-02941-3.

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**Author contributions** MFGS was the project leader. LLPM provided the binary vector. MFB performed in silico analysis, plant transformation, molecular assays, and helped by ITLT, performed gene expression analysis. FCM-G provided soybean genome datasets. RCT and MMCC performed the genome assembly and identification of *GmEXPA1* gene sequences. FBMA provided transcriptome datasets. MFB, DGP, ITLT, RAGM, and CEMP performed the production of *M. incognita* inoculum, and conducted and evaluated bioassays. CEMP, ITLT, and ACMMG supervised by the JAE performed the galls morphological analysis. AG, AFM, and MFB supervised by the MSB and EISF performed lignin content. MFB wrote the draft manuscript, while MFGS, MCMS, MSB, JAE, and EISF reviewed and provided intellectual inputs. All authors approved the final version.

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**Data availability** The partial genome sequences were provided into the NCBI database from GenBank numbers: GmEXPA1.1_PI595099: ON228172, GmEXPA1.2_PI595099: ON228173, GmEXPA1.1_BRS133: ON228176, and GmEXPA1.2_BRS133: ON228177. The nucleotide sequence can be accessed from https://www.ncbi.nlm.nih.gov/nuccore. The Sequence Read Archive (SRA) data from RNAseq are provided in the NCBI database from BioProject number: PRJNA75066. The BioProject can be accessed from https://www.ncbi.nlm.nih.gov/bioproject/. In addition, genome target sequence and transcriptome data, such as gene expression, were also provided as supplementary data.

## Declarations

**Conflict of interest** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Ethical approval** Not applicable.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

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