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RESEARCH PAPER

Endogenous soybean peptide overexpression: an alternative to protect plants against root-knot nematodes

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Abstract Nematodes are pathogens of many important crops, including soybean. The main species found in Brazil are root-knot (*Meloidogyne* spp.), cyst (*Heterodera glycines*), root lesion (*Pratylenchus brachyurus*) and reniform (*Rotylenculus reniformis*) nematodes. Ureases are traditionally known for catalyzing the hydrolysis of urea to ammonia and carbon dioxide. Besides the main function, they present other independent biological roles, including toxic activities against insects, specially Coleoptera and Hemiptera, and fungi. In previous work, the DNA sequence encoding an insecticidal peptide - named Jaburetox - was identified in a *Canavalia ensiformes* urease gene. The recombinant Jaburetox exhibited toxicity against insects. Subsequently, the DNA sequence corresponding to Jaburetox was identified as part of the soybean Eu4 urease gene, with the resulting peptide named Soyuretox. In the present study, explants of soybean were transformed with *Agrobacterium rhizogenes* and 'composite' plants produced consisting of wild-type shoots and transgenic hairy roots overexpressing Soyuretox. Thereafter soybean plants overexpressing Soyuretox were obtained through bombardment transformation.

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Due to similarity between nematode and insect digestion mechanisms, we challenged composite and whole-transgenic plants with the nematode *Meloidogyne javanica*. Hairy roots overexpressing Soyuretox exhibited a significant reduction (48 %; $p < 0.05$) in the average reproductive factor when compared with empty-vector transformed hairy roots. Transgenic plants overexpressing Soyuretox also exhibited significant reduction (37.5 %; $p < 0.05$) in reproductive factor when compared with non-transformed plants. This study demonstrates the potential of Soyuretox in confering resistance against nematodes, representing a new alternative control method for nematodes in economically important crops.

Introduction

Soybean (*Glycine max*) is affected by several abiotic and biotic stresses that limit the geographical distribution of the crop and lead to significant reductions in growth and productivity. Nematodes, causing yield losses of billions of dollars annually, represent a significant threat to global crop production (Bernard, Egnin, & Bonsi, 2017; Nicol et al., 2011).

In Brazil, diseases caused by nematodes are amongst the most damaging biotic stresses for soybean. The main species found in the country are *Meloidogyne spp*. (root-knot), *Heterodera glycines* (cyst), *Pratylenchus brachyurus* (root lesion) and *Rotylenchulus reniformis* (reniform) (Dias, Garcia, Silva, & Carniero, 2010). Root-knot and cyst nematodes are obligate biotroph sedentary nematodes and are considered the most damaging. As they induce the formation of complex feeding sites within the roots, they are very difficult to control (Jones et al., 2013). Plants infested by root-knot nematodes exhibit drastic morphological and physiological changes such as retarded growth, chlorosis and low yields.

The root-knot nematode life cycle, which can be completed in approximately 30 days under optimal conditions, includes six stages: egg, four juvenile stages and adult. Upon infection, pre-parasitic second-stage juveniles (J2) penetrate roots and migrate toward the vascular cylinder, where they induce the development of a multinuclear feeding site, which consists of several giant cells (Moens, Perry, & Star, 2010). The expansion and proliferation of neighboring cortical and pericycle cells leads to gall formation (Fuller, Lilley, & Urwin, 2008). After feeding, the larvae develop into third (J3) and fourth (J4) juvenile stages and finally into adults. Adult males are vermiform and leave the roots. Females continue to feed and grow, becoming pear-shaped (Chitwood & Perry, 2009). Mature females lay eggs in a protective gelatinous matrix, forming an egg mass, which are found on the root surface or are incorporated into galls, and can contain up to 1000 eggs (Moens et al., 2010).

Genetic engineering has proven a promising tool for the development of biotic and abiotic stress tolerance in crop plants. Regarding plant parasitic nematode resistance, current control strategies include natural resistance genes, overexpression of proteinase inhibitor coding genes and antinematodal proteins, and biotechnological tools, such as RNA interference to silencing nematode effectors (Ali et al., 2017). Considering the injury caused in soybean by nematodes and the occurrence of resistance breakage in current varieties, the identification of genes with the potential to confer resistance, plus the development of biotechnological strategies that will contribute to the production of highly resistant varieties, are both mandatory.

Plant ureases are multifunctional enzymes that are traditionally known to catalyze the conversion of urea to ammonia and carbon dioxide. These enzymes are found in virtually all plants, but are especially abundant in leguminosae and cucurbitaceae seeds. Three isozymes were described for *Canavalia ensiformis* (jack bean): JBU, canatoxin (CNTX) and JBUre-II (Mulinari et al., 2011). Soybean also produces three urease isoforms: the ubiquitous urease encoded by the *Eu*4 gene (Glyma.11G248700); the embryo-specific urease encoded by the *Eu*1 gene (Glyma.05G146000) (Goldraij, Beamer, & Polacco, 2003; Holland, Griffin, Meyer-Bothling, & Polacco, 1987) and the SBU-III urease-encoded by the *Eu*5 gene (Glyma.08G103000) (Wiebke-Strohm et al., 2016; Witte, 2011). Besides their role in nitrogen metabolism, it has been demonstrated that ureases can be toxic to fungi and insects (Kappaun, Piovesan, Carlini, & Ligabue-Braun, 2018). The domains conferring the insecticidal and fungicidal properties are independent of the enzyme activity (Becker-Ritt et al., 2007; Follmer, Real-Guerra, Wasserman, Olivera-Severo, & Carlini, 2004).

Insecticidal activity is mostly mediated through a proteolytic activation process that depends on the profile of the insect's digestive enzymes, which release toxic peptides (Carlini, Oliveira, Azambuja, Xavier-Filho, & Wells, 1997; Ferreira-DaSilva, Gombarovits, Masuda, Oliveira, & Carlini, 2000). *Canavalia ensiformis* (jack bean) and soybean ureases are particularly toxic to Coleoptera and Hemiptera, whose digestive tracts rely on proteolytic cathepsin-like enzymes (cysteine and aspartic proteases) (Stanisçuaski & Carlini, 2012). Similarly to insects, cysteine proteases have mostly been found to be expressed in gland or intestinal cells of nematodes and act as the main digestive enzymes (Dutta et al., 2015). Cathepsins have been demonstrated to have importance in the development and invasion process of plant parasitic nematodes, including *Meloidogyne* spp. (Wang, Cheng et al., 2018).

An insecticidal peptide of ~10 kDa was isolated from canatoxin of jack bean urease (Carlini & Ferreira-Da-Silva, 2000; Ferreira-DaSilva et al., 2000). Subsequently, the corresponding DNA sequence was identified in the *jbure-II* gene and the encoded peptide, called Jaburetox-2Ec (Mulinari et al., 2007) or simply Jaburetox (Postal et al., 2012). The recombinant Jaburetox exhibited toxicity against insects such as *Dysdercus peruvianus* and *Rhodnius prolixus*, including some species that were not affected by native ureases, such as the fall armyworm *Spodoptera frugiperda* (Stanisçuaski & Carlini, 2012). The DNA sequence corre-

sponding to Jaburetox was identified as part of the soybean *Eu4* gene, and the resulting peptide was named Soyuretox (Kappaun et al., 2018). This polypeptide is colinear to Jaburetox (Mulinari et al., 2007), sharing 72 % of identity considering the amino acid sequences. The biological properties were determined against yeasts and insects in order to compare to the Jaburetox's entomo- and fungitoxic activities (Carlini & Ligabue-Braun, 2016; Postal et al., 2012). Soyuretox presented biological activities against *Candida albicans* and *Rhodnius prolixus* and was not toxic to zebrafish larvae, demonstrating its' biotechnological potential (Kappaun et al., 2019).

In the present study, 'composite' plants (i.e. soybean plants consisting of wild-type shoots and transgenic hairy roots) and whole-transgenic plants overexpressing Soyuretox were produced, and challenged with the nematode *Meloidogyne javanica*. This is the first report providing evidence that the overexpression of Soyuretox in hairy roots and stable transgenic plants can be used to increase resistance against nematodes.

Materials and methods

Transformation vector

The Soyuretox-encoding sequence (242 bp) was amplified from a previously cloned pET23a::*Eu4* construct using a specific primer pair (Table 1). Start (ATG) and termination (TGA) codons and the CACC sequence (necessary for directional GATEWAY® cloning) were added to the primer sequences. The PCR mixture was prepared with 10 ng of recombinant plasmid, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.4 μM of each primer, 1 U Taq DNA polymerase (5 U/μl - Invitrogen) and autoclaved distilled water in a final volume of 25 μL. Reactions were hot-started (3 min at 94 °C) and subjected to 32 cycles as follows: 1 min at 95 °C, 1 min at 52 °C and 2 min at 72 °C.

The GATEWAY® system (Invitrogen) was used to recombine the PCR product into the pH7WG2D plant overexpression vector (Karimi, Inze, & Depicker, 2002). The T-DNA region of the resulting pH7WG2D::Soyuretox vector contained the Soyuretox-encoding sequence under the control of the CaMV 35S promoter (P35S), the hygromycin-phosphotransferase marker gene (*hpt*) and the green fluorescent protein reporter gene (*gfp*) (Supplementary Fig. S1). The amplicon identity was confirmed by sequencing using the P35S forward primer and Soyuretox reverse primer (Table 1).

Soybean transformation by *A. rhizogenes*

The pH7WG2D::Soyuretox and empty-pH7WG2D (control) plasmids were independently transformed into the *Agrobacterium rhizogenes* K599 strain. Single colonies of *A. rhizogenes* K599 were inoculated in 10 mL of Yeast Extract Peptone (YEP) liquid medium containing 100 mg/L rifampicin and 100 mg/L spectinomycin and incubated overnight under shaking at 28 °C. The pre-inoculum was inoculated in 500 mL of the same medium and incubated for an additional 24 h. Cultures were centrifuged at 500 rpm at 4 °C for 10 min, and pellets were resuspended in 8 mL of autoclaved Milli-Q water.

The soybean 'composite' plants presenting transgenic roots were obtained as previously described (Kuma et al., 2015), with minor modifications. Briefly, the radicle of seedlings (Williams-82 cultivar) was inoculated with 0.1 mL of *A. rhizogenes* culture using a syringe. The seedlings were transferred to a medium containing 15 μg/mL of hygromycin-B. After 10 days, the first selection of transformed roots was carried out by observing GFP expression under a fluorescence stereomicroscope Leica MZIII (Leica Microsystems GmbH, Wetzlar, Germany). GFP-negative roots were removed. Plants were recovered in a hydroponic system for 10 days, followed by a new GFP-positive selection under microscopy. 'Composite' plants that presented only transformed roots were transplanted into tubes containing autoclaved sand for bioassays with root-knot nematodes.

Soybean transformation by bombardment

Somatic embryogenesis was induced from IAS-5 cultivar immature cotyledons. Proliferating embryogenic tissues were submitted to transformation by particle bombardment. Transformation, selection, embryo histodifferentiation and plant regeneration were carry out according to the protocol described by Droste, Pasquali, and Bodanese-Zanettini (2002). After three months in hygromycin-B selection medium, pieces of green tissues (i.e. hygromycin-resistant) were visually selected and subcultivated individually for the establishment of transgenic lines. All plants regenerated from a piece of hygromycin-resistant tissue were considered as being clone plants derived from an independent transformation event. Control plants were obtained from embryogenic tissues bombarded with DNA-free particles and submitted to the same protocol without hygromycin selection.

Molecular analysis of transgenic hairy roots and plants

Total DNA was extracted from transgenic hairy roots in 'composite' plants exhibiting fluorescence signals of GFP and leaves from whole transgenic plants according to Doyle and Doyle (1987). All samples were PCR-screened for the presence of the P*35S*-Soyuretox chimeric gene using the primers described in Table 1. The PCR mixture contained 200−300 ng DNA, 1X PCR buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTP, 0.4 μM each primer, 1 U Taq DNA Polymerase (Invitrogen), and sterile distilled water to a final volume of 25 μL. Reactions were hot-started for 3 min at 94 °C and subjected to 32 cycles as follows: 1 min at 95 °C; 1 min at 52 °C and 2 min at 72 °C. PCR products were analyzed on a 1 % agarose gel containing ethidium bromide (0.01 mg/L).

Similarly, total RNA was extracted from the same plant material using TRIzol reagent (Invitrogen) and treated with DNAse I (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were obtained using 1 μg of DNA-free RNA, the M-MLV Reverse Transcriptase System™ (Invitrogen) and oligo (dT) primers. RT-qPCR was performed on a StepOnePlus Applied Biosystem Real-time Cycler™.

Table 1 Primer set designed for PCR and sequencing.

Target gene	Orientation	Sequence	Reference
Isolation of the coding sequence of Soyuretox (PCR)	Forward Reverse	5'-CACCATGGGTCCAGTTAATGAT-3' 5'-TCAGACTTTCCCACCTCCAAA-3'	This study
Confirmation of the condition of transgenic roots (PCR)	Forward Reverse	5'-CGCACAATCCCACTATCCTT-3' 5'-TCAGACTTTCCCACCTCCAAA-3'	This study
Confirmation of the presence *M. javanica* in roots (PCR)	Forward Reverse	5'-CAAAACCACGCGGCTTCGGC-3' 5'-TGGGGGTGCCCTTCCGTCAA-3'	This study
Cloning and expression of Soyuretox in *Escherichia coli*	Forward Reverse	5'-CAACATATGGGTCCAGTTAATGATTCTAATTGC-3'* 5'-CAAGCGGCCGCGACTTTCCCACCTC-3'*	This study
Soyuretox (RT-qPCR)	Forward Reverse	5'-ATTGCAGAGCAGCCATGAA-3' 5'-TGGCCCTACAACTGGTGAC-3'	This study
Actin II (RT-qPCR)	Forward Reverse	5'-CGGTGGTTCTAT CTTGGCATC-3' 5'-GTCTTTCGCTTCAA TAACCCTA-3'	Jian et al., 2008
Metalloprotease (RT-qPCR)	Forward Reverse	5'-ATGAATGACGGTTCCCATGTA-3' 5'- GGCATTAAGGCAGCTCACTCT -3'	Libault et al., 2008
Fbox protein (RT-qPCR)	Forward Reverse	5'-AGATAGGGAAATGTTGCAGGT-3' 5'-CTAATGGCAATTGCAGCTCTC-3'	Libault et al., 2008

* Restriction sites are underlined.

PCR-cycling conditions were: 5 min at 94 °C, followed by 40 repetitions of 10 s at 94 °C, 15 s at 60 °C and 15 s at 72 °C. A melting curve analysis was performed at the end of the PCR run, over the range of 55—99 °C, increasing the temperature stepwise by 0.1 °C every 1 s. Each 25-μL reaction comprised 12.5 μL of cDNA (1:50 dilution), 1x PCR buffer (Invitrogen), 2.4 mM $MgCl_2$, 0.024 mM dNTPs, 0.1 μM each primer, 2.5 μL of SYBR-Green (1:100,000, Molecular Probes) and 0.03 U of Platinum Taq DNA Polymerase (5 U/μL, Invitrogen). All PCR reactions were performed in technical quadruplicates. Reactions lacking cDNA were used as negative controls. Primers for the Soyuretox transcript detection were designed using Primer3 software (Table 1). The F-box protein and a Metalloprotease were used as reference for gene expression normalization (Jian et al., 2008; Libault et al., 2008). The gene expression data analyses were performed using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Root-knot nematode bioassays in soybean roots

M. javanica eggs were placed in a hatching chamber to obtain the infective juvenile stage (J2). After two days, larvae were separated from unhatched eggs and adjusted to a final concentration of 500 J2 larvae/mL. In the 'Composite' soybean plants assay, 500 J2 larvae were inoculated in the root system of each plant through a hole in the sand. Eleven 'composite' plants transformed with Soyuretox and ten with empty-vector were tested. In whole transformed plant assays, ten T_2 transgenic clonal plants (derived from the same transformation event) and six non-transformed plants were inoculated with 5000 J2 larvae/plant.

Forty-five days after inoculation, root tissues from both experiments were collected, washed and divided into two sets: one for molecular analyses and the second to estimate the nematode reproductive factor (RF).

PCR using primers for detection of the nematode 18S rRNA encoding-gene (Table 1) was employed to confirm *M. javanica* infection in root tissue. Female nematode DNA was used as a positive control. The PCR mixture was prepared as described above in the previous section. The amplification conditions used were a hot-start for 1 min at 94 °C and 30 cycles as follows: 30 s at 95 °C, 30 s at 64 °C and 1 min at 72 °C. PCR products were visualized on a 1 % agarose gel containing ethidium bromide (0.01 mg/L).

To determine the nematode final populations, roots and hairy roots were processed as previously described (Boneti & Ferraz, 1981). Briefly, the infected roots were weighted, cut in pieces, placed in a blender, immersed in a 0.5 % sodium hypochlorite solution and crushed. The resulting suspension was filtered through 60 and 500-mesh sieves, successively. The retained material in the 500-mesh sieve (which included eggs and J2 larvae) was washed with distilled water to remove the excess of hypochlorite and resuspended in 80 mL of distilled water. To measure the numbers of eggs and J2 individuals, 1 mL of the suspension was placed on a Peter's counting chamber and analyzed under optical microscopy (40×). The reproductive factor [RF = number of eggs and J2 in roots/Pi (initial population)] was obtained from the average of three counts/plant. The final RF was corrected by the root fresh weight of each plant.

Statistical analysis

A non-parametric *t* test was carried out to compare the Soyuretox transcript levels in hairy roots transformed with pH7WG2D::Soyuretox and non-transformed roots, as well as transformed *vs.* non-transformed whole plants roots.

The nematode RF values were compared among: (1) Soyuretox and empty-vector transformed hairy roots, and (2) Soyuretox transformed vs. non-transformed whole plants. One-way ANOVA followed by Tukey's test were performed using SPSS 18.0 software. Results with $p \leq 0.05$ were considered statistically significant.

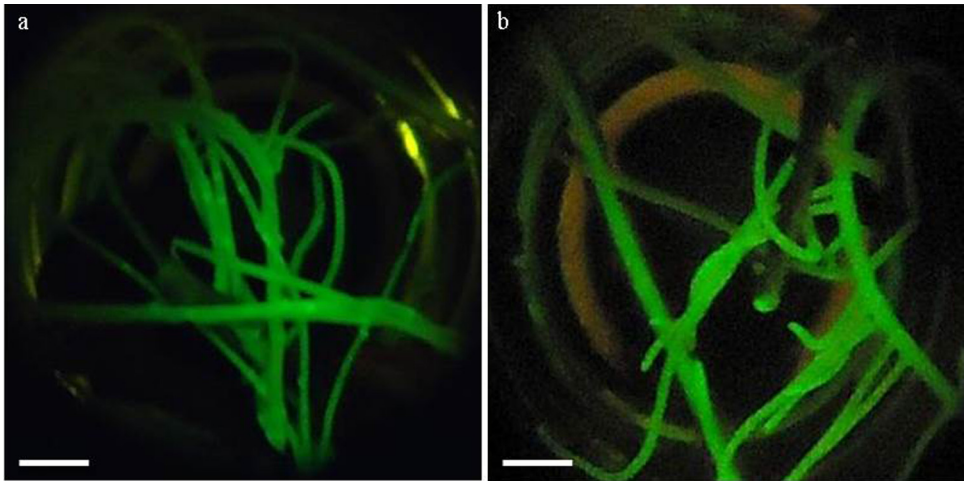

Figure 1 Detection of GFP in soybean hairy roots transformed with (a) pH7WG2D::Soyuretox and (b) empty-pH7WG2D. GFP expression was detected under blue light using a fluorescence stereomicroscope Leica MZIII (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a BP filter set containing a 488-ηm excitation filter and a 505−530 ηm emission filter. Bar =1 mm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Results

Generation and characterization of 'composite' and whole soybean transgenic plants

In order to assess whether Soyuretox overexpression would enhance resistance to nematodes, 'composite' and whole transgenic plants were obtained by *A. rhizogenes* and bombardment transformation, respectively.

After the selection period, hairy roots transformed with pH7WG2D::Soyuretox (expressing Soyuretox and GFP) and empty-pH7WG2D vector (expressing only GFP) were screened for GFP presence. Strong green fluorescence was evident in roots of the 21 'composite' plants, 11 carrying pH7WG2D::Soyuretox and 10 the empty-pH7WG2D, indicating the insertion and expression of the *gfp* gene harbored by the vector (Fig. 1). The presence of the 35S promoter and Soyuretox-enconding sequences in the genome of hairy roots was confirmed by PCR (data not shown). The transformation efficiency (i.e. number of positive 'composite' plants/number of seedlings subjected to transformation) obtained was 54 and 47 % for seedlings transformed with pH7WG2D::Soyuretox and empty-pH7WG2D, respectively. Ten clonal plants, derived from one independent transgenic event (cultivar IAS-5) carrying the pH7WG2D::Soyuretox construct were obtained by particle bombardment. The transgenic status of whole transformed plants was also confirmed by PCR (data not shown).

The Soyuretox relative expression varied in a range from 2 to 42-fold in transformed hairy roots when compared to non-transformed roots (Fig. 2a). In the same way, Soyuretox transcript levels were significantly higher in whole transgenic plants (2 to 21-fold) when compared with non-transformed plants (Fig. 2b). Basal transcript levels were detected in non-transformed hairy roots and plants, corresponding to endogenous *Eu4* gene expression.

Soyuretox overexpression confers resistance to root-knot nematode

Two bioassays were carried out aiming to evaluate the potential of Soyuretox in conferring resistance against nematodes. In the first, 'composite' plants containing only GFP-positive roots, harboring the empty vector or overexpressing the Soyuretox, were inoculated with *M. javanica* J2 larvae. Forty-five days after inoculation, 80 and 70 % of roots transformed with Soyuretox or empty-pH7WG2D, respectively, still exhibited GFP fluorescence (data not shown). Hairy roots overexpressing Soyuretox exhibited a significant reduction (48 %; $p < 0.05$) in the nematode reproductive factor when compared to empty vector-transformed roots (Fig. 3a; supplementary Table S1). In the second bioassay, whole transgenic plants overexpressing Soyuretox also exhibited significant reduction (37.5 %; $p < 0.05$) in the nematode's reproductive factor when compared to non-transformed plants (Fig. 3b; supplementary Table S2) 45 days after inoculation. These results indicate that the overexpression of Soyuretox is able to confer resistance to root-knot nematode in soybean.

All developmental stages of nematodes were found inside both Soyuretox-overexpressing and non-transformed roots (supplementary Fig. S2). The nematodes were almost exclusively detected in the root cortex.

Discussion

Although ureases are highly conserved molecules, one of the regions that exhibit remarkable variation in amino acid sequence corresponds to the N-terminal domain of Jaburetox (Mulinari et al., 2007). This portion of the polypeptide was later identified as the Jaburetox insecticidal domain (Martinelli et al., 2014). Jaburetox has also been described as fungitoxic to filamentous fungi and yeasts (Postal et al., 2012), although the structure-activity relationships implicated in this biological activity are not known yet. It is

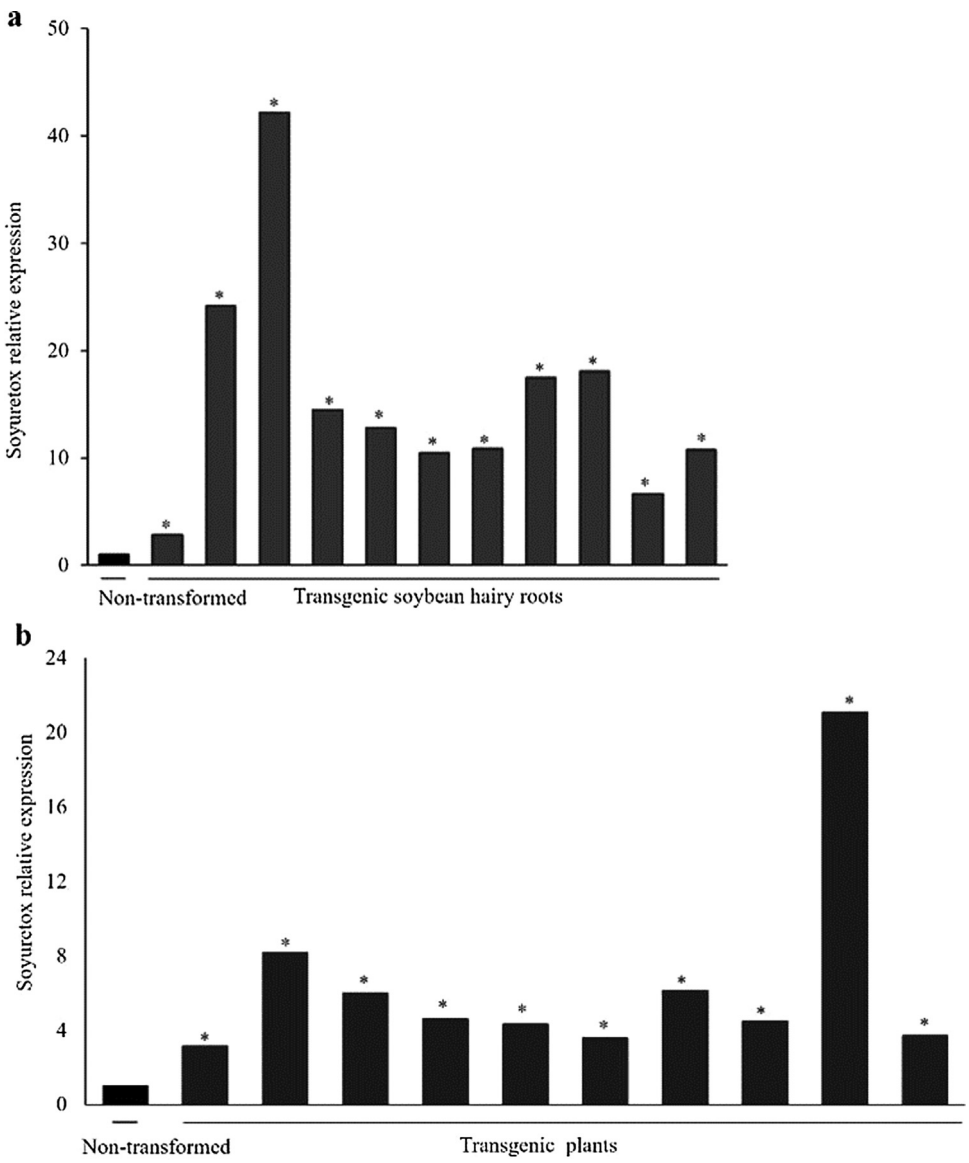

Figure 2 Soyuretox transcript levels (RT-qPCR). (a) Hairy roots of eleven 'composite' plants and a non-transformed plant (NT) from cv Williams82. (b) Ten transgenic whole-plants and a non-transformed plant (NT) from cv IAS-5. F-Box and Metalloprotease reference genes were used as internal control. Basal *Eu4* transcript levels detected in non-transformed hairy roots and leaves were used to normalize transcript accumulation in transgenic samples. The bars represent the means of four technical replicates. A non-parametric *t*-test ($p < 0.05$) was carried out to compare the Soyuretox transcript levels in transformed vs. non-transformed hairy roots and transformed vs. non-transformed plants. The symbol * indicates that values are significantly different from control sample (NT).

reasonable to expect that peptides collinear to Jaburetox that are derived from other plant ureases might possess different biological properties due to differences in the primary sequences of their N-terminal domains; for this reason, this is a topic worth investigating.

Given the very specialized feeding habits of root-knot nematodes, bioassays aiming to identify compounds with nematicidal properties often require a transformed plant expressing this compound in its roots. The *A. rhizogenes*-mediated transformation method allows the high-throughput production of transgenic hairy roots. This system has been widely used because it significantly reduces the time required to generate transgenic events compared to traditional methods for soybean transformation (Lin et al., 2013; Weber & Bodanese-Zanettini, 2011). Due to this feature, hairy roots have been used as a transgenic tool for the overexpression of proteins and secondary metabolites and for the study of gene function in plants (Guillon, Tremouillaux-Guiller, Pati, Rideau, & Gantet, 2006; Morriss, Studham, Tylka, & MacIntosh, 2017). In soybean, transgenic hairy root and 'composite' plant assays have been used to characterize the roles of genes involved in response to abiotic stresses, especially salt and drought tolerance (Li et al., 2018; Wang, Cheng et al., 2018; Wang, Chen et al., 2018; Cheng et al., 2019; Li et al., 2019). Regarding biotic stresses, nematode reproduction on soybean hairy roots was

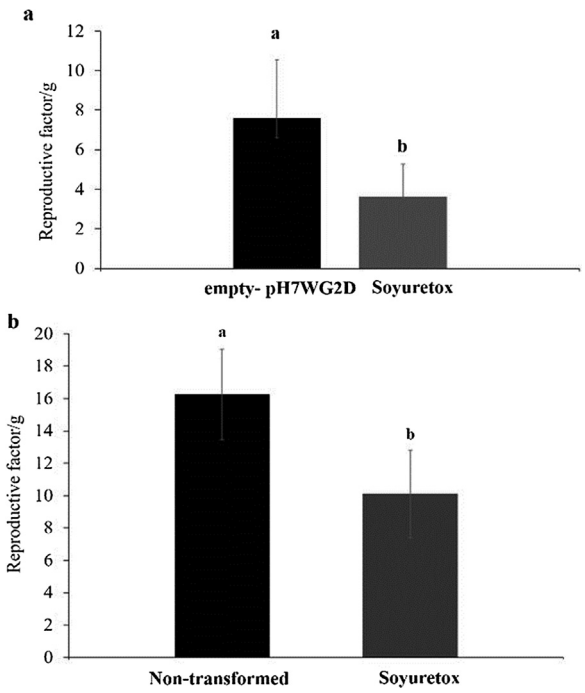

Figure 3 Reproductive factor of *M. javanica* per gram of roots 45 days after inoculation. The bars represent the nematode reproductive factor ± SD in: (a) hairy roots of 11 'composite' plants transformed with pH7WG2D::Soyuretox (overexpressing Soyuretox), 10 'composite' plants transformed with empty-pH7WG2D from cv Williams82. (b) Ten whole-transgenic plants transformed with pH7WG2D::Soyuretox (overexpressing Soyuretox) and six non-transformed plants (NT) from cv IAS-5. Bars with different letters differ according to ANOVA and Tukey's test at $p < 0.05$.

used to confirm that the *Rhg4* gene (for resistance to *Heterodera glycines*) confers resistance to this pathogen (Liu et al., 2012). Morriss et al. (2017) validated this method to study soybean aphid resistance genes. Authors showed that soybean aphid (leaf/stem herbivores) populations grows in hairy roots similarly to those growing on leaves. Our results corroborate that 'composite' plants are a good system for functional analysis of the resistance response to root-knot nematodes.

The increased resistance exhibited by Soyuretox-expressing hairy roots was validated using a stable whole-plant transgenic line. T_2 Soyuretox plants with a range of transgene expression also showed lower nematode reproductive factor when challenged with *M. javanica* compared to non-transformed plants. Proteins directly involved in inhibiting the nematode development on the plants are called anti-nematode proteins. The well-known lectins, certain antibodies, and Bt Cry proteins are examples of such proteins (Ali et al., 2017). Although genes encoding the Cry proteins efficient against Lepidoptera are quite common, those with nematicidal properties are rare (Gonçalves et al., 2014). Transgenic expression of Cry6A and Cry5B proteins in tomato hairy roots affected the reproduction of root-knot nematode *Meloidogyne incognita* (Li, Wei, Tan, & Aroian, 2007; Li et al., 2008).

Studies of soybean-nematode interactions have focused mainly on counting the number of galls and cysts (Ibrahim et al., 2011; Youssef, Margaret et al., 2013; Youssef, Kim, Haroon, & Matthews, 2013: Matthews et al., 2014). In the current study, preliminary visual observations revealed a higher number of galls in non-transformed roots (Supplementary Fig. 3). Because the number of eggs inside a gall varies, the reproductive factor provides a more precise result. This measure has been widely used in nematological studies to define resistance and susceptibility of plants to nematodes (Carneiro, Rading, Almeida, & Campos, 2000; Hamawaki et al., 2019; Lund, Mourtzinis, Conley, & Ané, 2018; Wilcken, Rosa, Higuti, Garcia, & Cardoso, 2010). Thus, the reproductive factor of *M. javanica* was determined by counting the number of eggs and nematodes after one cycle of replication (45 days). A reduction of 48 % and 37.5 % in the reproductive factor was observed in hairy roots and roots of whole transgenic plants overexpressing Soyuretox, respectively. Interestingly, the reduction did not exhibit a direct correlation with transgene transcript levels, indicating that a low amount of Soyuretox is sufficient to hinder nematodes.

In conclusion, the overexpression of Soyuretox in hairy roots and whole transgenic plants was found to increase the soybean resistance to root-knot nematodes. Decrease in the root-knot nematode reproductive factor brought about by Soyuretox expression can be valuable to integrated pest management systems against this important parasite, and perhaps other plant parasitic nematodes. Soyuretox could also be 'stacked' with, for example, Cry proteins, or combined with non-transgenic genotypes with nematode-resistant traits, providing a greater efficiency to nematode control. This strategy can contribute to reduce the use of chemical pesticides and negative environmental impact. In addition, the use of a native peptide derived from a soybean protein, such as Soyuretox, will likely facilitate the consumer's acceptance of genetically modified crops.

Author contribution

Conceived and performed the experiments: CR, BW-S, LAO-B, FCM-G, MCMC, VSLC, SMHS, WPD, CRC, MHB-Z. Wrote and revised the paper: CR, BW-S, LAO-B, RLMW, FCM-G, CRC, MHB-Z. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biori.2019.12.002.

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