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ORIGINAL ARTICLE

Overexpression of a soybean *Globin* (*GmGlb1-1*) gene reduces plant susceptibility to *Meloidogyne incognita*

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Abstract

Main conclusion **The overexpression of the *GmGlb1-1* gene reduces plant susceptibility to *Meloidogyne incognita*.**

Abstract *Non-symbiotic globin class #1* (*Glb1*) genes are expressed in different plant organs, have a high affinity for oxygen, and are related to nitric oxide (NO) turnover. Previous studies showed that soybean *Glb1* genes are upregulated in soybean plants under flooding conditions. Herein, the *GmGlb1-1* gene was identified in soybean as being upregulated in the nematode-resistant genotype PI595099 compared to the nematode-susceptible cultivar BRS133 during plant parasitism by *Meloidogyne incognita*. The *Arabidopsis thaliana* and *Nicotiana tabacum* transgenic lines overexpressing the *GmGlb1-1* gene showed reduced susceptibility to *M. incognita*. Consistently, gall morphology data indicated that pJ2 nematodes that infected the transgenic lines showed developmental alterations and delayed parasitism progress. Although no significant changes in biomass and seed yield were detected, the transgenic lines showed an elongated, etiolation-like growth under well-irrigation, and also developed more axillary roots under flooding conditions. In addition, transgenic lines showed upregulation of some important genes involved in plant defense response to oxidative stress. In agreement, higher hydrogen peroxide accumulation and reduced activity of reactive oxygen species (ROS) detoxification enzymes were also observed in these transgenic lines. Thus, based on our data and previous studies, it was hypothesized that constitutive overexpression of the *GmGlb1-1* gene can interfere in the dynamics of ROS production and NO scavenging, enhancing the acquired systemic acclimation to biotic and abiotic stresses, and improving the cellular homeostasis. Therefore, these collective data suggest that ectopic or nematode-induced overexpression, or enhanced expression of the *GmGlb1-1* gene using CRISPR/dCas9 offers great potential for application in commercial soybean cultivars aiming to reduce plant susceptibility to *M. incognita*.

Keywords *Glycine max* · *Glyma.11G121800* · New biotechnology tools · Phytoglobins · Plant-nematode interaction · Root-knot nematodes · PI595099 · BRS133

Abbreviations

DAI	Days after inoculation
Glb	Globin
J2	Second-stage juveniles
NO	Nitric oxide
pJ2	Parasitic second-stage juveniles
ppJ2	Pre-parasitic second-stage juveniles
Ev	Transgenic event

Introduction

Non-symbiotic globin proteins class #1 (Glb1) proteins have a high affinity to oxygen and NO and are differentially accumulated in different organs of both leguminous and non-nodulating plants (Garrocho-Villegas et al. 2007; Hill 2012).

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The *Glb1* genes are modulated by low oxygen levels, while Glb1 proteins are more related to NO turnover, acting as an NADH-dependent NO dioxygenase that metabolizes the NO to nitrate improving cellular homeostasis (Igamberdiev and Hill 2004; Perazzolli et al. 2004; Gupta and Igamberdiev 2016; Koltun et al. 2021). Consistently, *Glb1* genes are upregulated in response to hypoxia conditions possibly to modulate the NO scavenging (Nakayama et al. 2017; Koltun et al. 2021). Mainly the fact that although NO is important in signaling networks of various biological processes, including defense response to biotic and abiotic stresses, it is a highly reactive molecule with a high potential to damage cellular structures (Farnese et al. 2016).

Recently, it has been reported that plant infection by *Meloidogyne incognita* induces a higher accumulation of a globin protein in non-rhizobium-bacterized roots of a nematode-resistant cowpea cultivar (Oliveira et al. 2014). On the other hand, it is known that plant infection by nematodes induces a significant NO accumulation and results in *Glb1* gene expression upregulation in nematode-infected roots (Labudda et al. 2020). At the same time, NO at low levels acts as a signaling molecule to activate the defense response against nematodes, while at high levels becomes highly damaging to plant cells (Zhou et al. 2015). Therefore, it is plausible to link plant infection by nematodes, higher NO accumulation, and *Glb1* gene expression upregulation. However, the specific role of these plant globins, specially Glb1 proteins, linked with plant susceptibility to plant parasitic nematodes (PPNs) is not yet fully understood.

Root-knot nematodes (RKN) are obligate sedentary endoparasites of the genus *Meloidogyne* spp. (Trudgill and Blok 2001). *Meloidogyne incognita* is one of the most frequently reported species, causing significant damage to several economically important crops (Abad et al. 2008). Conceptually, its life cycle consists of the following stages: eggs, ppJ2 (pre-parasitic second-stage juveniles), pJ2 (parasitic second-stage juveniles), J3 and J4 juveniles, and females. The pJ2, J3, and J4 parasitic stages, and females are typically endophytes sedentary while eggs and ppJ2 are considered exophytes (Abad et al. 2008; Castagnone-Sereno et al. 2013). The restricted range of available control agents and resistant or tolerant soybean cultivars has limited the effectiveness of their control and management in the field (Seo and Kim 2014; Bernard et al. 2017). Therefore, there is a strong demand from farmers for new elite cultivars with some resistance level to nematodes. Interestingly, during a compatible interaction, RKN disrupts root cells by hyperactivating their cell cycle, increasing the size of parasitized cells until forming the called giant-feeding cells, causing cell hyperproliferation and resulting in the development of nematode feeding sites within root swellings called galls (Engler et al. 2015; Shukla et al. 2018). These nematode-infected roots are disrupted in the uptake capacity of water and nutrient which, consequently, causes a reduced plant growth and yield (Carneiro et al. 2002; Lu et al. 2014).

Herein, the *GmGlb1-1* (*Glyma.11G121800*) gene was identified as being upregulated in response to plant parasitism by *M. incognita*. Then, the *GmGlb1-1* gene was functionally characterized by overexpression in *Arabidopsis thaliana* and *Nicotiana tabacum* transgenic lines. The plant susceptibility to *M. incognita*, plant biomass and seed yield, plant growth, and root development under well-irrigated and flooding conditions, expression analysis of some genes involved in plant defense to oxidative stress, and activity of antioxidant enzymes were evaluated. The link between *GmGlb1-1* gene overexpression, reduced plant susceptibility to *M. incognita*, differential plant growth and higher production of axillary roots under flooding conditions, ROS production dynamics, NO scavenging, and cellular homeostasis maintenance in transgenic lines are discussed.

Materials and methods

Globin genes expression level in the soybean during infection by *M. incognita*

The *GmGlb1-1* gene (*Glyma.11G121800*; Suppl. Tables S1 and S2) was previously identified using a transcriptome (RNAseq) approach as an upregulated gene in the nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133 during plant infection by *M. incognita*. Its expression profile in leaves and roots of both soybean genotype and cultivar was evaluated by real-time PCR from plants in the opening stage of the second trifoliate. In this way, to validate this gene expression upregulation during plant infection by *M. incognita*, soybean plants of genotype PI595099 and cultivar BRS133 were inoculated with 1500 *M. incognita* ppJ2 race 1. Root and gall samples were collected at 0, 4, 8, 12, and 30 days after inoculation (DAI), and total RNA was purified with TRIzol Reagent (Invitrogen, Waltham, MA, USA). The RNA concentration was estimated using a spectrophotometer NanoDrop 2000 (Thermo Fisher Scientific) and RNA integrity was checked in agarose electrophoresis. The RNA samples were treated with RNase-free RQ1 DNase I (Promega), then 3 µg of DNase-treated RNA was used for cDNA synthesis using oligo-$(dT)_{20}$ primer and SuperScript III RT kit (Life Technologies, Carlsbad, CA, USA). In the end, the cDNA samples were diluted 1:10 (v:v) with nuclease-free water, while the real-time PCR assays were performed in Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Waltham, MA, USA) using 2 µL cDNA, 0.2 µM gene-specific primers (Suppl. Table S3), and GoTaq® qPCR Master Mix (Promega). The relative gene expression was calculated with the 2^-ΔCT formula using *GmCYP18* as an

endogenous reference gene (Suppl. Table S3). Three biological replicates for each transgenic line and five plants for each biological replicate were used. All cDNA samples were carried out in technical triplicate reactions. The primer efficiencies and target-specific amplification were confirmed by the occurrence of a single peak in the melting curve.

Sequence analysis of the soybean *Globin* genes

From genome sequencing of genotype PI595099 and cultivar BRS133, the *GmGlb1-1* gene sequences were retrieved using Williams 82 as a reference genome (Suppl. File S1). The raw sequences were mapped against the reference genome using the STAR program (Dobin et al. 2012), while resulting *bam* files were assembled using Trinity genome-guided v2.0.6 program (Grabherr et al. 2011). The *GmGlb1-1* gene sequences and features were retrieved of the Gmax_275_Wm82.a2.v1 annotation and *Glycine max* Wm82.a2.v1 datasets from Phytozome v.13 database (Schmutz et al. 2010; Goodstein et al. 2012) and searched against the assembled genomes using the Blastn program (Altschul et al. 1990). Phylogeny trees were generated with the Phylogeny.fr web service (Dereeper et al. 2008) using the maximum likelihood method, Approximate Likelihood-Ratio test (aLRT) SH-like branch support, and GTR (nucleotide) and WAG (amino acid) substitution model. Pairwise identity matrices from nucleotide and amino acid sequences were generated using the Sequence Demarcation Tool version 1.2 (Muhire et al. 2014). The positional conservation of globin-like superfamily (class1_nsHb_like cd:14784) was generated from multiple sequence alignment by Color Align Conservation software (Stothard 2000), while the presence of conserved domains was identified with the Conserved Domain Database tool (Marchler-Bauer et al. 2017). The in silico gene expression in different soybean organs and tissues was generated by the PhytoMine tool (https://phytozome.jgi.doe.gov/phytomine/begin.do).

Genetic transformation of *A. thaliana* and *N. tabacum*

The full-length cDNA (486 nucleotides; Suppl. File S1) of the *GmGlb1-1* gene was synthesized and cloned into a binary vector named p3300-35S::GmGlb1-1 by Epoch Life Science (Sugar Land, TX, USA). Subsequently, this binary vector was transfected into *Escherichia coli* and *Agrobacterium tumefaciens* strain GV3101. In this case, the *GmGlb1-1* gene was cloned under the control of enhanced CaMV 35S promoter and *in tandem* fused to the *GFP* reporter protein gene. The *bar/PAT* gene was used as a selectable marker gene also under the control of the CaMV 35S promoter (Fig. 1a). The *A. thaliana* ecotype Col-0 was genetically transformed by the floral dip method (Clough and Bent 1998), while *N. tabacum* var. SR1 was genetically transformed as described by Park et al. (1998) and Basso et al. (2020b). *N. tabacum* transgenic lines were in vitro regenerated under a selective medium containing 5 mg/L glufosinate-ammonium (Finale, Liberty Link, Bayer-Monsanto). The recovered transgenic plants were evaluated for transgene insertion by PCR using specific primers (Suppl. Table S3). In addition, QuickStix™ kit (Envirologix, Portland, ME, USA) was used for bar/PAT protein detection in leaves of these transgenic lines.

GmGlb1-1 gene expression in roots and leaves of *A. thaliana* and *N. tabacum* transgenic lines

Roots and leaves samples from three adult *A. thaliana* and *N. tabacum* transgenic lines (AtEv1 to AtEv3, and NtEv1 to NtEv3) were collected and total RNA was isolated using TRIzol Reagent (Invitrogen). Highly pure and DNA-free RNA samples were used for cDNA synthesis, while cDNA samples were diluted at 1:10 (v:v) and used in gene expression assays performed by real-time PCR, which were carried out as described above. In this case, the *AtActin 2* and *NtActin 4* were used as endogenous reference genes (Suppl. Table S3). Three biological replicates and three plants for each biological replicate were used, while all cDNA samples were carried out in technical triplicate reactions. The primer efficiencies and target-specific amplification were confirmed as described above. The relative gene expression values were calculated with the 2^-ΔΔCT formula (Schmittgen and Livak 2008). In addition, GmGlb1-1 protein fused to the GFP reporter protein was observed in roots of *A. thaliana* and *N. tabacum* transgenic lines using a fluorescence stereomicroscope (Leica M205 FA) with a GFP Long-Pass filter (excitation 395–455 and emission 480 nm).

Susceptibility level of *A. thaliana* and *N. tabacum* transgenic lines to *M. incognita*

The *M. incognita* ppJ2 race 3 inoculum was produced in tomato plants cv. Santa clara kept under greenhouse conditions. Nematode-infected roots were washed and processed as described by Hussey and Barker (1973). Then, *A. thaliana* plants were inoculated with 500 M. *incognita* ppJ2 suspended in distilled water, while 15 plants per transgenic or wild-type line were used, and the experiment was repeated 2 times. At 60 DAI, roots from inoculated plants were evaluated for the number of eggs, number of hatched ppJ2, number of galls and, then, the nematode reproduction factor was determined. The *M. incognita* reproduction factor in transgenic lines and wild-type plants was calculated as described by Oostenbrink (1966) and Windham and Williams (1987). In contrast, *N. tabacum* plants were inoculated with 1000 M.

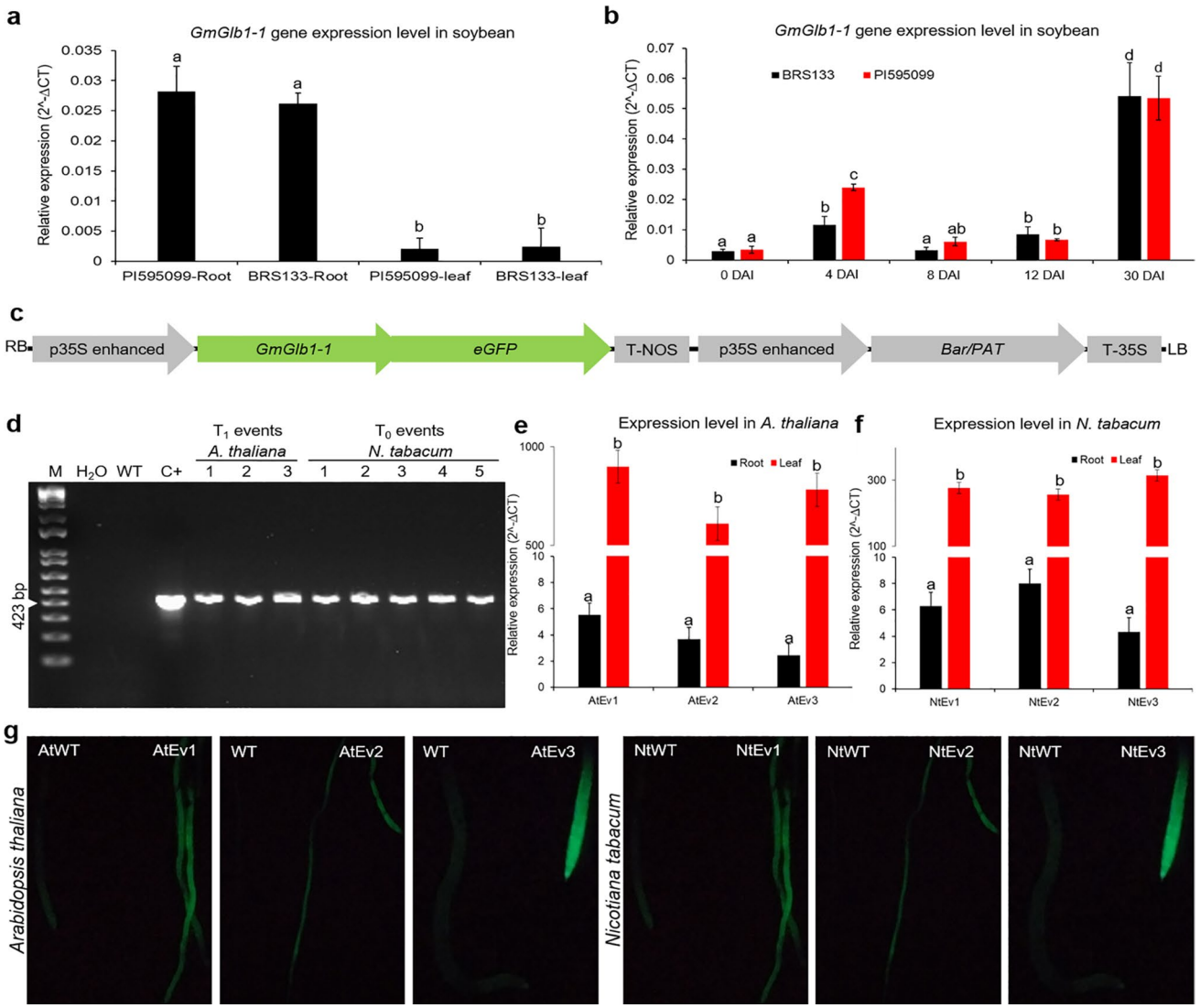


Fig. 1 Native expression level of *GmGlb1-1* (*Glyma.11G121800*) gene in soybean, T-DNA fragment used for plant transformation, confirmation of the presence and expression of the transgenes in plant tissues, and GFP fluorescence protein in *A. thaliana* and *N. tabacum* transgenic plants. **a** *GmGlb1-1* gene expression profile in leaves and roots of nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133. Error bars represent the standard deviation of three biological replicates consisting of four soybean plants each. Plants were evaluated in the opening stage of the second trifoliate. **b** *GmGlb1-1* gene expression profile in roots of nematode-resistant and nematode-susceptible plants at stages 0, 4, 8, 12, and 30 DAI. The relative expression values were calculated with the 2^-ΔCT formula and normalized with the *GmCYP18* as an endogenous reference gene (Suppl. Table S3). Error bars represent the confidence interval of three biological replicates, whereas each biological replicate consisted of five plants. Different letters indicate significant statistical differences between different times and cultivars after nematode inoculation based on Tukey's test at a 95% significance level. **c** Overview of T-DNA used in the binary vector for plant genetic transformation mediated by *A. tumefaciens*. **d** Molecular detection based on PCR assays of the minimal expression cassette (T-DNA) in *A. thaliana* and *N. tabacum* transgenic lines. The T_1 (*A. thaliana*) and T_0 (*N. tabacum*) transgenic lines were molecularly characterized using PCR assays with specific primers targeting the *GFP* gene present in the T-DNA. Molecular marker: 1.0 kb DNA ladder (Invitrogen, Cat. 10,787,018); WT: wild-type control plant used as a negative control in PCR assays and bioassays; C+: DNA plasmid used as a positive control in PCR assays. The *GmGlb1-1* gene expression profile was measured by real-time PCR assays in leaf and roots of *A. thaliana* (**e**) and *N. tabacum* (**f**) transgenic lines. The relative gene expression values were calculated with the 2^-ΔΔCT formula and normalized by amplification CT value in WT plants and with *AtActin 2* as an endogenous reference gene (Suppl. Table S3). Error bars represent confidence intervals corresponding to three biological replicates. Different letters indicate significant statistical differences between transgenic lines and wild-type plants based on Tukey's test at a significance level of 95%. **g** Ectopic accumulation of GFP protein in tandem fused for GmGlb1-1 protein in roots of *A. thaliana* and *N. tabacum* transgenic lines, monitored under a fluorescence stereomicroscope

incognita ppJ2, while 16 plants per transgenic or wild-type lines were used. At 60 DAI, roots from inoculated plants were evaluated for the number of eggs, number of hatched ppJ2, number of gall and, then, the nematode reproduction factor was determined. For gall morphology analysis, *A. thaliana* roots were collected at 10 and 45 DAI and fixed in 2% glutaraldehyde solution, subsequently dehydrated and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). These roots containing galls were sectioned to 3 µm, stained in 0.05% toluidine blue solution, and sections were mounted in Depex (Sigma-Aldrich). Microscopy analyses were performed under bright-field optics, while images were acquired using a digital camera Axiocam (Carl Zeiss, Oberkochen, Germany).

Plant growth, biomass, and seed yield of *A. thaliana* and *N. tabacum* transgenic lines

Plants from three to five transgenic lines (AtEv1 to AtEv3, and NtEv1 to NtEv5) were evaluated for growth, leaf and root biomass accumulation, and seed yield. Seeds from transgenic and wild-type *A. thaliana* lines were cultivated on Petri plates containing MS medium (Murashige and Skoog 1962). Two-week-old plants were transferred to 50 mL pots containing commercial substrate and maintained in a growth room at 22 °C, 70% relative humidity, and 16/8 h light/dark photoperiod. For the evaluation of seed yield, plants were kept in a growth room until the seed production and senescence stage, when the seeds were collected individually per plant. Meanwhile, for the evaluation of root and vegetative biomass, plants were grown in a growth room (*A. thaliana*) or greenhouse (*N. tabacum*) and at 40 days after planting of *A. thaliana* plants and at 30 days after planting of *N. tabacum* plants, the vegetative part or roots were collected individually from each plant and the fresh weight was determined. For the dry weight of vegetative biomass or roots, the material was dried by incubation in an oven at 60 °C for 2 days, then the dry weight was determined. For root architecture analysis, freshly germinated seeds were transferred to Petri plates containing MS medium supplemented with 7% Phytagel (Sigma-Aldrich) and 1% sucrose. Then, these Petri plates were maintained inverted in the same growth room. In contrast, seeds from *N. tabacum* transgenic plants were germinated in a commercial substrate, transplanted to 500 mL pots containing a mix of sand:soil:substrate (1:1:1, w/w/w), kept under greenhouse conditions, and evaluated 30 days after transplanting. For biomass and seed yield, 10 to 15 plants for each transgenic and wild-type line were screened, while root architecture analysis was used at least 20 plants for each transgenic line.

Defense-related gene expression profile in roots of *A. thaliana* and *N. tabacum* transgenic lines

Young roots of *A. thaliana* and *N. tabacum* transgenic lines kept in a growth room and greenhouse, respectively, were collected after 30 days. Total RNA was purified using TRIzol Reagent (Invitrogen) and DNA-free RNA was used for cDNA synthesis as described above. Then, the expression profile of *AtPRX17*, *AtRbohA*, *AtPRX45*, *AtPRX57* (Neuser et al. 2019), and *NtGST1* (Tang et al. 2017) genes was evaluated. The relative gene expression values were calculated using the 2^-ΔCT formula, while *AtActin 2* and *NtActin 4* were used as endogenous reference genes (Suppl. Table S3). All cDNA samples were carried out in technical triplicate reactions, while three biological replicates composed of four to 10 plants each were used for each treatment.

In situ hydrogen peroxide level in leaves of *N. tabacum* transgenic lines

For H_2O_2 staining in leaves, plants from three transgenic lines and wild-type (NtEv1 to NtEv3) were submerged for 24 h in 150 mM mannitol solution, and full-length leaves were excised and incubated for 8 h under agitation in 1 mg/mL 3,3-diaminobenzidine (DAB) solution pH 3.8. After DAB staining, these leaves were incubated in 100% ethanol until the complete removal of chlorophyll. Finally, stained leaves were rehydrated in 10% glycerol solution and photographed stereoscopically.

Antioxidant enzyme activity in leaves of *N. tabacum* transgenic lines

The superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) enzyme activities were determined from leaf protein extracts as described by Paes-de-Melo et al. (2021). The total protein concentration was previously determined by the Bradford method (Bradford 1976). All enzymatic activities were determined from three biological replicates composed of at least eight plants each, while each biological replicate was carried out in technical triplicates.

Results

GmGlb1-1 gene expression profile in non-transgenic soybean plants

Real-time PCR data revealed higher *GmGlb1-1* gene expression in roots than in leaves in both nematode-resistant

genotype PI595099 and nematode-susceptible cultivar BRS133 (Fig. 1a). This expression profile fits with information retrieved from the Phytozome database (using the Phytomine tool), indicating that the *GmGlb1-1* gene is highly expressed in soybean roots. In addition, in silico gene expression data showed that this gene is also strongly overexpressed in nodules and roots under symbiotic conditions or under higher nitrogen accumulation, contrasting with the basal or reduced expression level in other plant organs (Suppl. Fig. S1g and S1h). By using a transcriptome approach, we observed that the *GmGlb1-1* gene was upregulated in both genotype PI595099 and cultivar BRS133 during plant infection by *M. incognita* (Suppl. Tables S1 and S3). Then, soybean plants from genotype PI595099 and cultivar BRS133 were again inoculated with *M. incognita* ppJ2 race 1, and *GmGlb1-1* gene expression in inoculated and non-inoculated roots was evaluated by real-time PCR assays. These data confirmed that the *GmGlb1-1* gene expression was significantly modulated during soybean infection by *M. incognita*. Particularly, the *GmGlb1-1* gene expression was upregulated at 4 DAI in both soybean genotype and cultivar, but higher gene expression upregulation was observed in the nematode-resistant genotype PI595099 than in the nematode-susceptible cultivar BRS133. Furthermore, *GmGlb1-1* gene expression at 30 DAI was significantly higher in both genotype and cultivar compared to 0 DAI (Fig. 1b). Curiously, it is worth noting here that 4 DAI corresponds with the time point that *M. incognita* ppJ2 reaches and penetrates the soybean roots, while at 30 DAI, it fits with the nematode reproduction phase. Furthermore, the expression profile of the *GmGlb1-1* gene performed by real-time PCR consistently validated the RNAseq data (Suppl. Tables S1 and S3) and suggested that the *GmGlb1-1* gene expression can be correlated with the reduced susceptibility of soybean genotype PI595099 to *M. incognita*.

Sequence analysis of soybean *Globin* genes

The *GmGlb1-1* gene sequences were identified and retrieved from the nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133 genomes using Willians 82 as reference genome (Suppl. Fig. S1a to S1d; Suppl. File S1). A single *GmGlb1-1* gene copy was identified in the chromosome 11 of both genotype PI595099 and cultivar BRS133 (Suppl. File S1). A 100% identity was observed among the *GmGlb1-1* transcripts, CDS, and amino acid sequences from PI595099, BRS133, and Willians 82 cultivars (Suppl. Fig. S1a to S1c, Suppl. File S1). The genome analysis allowed identify four soybean leghemoglobin genes (*Glyma.10G198800, Glyma.10G199000, Glyma.10G199100,* and *Glyma.20G191200*), one *Glb1* gene (*Glyma.11G121800*), one *Glb2* gene (*Glyma.11G121700*), and one putative truncated globin pseudogene (*Glyma.10G198900*) in the nematode-resistant PI595099 genotype and in the nematode-susceptible cultivar BRS133 (Suppl. Table S2). Interestingly, was observed that the *GmGlb1-1* gene is strongly upregulated in roots and nodules and almost not expressed in other plant organs (Suppl. Fig. S1g and S1h; Suppl. Table S4). Regarding the *Globin* genes, our data revealed that not all these genes were significantly modulated during plant infection by *M. incognita*, while one of the highlights was the *GmGlb1-1* gene (Suppl. Table S4; Suppl. Fig. S1g and S1h). The amino acid sequence analysis showed that the *GmGlb1-1* gene contains a typical cd14784 domain that belongs to the globin-like superfamily (Suppl. Fig. S1e and S1f). Additional nucleotide and amino acid sequences and phylogenetic analysis suggested that the *GmGlb1-1* gene had a higher sequence identity and evolutionary linkage to *Globin* genes from *Vigna unguiculata* (*Vigun11g171400*) and *Phaseolus vulgaris* (*Phvul.011G048700*) (Suppl. Fig. S2a to S2f). In addition, sequence features confirmed that the *GmGlb1-1* gene is highly conserved in three soybean PI595099, BRS133, and Willians 82 cultivars studied here. Based on these previous results, was selected the *GmGlb1-1* gene for constitutively overexpression in *A. thaliana* and *N. tabacum* transgenic lines and to evaluate subsequently the role of this gene in reduced plant susceptibility to *M. incognita*.

A. thaliana and *N. tabacum* transgenic lines overexpressing the *GmGlb1-1* gene

A total of 12 *A. thaliana* (Suppl. Fig. S3a) and 22 N. *tabacum* transgenic lines (Suppl. Fig. S3b) constitutively overexpressing the *GmGlb1-1* gene were successfully generated (Fig. 1c). Then, three *A. thaliana* (AtEv1 to AtEv3) and five *N. tabacum* transgenic lines (NtEv1 to NtEv5) were selected for all subsequent analyses according to the higher *GmGlb1-1* gene expression level (Suppl. Fig. S3a and S3b; Fig. 1d). Subsequently, real-time PCR data showed higher *GmGlb1-1* gene expression in leaves than in roots of selected *A. thaliana* (Fig. 1e) and *N. tabacum* (Fig. 1f) transgenic lines. In addition, the *GmGlb1-1* gene overexpression in tandem fused to the *GFP* gene was confirmed by the simultaneous accumulation of the GmGlb1-1/GFP proteins in transgenic roots (Fig. 1g).

Reduced susceptibility of *A. thaliana* and *N. tabacum* transgenic lines to *M. incognita*

A. thaliana (AtEv1 to AtEv3) and *N. tabacum* (NtEv1 to NtEv5) transgenic lines were challenged with *M. incognita* ppJ2 race 3 to evaluate the potential role of the GmGlb1-1 protein in the reduced plant susceptibility to nematode. Interestingly, all *A. thaliana* transgenic lines showed a significant reduction in the number of eggs and number of hatched ppJ2

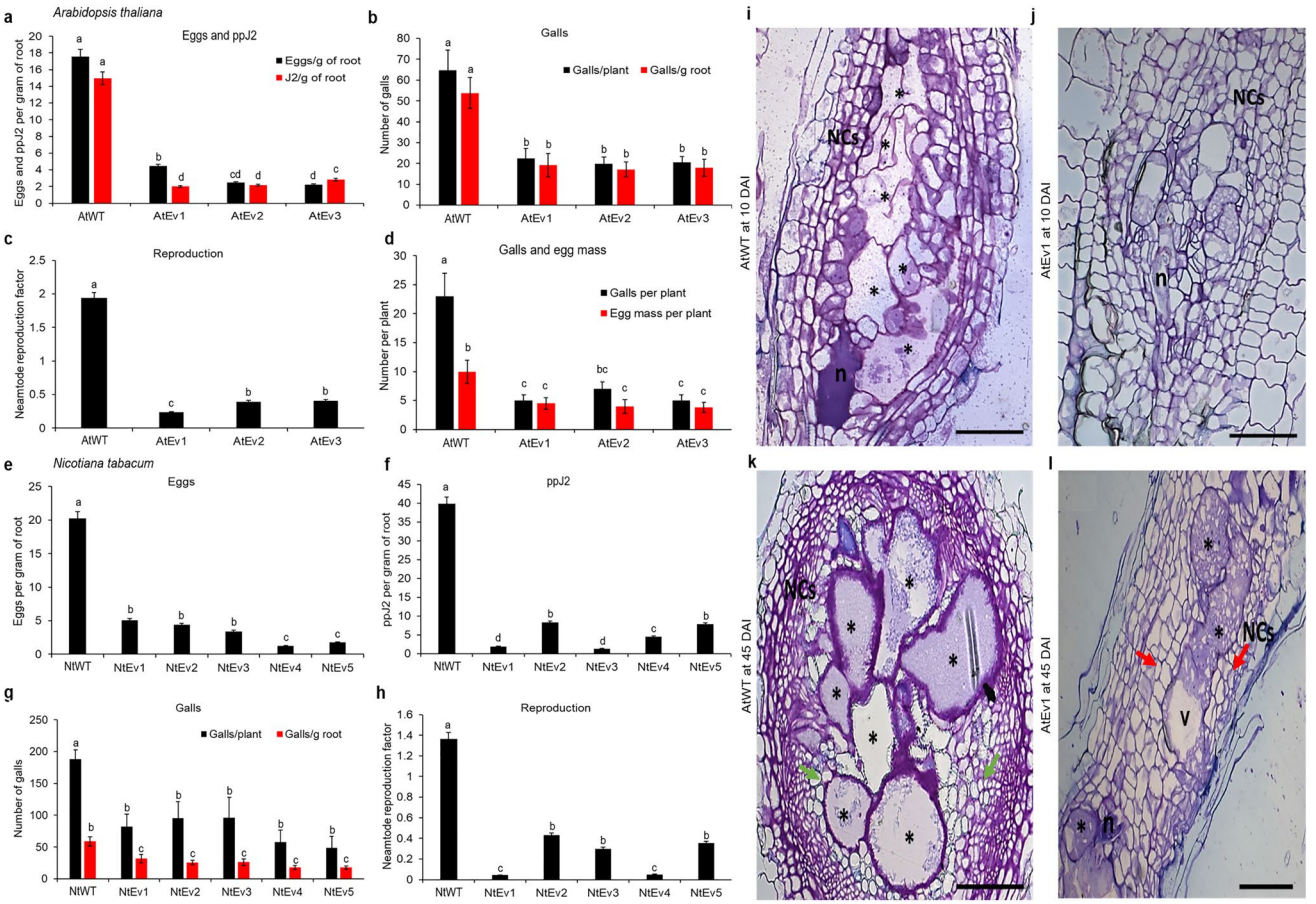
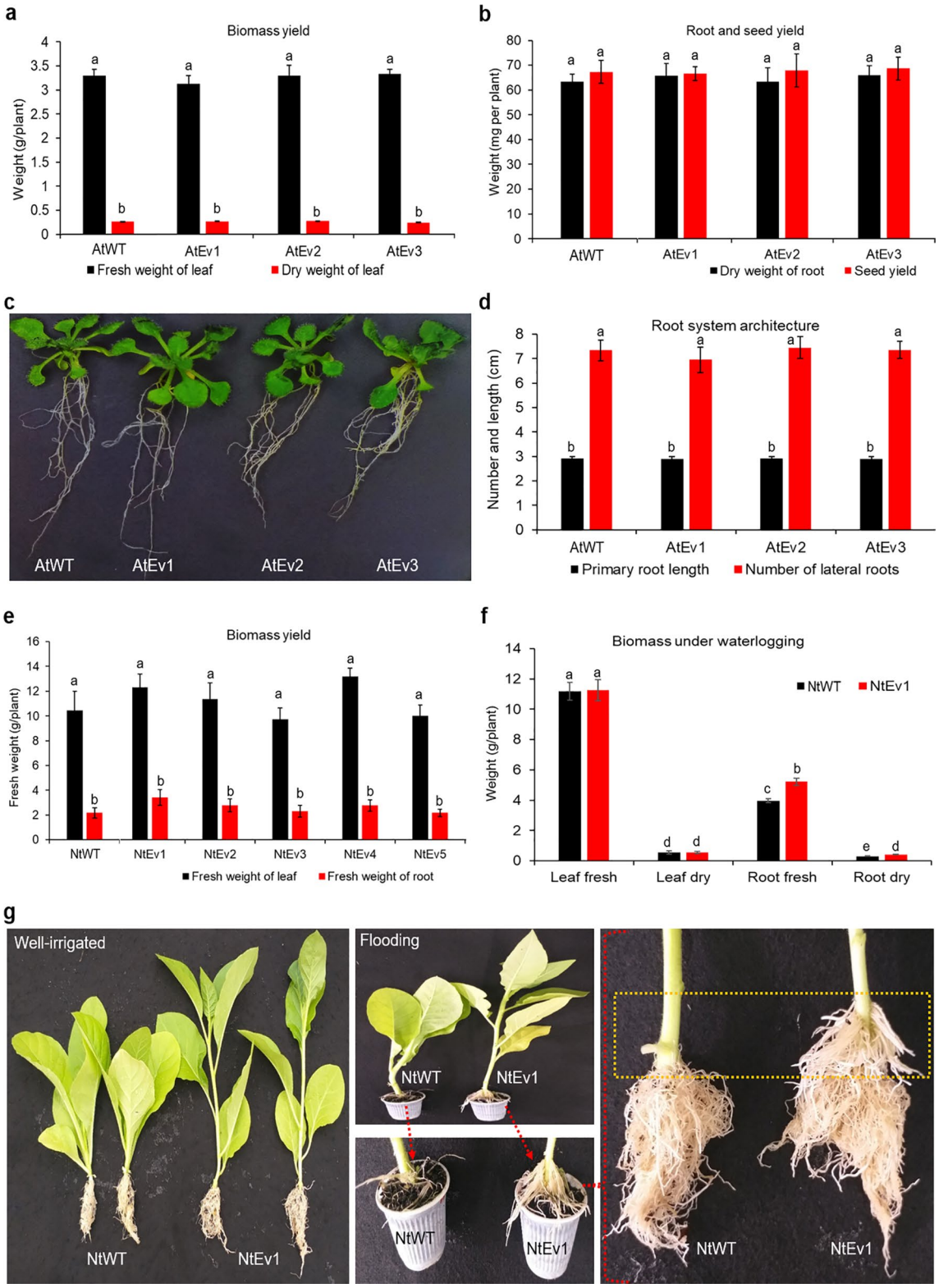


Fig. 2 Susceptibility level to *M. incognita* of *A. thaliana* and *N. tabacum* transgenic lines overexpressing the *GmGlb1-1* (*Glyma.11G121800*) gene. **a** Number of eggs per gram of root and number of ppJ2 per gram of root, **b** number of galls per plant or per gram of root, and **c** nematode reproduction factor in *A. thaliana* transgenic lines inoculated with *M. incognita* ppJ2 race 3 compared with wild-type plants (WT). **d** Number of galls per plant and number of egg mass per *A. thaliana* plant inoculated with *M. incognita* ppJ2 strain Morelos at 45 DAI. Error bars represent the confidence interval corresponding to 12 biological replicates ($n = 12$ plants). Different letters indicate significant differences according to Tukey's test at 95% 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 gram of root, and **h** nematode reproduction factor in *N. tabacum* transgenic lines after inoculation with *M. incognita* ppJ2 race 3. Error bars represent confidence intervals corresponding to three technical replicates from 15 plants each. Different letters indicate significant differences according to Tukey's test at 95% significance level. Susceptibility level to *M. incognita* ppJ2 strain Morelos and galls histological analysis from *A. thaliana* transgenic lines overexpressing the *GmGlb1-1* gene compared with wild-type plants at 10 and 45 DAI. Galls morphology in **i** wild-type plants at 10 DAI, **j** transgenic lines at 10 DAI, **k** wild-type plants at 45 DAI (green arrows point to a large number of NCs), and **l** transgenic lines at 45 DAI (red arrows point to a reduced number of NCs). Bright-field images of toluidine blue-stained gall sections. *Giant cell; *NCs* neighboring cells, *n* nematode, *v* vacuoles. Bars = 50 µm

(Fig. 2a). As well, the number of galls and the nematode reproduction factor were significantly decreased between 80 and 90% in transgenic lines compared with wild-type plants (Fig. 2b and c). In addition, *A. thaliana* transgenic lines were also evaluated for plant susceptibility to *M. incognita* ppJ2 strain Morelos (Koutsovoulos et al. 2018). Consistently, a reduced susceptibility level of transgenic lines to *M. incognita* strain Morelos was observed (Fig. 2d). In addition, the galls morphology of these *A. thaliana* transgenic lines inoculated with *M. incognita* ppJ2 strain Morelos was evaluated at 10 and 45 DAI. These data showed that galls formed in roots of transgenic lines were smaller than wild-type plants, while that *M. incognita* when infecting transgenic lines exhibited delayed development. In addition, assays using five *N. tabacum* transgenic lines also revealed a reduction in the number of egg masses and number of hatched ppJ2, suggesting a reduced plant susceptibility to *M. incognita* (Fig. 2e and f). Similarly, were also observed a reduced number of galls per plant or per gram of roots and reduced nematode reproduction factor, ranging from 50 to 90% compared with wild-type plants (Fig. 2g, h). As well, giant cells were visibly smaller and often more vacuolated, and the reduced numbers of neighboring cells likely contributed to reduced root swelling (Fig. 2i–m). Therefore, these collective data suggested


a
Biomass yield
Weight (g/plant)
AtWT
AtEv1
AtEv2
AtEv3
Fresh weight of leaf
Dry weight of leaf
b
Root and seed yield
Weight (mg per plant)
Dry weight of root
Seed yield
c
d
Root system architecture
Number and length (cm)
Primary root length
Number of lateral roots
e
Biomass yield
Fresh weight (g/plant)
NtWT
NtEv1
NtEv2
NtEv3
NtEv4
NtEv5
Fresh weight of leaf
Fresh weight of root
f
Biomass under waterlogging
Weight (g/plant)
Leaf fresh
Leaf dry
Root fresh
Root dry
g
Well-irrigated
Flooding

◂**Fig. 3** Evaluation of yield penalties and plant growth in uninfected *A. thaliana* and *N. tabacum* transgenic lines under growth room or greenhouse conditions. **a** Fresh and dry vegetative biomass of three *A. thaliana* transgenic lines. Error bars represent the confidence interval corresponding to 15 biological replicates ($n = 15$ plants). **b** Root dry weight and seed yield of three *A. thaliana* transgenic lines. Error bars represent the confidence interval corresponding to 15 biological replicates ($n = 15$ plants). **c** and **d** Primary root length and number of lateral roots of three *A. thaliana* transgenic lines compared with wild-type plants (WT) grown in vitro under growth room conditions. Error bars represent the confidence interval corresponding to 20 biological replicates ($n = 20$ plants). **e** Fresh weight of vegetative biomass and root in five *N. tabacum* transgenic lines kept under well-irrigated conditions compared with wild-type plants. Error bars represent the confidence interval corresponding to 16 biological replicates ($n = 16$ plants). **f** Fresh and dry weight of vegetative biomass and root in *N. tabacum* transgenic line kept under flooding conditions compared with wild-type plants. Error bars represent the confidence interval corresponding to 12 biological replicates ($n = 12$ plants). Statistical differences were evaluated with Tukey's test at 95% significance level. **g** Phenotype of *N. tabacum* transgenic line and wild-type plants kept well-irrigated or under flooding conditions

that *GmGlb1-1* gene overexpression might be involved in the reduced plant susceptibility to *M. incognita*.

A. thaliana and *N. tabacum* transgenic lines showed no significant yield penalty

A. thaliana and *N. tabacum* transgenic lines overexpressing the *GmGlb1-1* gene were evaluated under growth room or greenhouse conditions, respectively, for plant growth, biomass accumulation, and seed yield. The uninfected *A. thaliana* transgenic lines showed similar leaf and root biomass yield, seed yield, primary root length, and the number of lateral roots compared with wild-type plants (Fig. 3a–d). Similarly, *N. tabacum* transgenic lines showed also equivalent vegetative and root biomass yield compared with wild-type plants (Fig. 3e). However, *N. tabacum* transgenic plants under flooding showed higher root fresh biomass yield compared with wild-type plants (Fig. 3f), while uninfected transgenic lines under well-irrigated conditions showed plants with more elongated growth (Fig. 3g). In addition, these same transgenic lines when maintained under flooding conditions developed a greater amount of axillary roots (Fig. 3g). Given this, these data suggested that *GmGlb1-1* gene overexpression does not result in an apparent yield penalty in transgenic lines under growth room or greenhouse conditions, but significant changes in plant resilience may have been altered or improved.

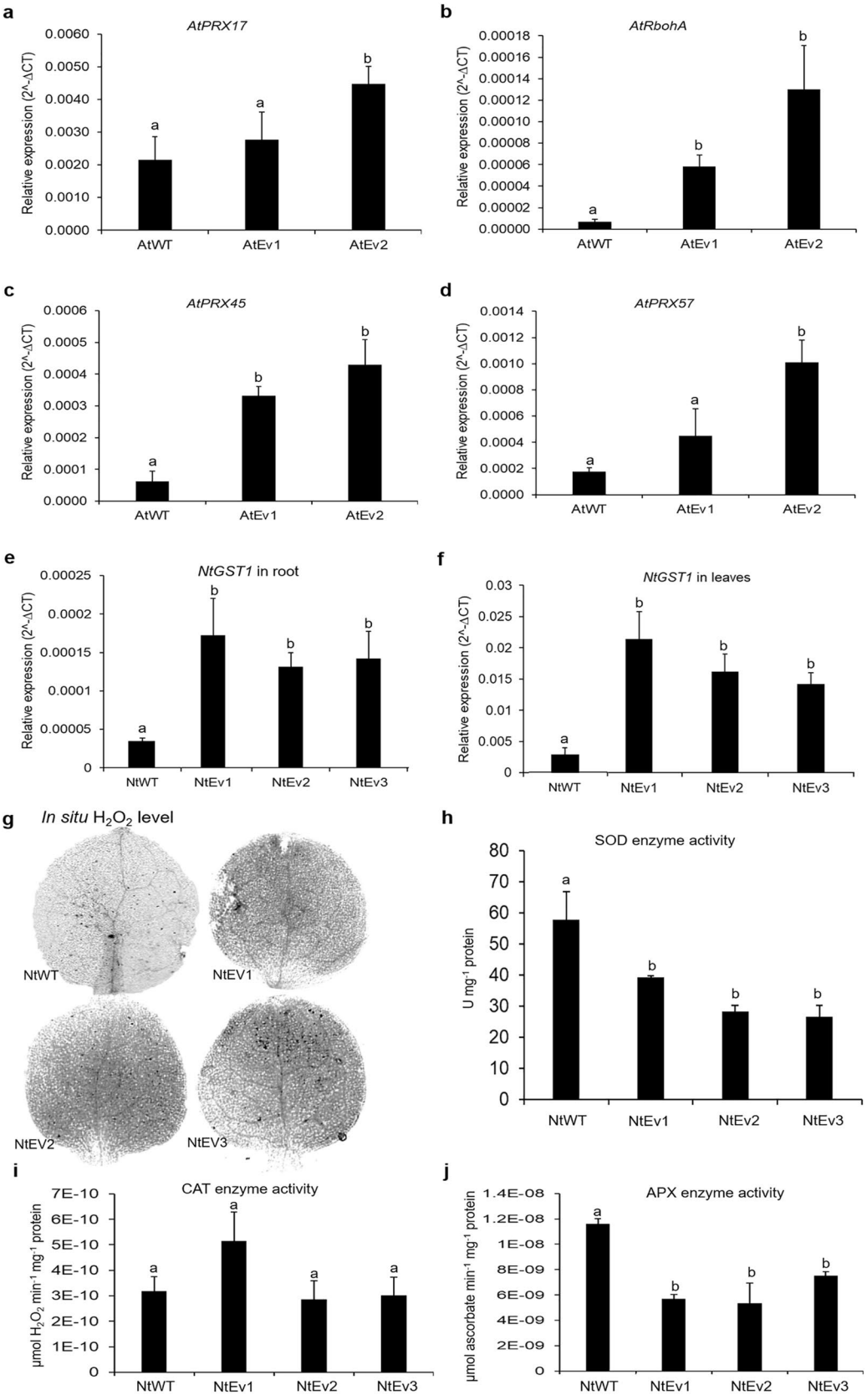
Defense genes expression profile in roots of *A. thaliana* and *N. tabacum* transgenic lines

Some marker genes involved in different biological processes were monitored by real-time PCR in *A. thaliana* and *N. tabacum* transgenic lines. The *AtPRX17*, *AtRbohA*, *AtPRX45*, and *AtPRX57* genes showed higher expression level in at least one transgenic line compared with wild-type plants (Fig. 4a–d). In contrast, the *NtGST1* gene was significantly more expressed in roots and leaves of *N. tabacum* transgenic lines than in wild-type plants (Fig. 4e and f). These significant increases in the gene expression level suggested that a plant defense response to oxidative stress may have occurred in these transgenic lines. These gene expression data were supported by a higher H_2O_2 accumulation in leaves of transgenic lines (Fig. 4g). Accordingly, the SOD enzyme activity was significantly reduced in leaves of *N. tabacum* transgenic lines (Fig. 4h), CAT enzyme activity was not altered (Fig. 4i), while APX enzyme activity was also significantly reduced in leaves of transgenic lines (Fig. 4j). Therefore, a significant increase in ROS accumulation and higher oxidative activity observed in transgenic lines suggested that this effect may be one of the major factors that hampered the plant parasitism by *M. incognita*.

Discussion

The soybean crop is of great socio-economic importance and is one of the most notable commodities worldwide (Hartman et al. 2011; Hamawaki et al. 2019). However, this crop faces increasing challenges regarding yield losses caused by PPNs (Bernard et al. 2017; Gillet et al. 2017; Mejias et al. 2019). The root-knot nematode (RKN) *M. incognita*, the cyst nematode *Heterodera glycines*, and reniform nematode *Rotylenchulus reniformis* are the major nematode species of economic importance in soybean (Basso et al. 2020b; Tylka and Marett 2021; Fragoso et al. 2022). The most effective management ways in the field are by use of soybean cultivars with reduced susceptibility or by rotation of these cultivars carrying different resistance sources (Basso et al. 2019, 2020a; Bellafiore et al. 2008). However, there are few soybean cultivars currently available commercially with a level of resistance or tolerance to these PPNs (Ali et al. 2017). Until the moment, much is known about soybean genes and proteins that are directly linked to the plant defense mechanisms against nematodes, but it is also known that structural proteins can act secondarily in plant defense (Mendes et al. 2021a).

Thus, aiming to prospect new soybean molecules such as gene and promoter sequences that act in plant defense against RKNs, this study conducted a transcriptome (RNAseq) analysis during soybean infection by *M. incognita* using the contrasting non-transgenic lines: nematode-resistant genotype PI595099 and nematode-susceptible cultivar BRS133 (Mendes et al. 2021a, b). Among the differentially expressed genes in response to plant infection by *M. incognita* using the genotype PI595099 as a reference associated with plant resistance, our results invited us to


a
AtPRX17
Relative expression (2^-ΔCT)
0.0060
0.0050
0.0040
0.0030
0.0020
0.0010
0.0000
AtWT
AtEv1
AtEv2
b
AtRbohA
0.00018
0.00016
0.00014
0.00012
0.00010
0.00008
0.00006
0.00004
0.00002
0.00000
c
AtPRX45
0.0006
0.0005
0.0004
0.0003
0.0002
0.0001
0.0000
d
AtPRX57
0.0014
0.0012
0.0010
0.0008
0.0006
0.0004
0.0002
0.0000
e
NtGST1 in root
0.00025
0.0002
0.00015
0.0001
0.00005
0
NtWT
NtEv1
NtEv2
NtEv3
f
NtGST1 in leaves
0.03
0.025
0.02
0.015
0.01
0.005
0
g
In situ H2O2 level
NtWT
NtEV1
NtEV2
NtEV3
h
SOD enzyme activity
U mg-1 protein
80
70
60
50
40
30
20
10
0
i
CAT enzyme activity
μmol H2O2 min-1 mg-1 protein
7E-10
6E-10
5E-10
4E-10
3E-10
2E-10
1E-10
0
j
APX enzyme activity
μmol ascorbate min-1 mg-1 protein
1.4E-08
1.2E-08
1E-08
8E-09
6E-09
4E-09
2E-09
0

◂**Fig. 4** Gene expression profile determined by real-time PCR in roots of three uninfected *A. thaliana* and *N. tabacum* transgenic lines compared with wild-type plants (WT). Expression profile of genes associated with ROS detoxification: **a** *AtPRX17*, **b** *AtRbohA*, **c** *AtPRX45*, **d** *AtPRX57* in *A. thaliana*, and **e** *NtGST1* in roots and **f** *NtGST1* in leaves of *N. tabacum* transgenic lines. Relative gene expression values were calculated with the 2^-ΔCT formula and normalized with *AtActin 2* and *NtActin 4* as endogenous reference genes (Suppl. Table S3). Error bars represent confidence intervals corresponding to three biological replicates consisting of four to six plants each. Different letters on the bars indicate significant statistical differences according to Tukey's test at 95% significance level. Hydrogen peroxide (H_2O_2) level and antioxidant enzyme activity in plants from three uninfected *N. tabacum* transgenic lines compared to wild-type plants. **g** In situ H_2O_2 detection by 3-diaminobenzidine (DAB) staining in leaves of transgenic lines. It is worth emphasizing here, in the peroxidase reaction in transgenic leaves, DAB serves as a hydrogen donor in the H_2O_2 presence. The oxidized DAB forms an insoluble brown end-product whose color intensity correlates with H_2O_2 concentration in the leaf tissue. Activity of **h** superoxide dismutase (SOD), **i** catalase (CAT), and **j** ascorbate peroxidase (APX) enzymes determined in total protein extract of leaves

further investigate the biological role of the *GmGlb1-1* gene. The in silico transcriptome data retrieved from Phytozome database v.13 showed that the *GmGlb1-1* gene expression was strongly higher in roots under symbiotic conditions or nodules, while a very low number or absence of its transcript was detected in other plant vegetative organs (*e.g.*, absence in shoots and very low transcripts in stems). Given these previous considerations, based on gene expression in different soybean tissues and using genome data mining were confirmed the presence of four soybean leghemoglobin genes, one Glb1 class #1 (*GmGlb1-1*) gene, one Glb2 class #2 gene, and one putative truncated globin pseudogene in the genome of nematode-resistant genotype PI595099 and the nematode-susceptible cultivar BRS133. In accordance with our data, Koltun et al. (2021) also identified these seven *Globin* genes in the soybean genome and their phylogenetic analyses showed that *GmGlb1-1* and *Glyma.11G121700* (named here as *Glb2-1* gene) genes were grouped as Glb1 class #1 and Glb2 class #2, respectively. Interestingly, in our study, the *GmGlb1-1* gene was upregulated in the nematode-resistant genotype PI595099 compared to the nematode-susceptible cultivar BRS133 during the period of penetration and migration of *M. incognita* pJ2. The *GmGlb1-1* gene expression upregulation in the nematode-resistant genotype at these early infection stages suggested its implication in reducing the plant susceptibility to *M. incognita*. Previous studies suggested that some *Globin* genes could be also induced during nematode infection (Oliveira et al. 2014). In the first moment, *GmGlb1-1* gene expression upregulation observed during soybean infection by *M. incognita* can be speculated due to oxygen consumption by nematode during its parasitism, which can be an attempt by the plant to balance this oxygen reduction. In this sense, Valliyodan et al. (2014) and Ma et al. (2019) suggested that the *GmGlb1-1* gene may be associated with response to hypoxia in soybean through the globin protein synthesis for oxygen buffering. Previous reports showed that soybean plants under flooding conditions resulted in a strong oxygen levels reduction in roots and, consequently, showed also *GmGlb1-1* gene expression upregulation (Nakayama et al. 2017; Koltun et al. 2021). Probably, this *GmGlb1-1* gene expression upregulation may be suggested as a plant defense mechanism that leads to the NO scavenging which accumulated as a consequence of plant infection by *M. incognita* (Sato et al. 2019).

To better understand these observations and improve the understanding of the action mode of the GmGlb1-1 protein were generated *A. thaliana* and *N. tabacum* transgenic lines overexpressing the *GmGlb1-1* gene. Transgenic lines with higher *GmGlb1-1* gene expression level grown under growth room or greenhouse conditions were challenged with *M. incognita* ppJ2 race 3. Both *A. thaliana* and *N. tabacum* transgenic lines showed a significant reduction in the plant susceptibility level to *M. incognita*. Consistent with this observation, gall morphology data revealed undersized feeding sites with more vacuolated giant cells typically observed in incompatible interactions, consequently affecting nematode development (Junior et al. 2017). Since Glb1 proteins have a high affinity for oxygen, it was initially hypothesized that constitutive overexpression of the *GmGlb1-1* gene could lead to an unbalance in oxygen levels in non-stressed transgenic roots (Singh and Varma 2017). This effect could result in activation of defense response similar to hypoxia stress, consequently, might impair plant infection by nematodes and, possibly, reduce the plant growth and yield. Curiously, uninfected *A. thaliana* and *N. tabacum* transgenic lines kept under growth room or greenhouse conditions showed equivalent biomass and seed yields. However, *N. tabacum* transgenic lines showed plants with more elongated growth when under well-irrigated conditions, while that transgenic plants under flooding/hypoxia were able to keep the more elongated growth and developed also a significantly greater amount of axillary roots. These collective data showed that these transgenic lines have remarkable physiological changes and improved resilience to stress conditions. These data fit what was observed in tobacco plants overexpressing a *Globin* gene from the bacteria *Vitreoscilla* sp. (Holmberg et al. 1997; Bülow et al. 1999) and a *Globin* gene from humans (Dieryck et al. 1997), such as enhanced growth and metabolites production. Similarly, Hebelstrup et al. (2006) showed that an *A. thaliana* transgenic line with *Glb1* gene knockout had high NO accumulation and abnormal plant development, indicating the importance of this Glb1 protein for NO scavenging. In accordance, Hebelstrup and Jensen (2008) showed also that an *A. thaliana* transgenic line overexpressing a *Glb1* gene has improved NO scavenging and, consequently, improved plant development. Comparatively, Barata et al. (2000) targeting a soybean globin protein

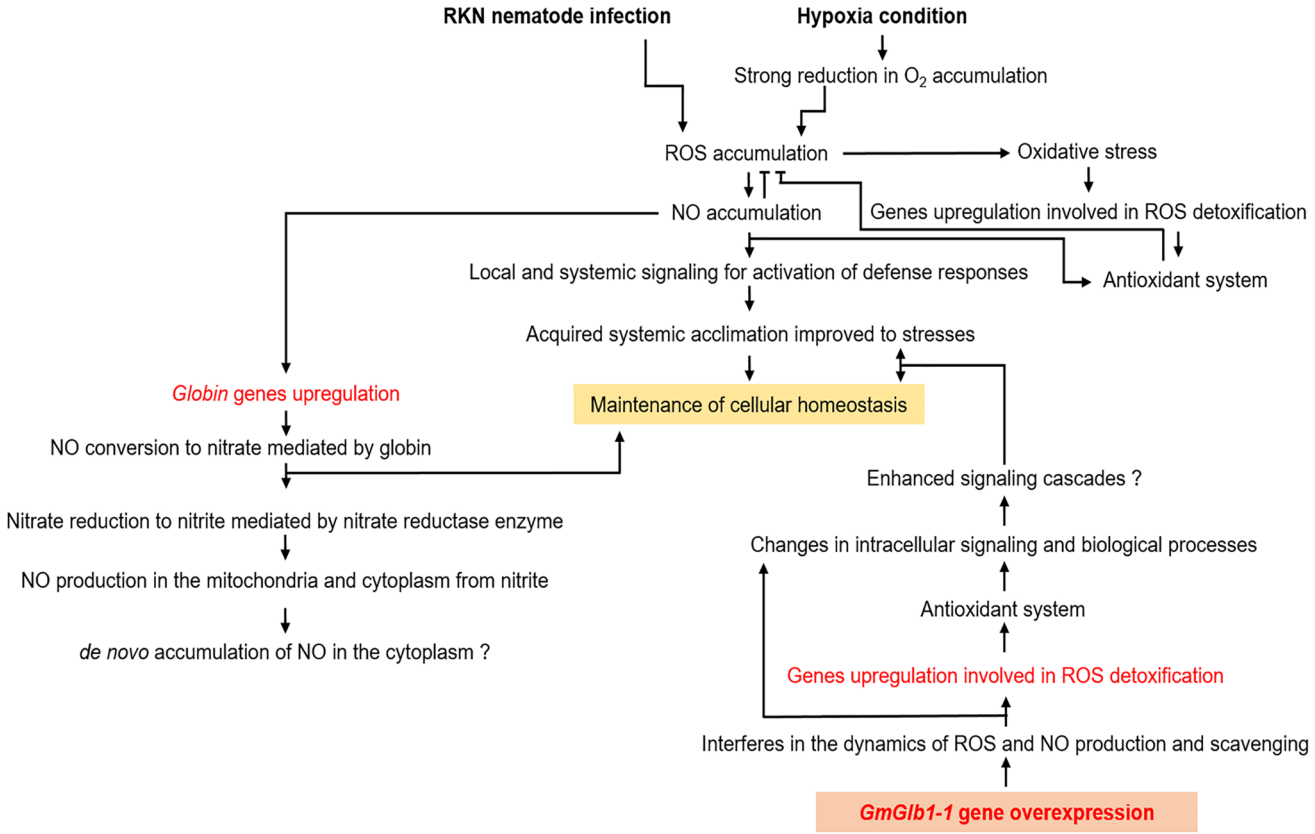


Fig. 5 Action mode proposed by the *GmGlb1-1* gene upregulated in *A. thaliana* and *N. tabacum* transgenic plants. The *M. incognita* infection, as well as, hypoxia conditions which drastically reduce oxygen (O_2) levels in roots, results in higher reactive oxygen species (ROS) and nitric oxide (NO) accumulation in stressed plants, which in *trans* leads to an increased or decreased acquired systemic acclimation to stresses and, later on, increased or reduced maintenance of cellular homeostasis. To efficient NO scavenging highly accumulated in consequence of these stresses, some *Glb1* genes (such as *GmGlb1-1*) are upregulated (Nakayama et al. 2017; Koltun et al. 2021). The Glb1 proteins (such as GmGlb1-1) act in NO conversion to nitrate, consequently, improving the maintenance of cellular homeostasis (Igamberdiev and Hill 2004; Perazzolli et al. 2004; Gupta and Igamberdiev 2016). So, we speculated that the *GmGlb1-1* gene overexpression can interfere in the dynamics of ROS and NO, reducing the plant susceptibility to *M. incognita* and, potentially, to hypoxia conditions

to tobacco chloroplasts did not produce any significant alteration in photosynthesis, starch, sucrose, and enzymes involved in aerobic metabolism. Besides, additional reports targeting a soybean globin protein to potato chloroplasts reduced growth and tuber production in transgenic lines (Chaparro-Giraldo et al. 2000; Bonna et al. 2008). Similarly, Dmitryukova et al. (2011) also demonstrated that *N. tabacum* transgenic lines overexpressing a *Globin* gene were more tolerant to the adverse effects caused by heavy metals, but these plants showed reduced growth.

On the other hand, several genes that act in oxidative stress responses, such as *AtPRX17*, *AtRbohA*, *AtPRX45*, *AtPRX57*, and *NtGST1* were upregulated in *A. thaliana* and *N. tabacum* transgenic lines. Particularly, these AtPRX proteins (class III plant peroxidases) catalyze the H_2O_2 reduction and produce ROS such as ^{-}OH or HOO^{-} (Cosio et al. 2017). In contrast, the AtRbohA protein act on ROS production during the plant defense response sustaining rhizobial invasion and nodule formation (Arthikala et al. 2017). Similarly, NtGST1 protein is commonly induced by diverse biotic and abiotic stimuli and it is important to protect the plant cells from oxidative damage (Hasan et al. 2021). Thus, we speculated based on our data and previous results (Nakayama et al. 2017; Koltun et al. 2021) that overexpressing the *GmGlb1-1* gene can interfere with the dynamic of ROS production and NO scavenging, leading to changes in intracellular signaling and biological processes, enhancing plant resilience to biotic or abiotic stresses, and improving cellular homeostasis (Fig. 5). This hypothesis was supported by the higher H_2O_2 accumulation and lower activity of SOD and APX antioxidant enzymes observed in transgenic lines. Regarding the higher oxidative activity in these transgenic lines, two hypotheses can be made for this observation: (*i*) the improved NO scavenging may reduce the inhibitory effect of NO on ROS production, and (*ii*) GmGlb1-1 protein highly accumulated resulted also in higher ROS production due to its potential involvement in other biological processes.

Importantly, Koltun et al. (2021) showed also that the *GmGlb1-1* gene was upregulated in soybean roots under flooding conditions, whereas composite soybean roots overexpressing the *GmGlb1-1* gene showed expression upregulation of several other genes involved in antioxidant responses, hypoxia-related genes, lower activity of antioxidant enzymes, and significant changes in H_2O_2 accumulation. Thus, we speculated that this more prominent oxidative stress observed in *A. thaliana* and *N. tabacum* transgenic lines is far less harmful to these plants than for the nematode establishment and development during plant parasitism. This observation was supported by the analysis of galls morphology which showed that at 10 DAI the nematodes that parasitize these transgenic lines have their development significantly compromised. In addition, the higher H_2O_2 accumulation in these transgenic lines and/or triggered by the nematode infection can increase the extracellular H_2O_2-triggered programmed cell death rate, which impairs the plant infection success by *M. incognita* (Gechev and Hille 2005). At the same time, better NO scavenging in transgenic lines enables these plants to maintain better cellular homeostasis, mainly when under stress. Overall, these collective data revealed the potential role of GmGlb1-1 protein in soybean during plant parasitism by *M. incognita*.

In conclusion, herein was demonstrated that the *GmGlb1-1* gene was upregulated in soybean during plant infection by *M. incognita,* and was confirmed in model plants that this gene can be closely associated with reduced plant susceptibility to this nematode. So that, constitutive overexpression of the *GmGlb1-1* gene in transgenic lines reduced significantly plant susceptibility to *M. incognita*. In addition to the putative increased NO scavenging provided by higher GmGlb1-1 protein accumulation, the higher H_2O_2 accumulation and lower SOD and APX enzyme activity in transgenic lines revealed higher oxidative activity in these transgenic lines, which can explain in parts the reduced plant susceptibility to *M. incognita*. The improved NO scavenging can be speculated as the major effect conferred by the *GmGlb1-1* gene overexpression and, then, associated with the reduced plant susceptibility. Finally, was highlighted the potential use of the *GmGlb1-1* gene for the genetic engineering of soybean cultivars. Therefore, soybean transgenic plants overexpressing the *GmGlb1-1* gene under the control of constitutive or nematode-induced promoters or modulation of *GmGlb1-1* gene expression through intervention in its promoter by CRISPR/dCas9 strategy can be an interesting strategy to reduce plant susceptibility to *M. incognita*.

Author contribution statement MFGS was the project leader. MFB performed in silico analysis, plant transformation, and molecular assays. FCM-G provides access to the genome raw data from the soybean contrasting cultivars. MMCC and RCT performed the assembly of genomes from contrasting soybean cultivars and the promoter and CDS sequences identification of the *GmGlb1-1* gene. FBMA provided access to soybean RNAseq datasets. LLPM designed and provided the binary vector. MFB, ITLT, MCMS, RAGM, CEMP, and MRN performed the production of *M. incognita* ppJ2 inoculum and evaluated all the bioassays. CEMP, ITLT, ACMMG, and JAE, performed the galls morphology analysis. ITLT, AG, AFM, and BPM supervised by JAE performed in situ oxygen species analysis and antioxidant enzyme activity. MSB, EISF, VF, and AEP provided intellectual inputs to the work. MFB wrote the manuscript while MFGS and JAE reviewed it. All authors approved the final version.

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Data availability statement The partial genome sequences are provided in the NCBI database from GenBank accession number: GmGlb1.1_PI595099: ON228174 and GmGlb1-1_BRS133: ON228175. The nucleotide sequence can be accessed at 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 at https://www.ncbi.nlm.nih.gov/bioproject/. In addition, genome target sequence and transcriptome data, such as gene expression, are also provided by authors as supplementary data.

Declarations

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

Consent for publication or ethical approval and consent to participate Not applicable.

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