The soybean Rhg1 amino acid transporter gene alters glutamate homeostasis and jasmonic acid-induced resistance to soybean cyst nematode

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SUMMARY

Rhg1 (resistance to Heterodera glycines) is an important locus that contributes to resistance against soybean cyst nematode (SCN: Heterodera glycines Ichinohe), which is the most economically damaging disease of soybean worldwide. Simultaneous overexpression of three genes encoding a predicted amino acid transporter, an α-soluble N-ethylmaleimide-sensitive factor attachment protein (α-SNAP) and a predicted wound-induced protein resulted in resistance to SCN provided by this locus. However, the roles of two of these genes (excluding α-SNAP) remain unknown. Here, we report the functional characterization of Glyma.18G022400, a gene at the Rhg1 locus that encodes the predicted amino acid transporter Rhg1-GmAAT. Although the direct role of Rhg1-GmAAT in glutamate transport was not demonstrated, multiple lines of evidence showed that Rhg1-GmAAT impacts glutamic acid tolerance and glutamate transportation in soybean. Transcriptomic and metabolite profiling indicated that overexpression of Rhg1-GmAAT activated the jasmonic acid (JA) pathway. Treatment with a JA biosynthesis inhibitor reduced the resistance provided by the Rhg1-containing PI88788 to SCN, which suggested that the JA pathway might play a role in Rhg1-mediated resistance to SCN. Our results could be helpful for the clarification of the mechanism of resistance to SCN provided by Rhg1 in soybean.

Keywords: amino acid transporter, glutamate, jasmonic acid, Rhg1, soybean, soybean cyst nematode.

INTRODUCTION

Soybean cyst nematode (SCN; Heterodera glycines Ichinohe) is an important plant-parasitic pest in soybean [Glycine max (L.) Merrill] and causes substantial damage to soybean production worldwide (Niblack et al., 2006). SCNs are obligate endoparasites that penetrate soybean roots and reprogram host root cells to form multinucleate cells, termed syncytia, which subsequently rob the infected plant of its nutrients for their own growth and reproduction (Gheysen and Mitchum, 2011). Other than rotation with non-host crop plants, the breeding of SCN-resistant soybean varieties is the most effective and environmentally friendly technique for the control of SCN (Cook, 2004).

The Rhg1 (resistance to Heterodera glycines) locus, sometimes in combination with the Rhg4 locus, provides the strongest known resistance to SCN (Brucker et al., 2005; Caldwell et al., 1960; Tylka et al., 2010; Webb et al., 1995) and has been widely used in the breeding of SCN-resistant soybean cultivars. Two different alleles exist at the Rhg1 locus: Rhg1-a, which is from Peking, and Rhg1-b, which is from PI88788 (Concibido et al., 2004; Kim et al., 2010). These two alleles have distinctive mechanisms of resistance to SCN. Rhg1-a epistatically interacts with Rhg4 in providing resistance to SCN race 3 (HG type 0), whereas Rhg1-b functions independently (Brucker et al., 2005; Meksem et al., 2001). Three genes are responsible for the resistance provided by Rhg1-b: Glyma.18G022400 (Rhg1-GmAAT), which encodes a predicted amino acid transporter; Glyma.18G022500 (GmSNAP18), which encodes a predicted α-soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein (α-SNAP); and Glyma.18G022700 (Rhg1-GmWI12), which encodes a putative wound-induced protein (Cook et al., 2012). The overexpression of any one of these three genes cannot provide resistance to SCN in soybean. However, the simultaneous overexpression of all three genes has been shown to account for resistance to SCN at Rhg1 (Cook et al., 2012). Recently, the function of GmSNAP18 by itself at Rhg1-b has been investigated (Bayless et al., 2016; Liu et al., 2017); this gene encodes a dysfunctional α-SNAP variant and results in the interruption of NSF function and vesicle trafficking in soybean. For the other two
genes, Rhg1-GmAAT and Rhg1-GmWI12, resistance is attributable to elevated expression due to the increased copy number rather than nucleotide mutations. However, the characterization of these two genes, as well as the understanding of how all three genes coordinately function to provide resistance to SCN, is lacking.

Amino acids represent the main form of nitrogen transported by xylem and phloem among different organs in plants. In the roots, xylem loading involves membrane export from the stele cells into the xylem sap. In the shoots, apoplastic phloem loading from the leaf parenchyma involves export and import steps across membranes (Simpson, 1986). At the cellular level, amino acid transport across membranes is mediated by importers, exporters and facilitators (Okumoto and Pilot, 2011). Importers mediate transport towards the cytosol; exporters mediate transport away from the cytosol; in addition, importers and exporters mediate transport from or to the apoplasm, vacuoles or intracellular vesicles, respectively. More than 20 amino acid importers have been isolated and characterized, most of which are recognized as amino acid permeases (AAPs), cationic amino acid transporters (CATs) and lysine (Lys)/histidine (His) transporters (LHTs). All of these transporters mediate the proton-dependent import of amino acids into the cell and have important biological functions in plants (Chen and Bush, 1997; Chen et al., 1993, 1995; Hirner et al., 2006; Lee and Tegeder, 2004; Simpson, 1986). For example, AtAAP1 and AtLHT1 are responsible for the uptake of amino acids into root and mesophyll cells (Hirner et al., 2006; Lee et al., 2007; Svennerstam et al., 2008, 2011). AtAAP2 and AtAAP8 are involved in the accumulation of amino acids in developing seeds (Okumoto et al., 2002; Schmidt et al., 2007). In contrast with amino acid importers, few exporters have been identified (Okumoto and Pilot, 2011). The amino acid polyamine transporter (APC) bidirectional amino acid transporter 1 (BAT1) may be an amino acid exporter. BAT1 exports glutamate and Lys and mediates the influx of γ-aminobutyric acid (GABA), alanine (Ala) and arginine (Arg) (Dundar and Bush, 2009; Michaeli et al., 2011). A member of the drug and metabolite transporter superfamily, siliques are red1 (SiAR1), functions as a bidirectional amino acid transporter domain (from position 22 to 426). The alignment of protein sequences revealed that Rhg1-GmAAT is predicted to encode an amino acid transporter gene at Rhg1, Pfam on UniProt (the universal protein resource) predicted that the Glyma.18G022400 gene, referred to as Rhg1-GmAAT, at Rhg1 encoded a 436-amino-acid protein that contained one amino acid transporter domain (from position 22 to 426). The alignment of protein sequences revealed that Rhg1-GmAAT shared 58.72% amino acid identity with Arabidopsis vacuolar amino acid transporter 6C (AVT6C) and 58.31% amino acid identity with AtAVT6D. AtAVT6C and AtAVT6D are homologous to Saccharomyces cerevisiae AVT6 (Fig. S1, see Supporting Information), a vacuolar exporter of Glu and aspartic acid (Asp) (Chahomchuen et al., 2009).

To determine the expression patterns of Rhg1-GmAAT, we first used real-time quantitative reverse transcription-PCR (qRT-PCR) to calculate the transcript abundance of Rhg1-GmAAT in various tissues and treatments. Expression of Rhg1-GmAAT in providing resistance to SCN and will be helpful in revealing the functional mechanism of Rhg1.
transcription-polymerase chain reaction (qRT-PCR) to measure Rhg1-GmAAT expression. Rhg1-GmAAT was ubiquitously expressed in the roots, stems, leaves, flowers, cotyledons and stem apex, with the expression of Rhg1-GmAAT being highest in the roots, stems and cotyledons (Fig. 1a). To investigate the expression pattern of Rhg1-GmAAT in more detail, we fused the Rhg1-GmAAT native promoter to a β-glucuronidase (GUS) reporter and transformed the construct into Arabidopsis. Histochemical analyses of the transgenic Arabidopsis revealed that the promoter of the Rhg1-GmAAT gene was active in the vasculature throughout the whole plant (i.e. roots, leaves, flowers, stems and siliques) (Fig. 1b–g). We also performed a histochemical analysis with the P_GmAAT-GUS construct in transgenic soybean hairy roots. GUS staining revealed a relatively strong signal in the stele of the root.

![Figure 1](image1.png)

**Fig. 1** Expression and subcellular localization of Rhg1-GmAAT. (a) The expression patterns of Rhg1-GmAAT were determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and normalized to SKIP16. The values are the means ± standard deviations (SDs) (n = 3). (b–g) Histochemical analysis of β-glucuronidase (GUS) activity from Arabidopsis plants expressing P_GmAAT-GUS. GUS staining in a 2-week-old cotyledon (b), 2-week-old stem (c), 2-week-old root (d), leaf of a 35-day-old plant (e), flower of a 35-day-old plant (scale bar, 10 mm) (f) and silique of a 35-day-old plant (g). (h, i) Histochemical analysis of GUS activity in soybean hairy roots expressing P_GmAAT-GUS. GUS staining in the stele (h) and within a root cross-section (i). (j) Subcellular localization via a P35S-GmAAT-GFP fusion protein in tobacco epidermal leaf cells. From left to right: 4,6-diamidino-2-phenylindole (DAPI)-stained nuclear DNA, green fluorescent protein (GFP) fluorescence, bright-field and overlay panels. Nucleus (N) and plasma membrane (P) are indicated by white and yellow arrows, respectively. Unless otherwise specified, scale bar = 200 μm. [Colour figure can be viewed at wileyonlinelibrary.com]

![Figure 2](image2.png)

**Fig. 2** Growth of plants subjected to toxic levels of glutamic acid (Glu) or the toxic Glu analogue N-methyl sulfoximine (MSX). (a) Col-0 and Rhg1-GmAAT-OX (Rhg1-GmAAT-overexpressing) Arabidopsis lines were grown for 3 weeks on half-strength Murashige and Skoog medium containing 75 mM Glu. (b) The wild-type (cultivar Tianlong 1) and Rhg1-GmAAT-OX soybean lines were grown for 3 days in quarter-strength Murashige and Skoog liquid medium containing 50 mM Glu. (c, d) Two soybean near-isogenic lines (NIL-S and NIL-R) were grown for 3 days in quarter-strength Murashige and Skoog liquid medium containing either 50 mM Glu (c) or 10 μM MSX (d). Fresh weights were measured. The values are the means ± standard deviations (SDs) (n = 4). *0.01 < P < 0.05, ** P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test). The values above the columns provide the percentage of inhibition compared with that of untreated plants. WT, wild-type.
transgenic hairy roots (Fig. 1h) and a highly enriched signal in the vascular tissues and pericycle (Fig. 1i). To verify this observation, more experiments are needed in the future. GUS expression supports the assumed role of Rhg1-GmAAT in the transportation of amino acids between the organs of the plant via the vascular system. Amino acids are transported not only across the plasma membrane, but also in and out of all cellular compartments; as such, by overexpression of a P35S-GmAAT-green fluorescent protein (GFP) construct in tobacco epidermal leaf cells, onion epidermal cells and soybean hairy roots, we analysed the cellular localization of the overexpressed Rhg1-GmAAT (Figs 1j and S2, see Supporting Information). For nucleus staining, the reagent 4,6-diamidino-2-phenylindole (DAPI) (Sigma, USA) was used. The green signal of P35S-GmAAT-GFP overlapped the blue signal of DAPI (Figs S1j and S2a). To verify the plasma membrane localization, we induced plasmolysis of onion epidermal cells expressing P35S-GmAAT-GFP by treatment with a high concentration of NaCl solution. P35S-GmAAT-GFP was observed in the plasma membrane after plasmolysis (Fig. S2a). Therefore, we speculated that P35S-GmAAT-GFP was located in both the nucleus and plasma membrane. As there was no clear difference between the control P35S-GFP and fusion protein P35S-GmAAT-GFP images, P35S-GmAAT-GFP might also exist in the cytoplasm.

Glutamate may be the substrate of Rhg1-GmAAT in vivo

To analyse the substrate of Rhg1-GmAAT in plants, we performed a growth inhibition assay which involved the evaluation of the tolerance of plants to excess amounts of different amino acids. Exogenous treatment with toxic level of amino acids caused a feedback inhibition of the biosynthesis of the shared precursors of other amino acids in vivo, resulting in stunted plant growth (Less and Galili, 2008). Two transgenic Arabidopsis lines, at-1 and at-3 (Fig. S3a, see Supporting Information), overexpressing Rhg1-GmAAT (Rhg1-GmAAT-OX), and a control line ecotype Columbia-0 (Col-0), were sown on medium supplemented with excess amounts of different amino acids, as described by Lee et al. (2007). The Rhg1-GmAAT-OX lines grew better than the Col-0 line only on medium containing Glu (Fig. 2a). Under other experimental conditions, all plants exhibited severely stunted growth (Fig. S4, see Supporting Information). Tolerance to excess Glu suggested that Rhg1-GmAAT might specifically impact glutamate transportation in plants.

To further investigate the function of Rhg1-GmAAT in soybean, two homozygous transgenic soybean Rhg1-GmAAT-OX lines (gm-2 and gm-3) in the cultivar Tianlong 1 background (Fig. S3b) were generated, and a growth inhibition assay was also conducted. Toxic levels of Glu, Gln, Asp and glycine (Gly) were used to treat the WT (cultivar Tianlong 1) and Rhg1-GmAAT-OX plants. At 72 h after treatment, the WT plants were wilted and their fresh weight had decreased by 47.7%. In contrast, the fresh weight of Rhg1-GmAAT-OX plants decreased by only 18.9% and 17.7% (Fig. 2b). No difference between the WT and transgenic lines was observed in response to Gln, Asp and Gly treatments (Fig. S5, see Supporting Information). The growth of all plants was severely affected. Obviously, in soybean, overexpression of Rhg1-GmAAT enhances the tolerance of seedlings specifically to excess amounts of Glu.

The more tolerant phenotype could possibly be caused by the constitutive overexpression of Rhg1-GmAAT rather than by its true function in vivo. To check this possibility, we compared the tolerance to Glu of a pair of near-isogenic lines (NILs) for Rhg1, NIL-R and NIL-S. Rhg1-GmAAT in the NIL-R lines was expressed three- to four-fold more strongly than in the NIL-S lines (Fig. S3c). NIL-S and NIL-R were susceptible and resistant to SCN, respectively (Fig. S6, see Supporting Information). After being treated with 50 mM Glu, the fresh weights of NIL-S plants were significantly decreased (P = 4.41E-04). In comparison, the fresh weights of NIL-R plants were nearly unchanged (Fig. 2c). To further confirm the natural function of Rhg1-GmAAT, NIL-S and NIL-R plants were treated with 10 μm methionine sulfoximine (MSX), which is a toxic analogue of Glu (Rawat et al., 1999); at low levels (1 μM), MSX is toxic to Arabidopsis Col-0 (Perchlik et al., 2014). The fresh weights of MSX-treated NIL-R plants decreased by 8.95%, whereas that of MSX-treated NIL-S plants decreased by 34.5% (Fig. 2d). The more tolerant phenotype could possibly be caused by the constitutive overexpression of Rhg1-GmAAT in plants and may provide tolerance to toxic levels of exogenous Glu.

The expression of glutamate receptor-like genes and Gln synthetase genes is sensitive to glutamate uptake (Masclaux-Daubresse, 2006). In Arabidopsis, expression of the glutamate receptor gene AtGLR2.7 (AT2G29120) and the Gln synthetase gene AtGSR1 (AT5G37600) was analysed in roots of 2-week-old Col-0 and Rhg1-GmAAT-OX line at-3 growing on medium with or without 75 μM Glu. In soybean, the expression levels of the glutamate receptor-like gene Glyma.06G233600 and Gln synthetase PR-2 gene Glyma.07G04500 were detected in 50 mM Glu-treated soybean roots of the WT (cultivar Tianlong 1) and Rhg1-GmAAT-OX line gm-3 at 2 days post-treatment. As expected, the treatment of Glu induced the expression of these sensor genes (Fig. 3) in both plant species. Compared with WT plants, Rhg1-GmAAT-OX plants demonstrated higher expression levels of the sensor genes under conditions with or without a toxic level of Glu (P < 0.05). These results indicate that the overexpression of Rhg1-GmAAT might enhance the accumulation of glutamate in roots.
The amino acid transporter gene at Rhg1

Overexpression of Rhg1-GmAAT increases the transportation of glutamate from shoots to roots in soybean

Because the promoter of Rhg1-GmAAT was active in the root vascular tissue, the expression of Rhg1-GmAAT was expected to affect amino acid transport. Excess amino acids synthesized in the leaves are exported via the phloem (Tegeder et al., 2012). To investigate the effects of increased expression of Rhg1-GmAAT on phloem amino acid transport, we used high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) to examine the amino acid content in the phloem exudates of Rhg1-GmAAT-OX and WT plants (cultivar Tianlong 1). Compared with WT plants, Rhg1-GmAAT-OX plants exhibited relatively higher amounts of free Glu ($P = 0.003$) and Gly ($P = 0.025$) in the leaf phloem (Fig. 4a), suggesting that the transportation of glutamate and Gly from the shoots to the roots might be enhanced by the overexpression of Rhg1-GmAAT. Amino acids in the xylem are mainly derived from the roots (Ortiz-Lopez et al., 2002). To investigate the transportation of amino acids from the roots to the shoots, we compared the free amino acid contents in the root xylem sap between Rhg1-GmAAT-OX and WT (cultivar Tianlong 1) plants, and detected similar amounts of individual amino acids between the two (Fig. 4b), suggesting that the transportation of glutamate from the roots to the shoots was not affected by the overexpression of Rhg1-GmAAT. The above results imply that Rhg1-GmAAT may positively regulate the transportation of glutamate from the shoots to the roots.

By using HPLC-MS/MS, we then compared the amounts of free amino acids between the roots and leaves. Compared with the leaves, the roots of Rhg1-GmAAT-OX plants presented a 2.88-fold increase in Glu content and a 21.4%–75.3% decrease in several amino acid contents, including those of Met, tyrosine (Tyr), leucine (Leu), Ala, isoleucine (Ile), phenylalanine (Phe), valine (Val), Trp, Gln, threonine (Thr), Gly and serine (Ser). No significant differences were observed in the total amounts of free amino acids, which were obtained by summing the individual
amino acids (Fig. 4c; Table S1, see Supporting Information). In contrast, the Glu contents of the Rhg1-GmAAT-OX leaves were reduced by 39.7% compared with those of WT. However, no significant differences were observed in the other measured individual free amino acids (Fig. 4d; Table S1). The data confirm that the overexpression of Rhg1-GmAAT enhances the transportation of glutamate from the shoots to the roots and consequently increases the glutamate content in the roots.

Overexpression of Rhg1-GmAAT activates the JA pathway in soybean

Endogenous and exogenous amino acids are key players in plant defence against pathogens (Seifi et al., 2013). Previous studies in citrus have suggested that the accumulation of Glu induces the JA pathway and subsequently activates the systemic defence response to herbivore attack (Agut et al., 2016). JA synthesis starts with linolenic acid, which is converted to OPDA via the
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...octadecanoid pathway; this conversion occurs via the sequential action of the chloroplast-localized enzymes lipoygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC), after which OPDA is then converted to JA in the peroxisome by OPDA reductase3 (OPR3) (Taki et al., 2005; Wasternack and Hause, 2013). GmLOX (type II 13-LOX) (Song et al., 2016) and GmAOS1 (Kongrit et al., 2007) serve critical functions in JA biosynthesis. To determine whether the JA pathway can be induced by enhanced applications of Glu in soybean, we treated the roots of soybean seedlings with 5 mM Glu for 24 h and used real-time qRT-PCR to measure the expression of GmLOX (Glyma.03G264300) and GmAOS1 (Glyma.14G078600). The results showed that, compared with those in control seedlings, the expression levels of GmLOX and GmAOS1 in treated seedlings increased by up to approximately 91-fold and four-fold, respectively (Fig. 5a). These observations indicate that JA biosynthesis is activated in response to exogenous Glu applications to soybean roots. Considering the increased accumulation of free Glu in the roots of Rhg1-GmAAT-OX plants, we inferred that JA biosynthesis might be activated in vivo in Rhg1-GmAAT-OX plants.

To support this inference, we compared the transcriptomes between the roots of 3-week-old Rhg1-GmAAT-OX line gm-3 and WT (cultivar Tianlong 1) plants. A total of 1607 differentially expressed genes (DEGs) with two-fold cut-off values were identified [false discovery rate (FDR) < 0.05]. Enrichment analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG)...

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**Fig. 5** Expression of a set of jasmonic acid (JA) biosynthesis and JA-responsive genes. (a) JA biosynthesis genes expressed in the roots of glutamic acid (Glu)-treated soybean. Roots were treated with quarter-strength Murashige and Skoog medium that either lacked nitrogen (control) or was supplemented with 5 mM Glu for 24 h. JA biosynthesis genes (b) and JA-responsive genes (c) expressed in the roots of the wild-type (cultivar Tianlong 1) and Rhg1-GmAAT-overexpressing (Rhg1-GmAAT-OX) line gm-3. (d) JA biosynthesis genes expressed in the roots of Williams 82 and PI88788. The expression levels of the selected genes were assayed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and were normalized to SKIP16. The values are the means ± standard deviations (SDs) (n = 3). **0.01 < P < 0.05, ** P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test). WT, wild-type; OX, Rhg1-GmAAT-OX line gm-3.
suggested that the plant–pathogen interaction pathway was the most enriched pathway for these DEGs in Rhg1-GmAAT-OX plants (Fig. S7, see Supporting Information). In addition, a set of up-regulated JA-related genes was identified in Rhg1-GmAAT-OX plants (Table S2, see Supporting Information). The expression levels of the JA biosynthesis genes (GmLOX, GmAOS1 and GmOPR3) and JA signalling pathway genes [GmPDF1.2 (Glyma.18G027700), GmJAZ1 (Glyma.11G038600) and GmbHLH35 (Glyma.17G058600)] were detected and verified by qRT-PCR (Fig. 5b,c). These results suggest that the JA pathway may be up-regulated by the overexpression of Rhg1-GmAAT in soybean.

As the JA pathway was up-regulated by the overexpression of Rhg1-GmAAT, we performed a metabolite analysis of the roots of transgenic plants to determine whether the overexpression of Rhg1-GmAAT induced the JA content. A total of 545 compounds were detected in the metabolite assay; 36 significant differences occurred, 29 of which were suppressed and seven of which were induced. Four kinds of plant hormones changed significantly; JA was the only induced hormone (Table S3, see Supporting Information). To verify these results, an absolute quantitative technique was used to analyse the JA content in the roots of both the WT (cultivar Tianlong 1) and Rhg1-GmAAT-OX soybean plants. Compared with WT roots, the JA and JA-Ile contents in the Rhg1-GmAAT-OX roots significantly increased by up to 1.717-fold (P = 1.92E-06) and 1.789-fold (P = 5.22E-07), respectively (Fig. 6). MeJA was not detected in any of the samples. These results support the hypothesis that Rhg1-GmAAT positively affects the accumulation of JA.

To study the changes in the JA biosynthesis pathway in populations that exhibit a naturally high expression of Rhg1-GmAAT, the transcripts of Rhg1-GmAAT and the JA biosynthesis gene GmLOX were measured in Williams 82 (PI518671) and PI88788, which contain one and eight copies of Rhg1, respectively (Lee et al., 2015). Consistent with the copy number results, PI88788 exhibited progressively higher transcript levels of Rhg1-GmAAT and GmLOX than did Williams 82 (Figs 5d and S3d). Furthermore, JA content was compared between a bulk (Bulk-R) of naturally resistant soybean varieties [PI437655, PI495017C, PI209332, PI438503A and PI467312J] and a bulk (Bulk-S) of susceptible soybean varieties [Hutcheson (PI518664), Magellan (PI595362) and Williams 82]. The content of JA in Bulk-R was 1.84 times (P = 1.37E-07) that of JA in Bulk-S. In detail, PI88788 contained 2.92 times (P = 2.17E-10) more JA than Hutcheson (Fig. S8, see Supporting Information). These observations accord with the hypothesis that JA is involved in SCN resistance.

**Inhibition of the JA pathway reduces the resistance to SCN in Rhg1-containing soybean germplasm**

As the JA pathway was activated in naturally resistant germplasm, we sought to confirm the involvement of the JA pathway in conferring resistance to SCN. As such, by application of the JA biosynthesis inhibitor n-propyl gallate (nPG) (Bruinsma et al., 2010; Pena-Cortés et al., 1993) to inhibit JA biosynthesis (Fig. S9, see Supporting Information), the changes in resistance to SCN in PI88788 were evaluated. As shown in Fig. 7a, the average numbers of cysts in mock-treated Hutcheson, mock-treated PI88788 and nPG-treated PI88788 were 32, 1.8 and 5.4, respectively. nPG treatment increased the cysts in PI88788 from 5.6% to 16.9%, with the number of cysts on Hutcheson being 100% (P = 0.02). As expected, inhibition of JA makes soybean more susceptible (Kammerhofer et al., 2015; Li et al., 2018; Zhang et al., 2017).

No difference in resistance to SCN was observed between Rhg1-GmAAT-OX plants and cultivar Tianlong 1 control plants (Fig. 7b), which indicated that overexpression of Rhg1-GmAAT alone in soybean could not provide resistance to SCN. These results were consistent with those in previous reports by Cook et al. (2012), in that three genes rather than one were responsible for resistance to SCN at Rhg1.

**DISCUSSION**

In this study, we explored the function of Rhg1-GmAAT, one of three genes at Rhg1, which is a major quantitative trait locus involved in resistance to SCNs (Cook et al., 2012). Based on analyses of sequence and expression patterns, Rhg1-GmAAT was predicted to encode an amino acid transporter, which was expressed mostly in the vasculature (Fig. 1b–i). Subcellular localization revealed that Rhg1-GmAAT was located in the plasma membrane and nucleus, and might also exist in the cytoplasm.
The amino acid transporter gene at Rhg1 (Figs 1j and S2). Some transporters located in the plasmalemma are thought to be involved in amino acid absorption and distribution (Ladwig et al., 2012; Lee et al., 2007; Su et al., 2004; Tegeder et al., 2012). A growth inhibition assay was performed to evaluate the tolerance of plants to toxic levels of amino acids, and found that Rhg1-GmAAT might impact the transportation of glutamate (Figs 2, 3, S4 and S5). In addition to the role of Rhg1-GmAAT as an amino acid transporter, the nuclear localization of Rhg1-GmAAT suggested that this protein might have other functions. For example, when localized in the plasma membrane, the mammalian amino acid transporter PAT1 (SLC36A1) functions in amino acid transportation, but, when localized in the nucleus, PAT1 functions as an amino acid sensor (Chen et al., 2003; Jensen et al., 2014). Whether Rhg1-GmAAT functions as an amino acid sensor is an interesting question to be answered.

The measurements of free Glu contents in the phloem exudates and xylem sap showed that overexpression of Rhg1-GmAAT could enhance the long-distance transportation of glutamate from the shoots to the roots without affecting the transportation from the roots to the shoots; this phenomenon was further demonstrated by the increased content of free Glu in the roots and the decreased content in the shoots in transgenic Rhg1-GmAAT-OX plants (Fig. 5; Table S1). These results also support the hypothesis that Rhg1-GmAAT functions as an amino acid transporter in vivo. As a signalling molecule, glutamate metabolism can regulate plant hormone pathways (Kadotani et al., 2016; Liu et al., 2010; Seifi et al., 2013). Kadotani et al. (2016) reported that exogenous Glu induced blast resistance partially dependent on salicylic acid (SA) signalling. Agut et al. (2016) reported that, in Citrus aurantium (sour orange), the roots exported Glu to the shoots to trigger the transcription of glutamate receptors in order to induce JA biosynthesis to increase resistance against spider mites. In this study, the treatment of soybean roots with Glu significantly activated the transcription of JA biosynthesis genes (Fig. 5a), which suggested that the increased Glu content might stimulate JA biosynthesis in vivo in soybean. We then performed RNA-sequencing (RNA-Seq) and metabolite analyses of the roots of transgenic Rhg1-GmAAT-OX soybean plants; the results confirmed our speculation (Figs 5, 6, S7; Tables S2 and S3).

JA has been implicated in resistance to nematodes (Heil and Ton, 2008; Schilmiller and Howe, 2005). In soybean, the majority of JA pathway components are suppressed during compatible soybean SCN infection (Ithal et al., 2007a). The up-regulation of soybean LOX genes, which are key for JA biosynthesis, has been reported in syncytia induced by SCN on Peking and PI88788 plants (Klink et al., 2009, 2010). Matthews et al. (2014) overexpressed three Arabidopsis genes (AtAOS, AtAOC, AtJAR) involved in JA/JA-Ile production in soybean, which improved resistance to SCN (Lin et al., 2013). To confirm whether the JA pathway is involved in Rhg1-mediated resistance to SCN, we used a JA biosynthesis inhibitor, nPG, to inhibit the JA pathway in the naturally SCN-resistant soybean accession PI88788. Compared with the mock-treated control roots, the nPG-treated roots presented significantly more female cysts (Fig. 7a), indicative of a role for the JA pathway in Rhg1-mediated resistance to SCN.

Recently, the JA pathway has been discovered to consist of two antagonistic branches: one branch is characterized by the basic helix-loop-helix leucine zipper transcription factor (TF) MYC2, whereas the other is characterized by the apetala2/ethylene response factor (AP2/ERF) domain TF ORA59 (Verhage et al., 2013).
The MYC2 branch can be induced by damage-triggered immunity responses and is marked by the strong induction of the marker gene vegetative storage protein 2 (VSP2) in Arabidopsis. In Arabidopsis, the ERF branch is induced by oral secretions from Pieris rapae larvae; PDF1.2 is the marker gene (Verhage et al., 2011). During the early stages of nematode parasitism in Arabidopsis, phosphatase AP2C1 negatively regulates the mitogen-activated protein kinases (MAPKs) MPK3 and MPK6; these kinases are two positive regulators of plant defence responses and control ET and JA biosynthesis (Sidonskaya et al., 2016). The enhanced susceptibility of mpk3 and mpk6 plants to sugar beet cyst nematode (Heterodera schachtii) indicates a positive role of stress-activated MAPKs in plant immunity against nematodes (Sidonskaya et al., 2016). In addition, the up-regulation of VSP2, a marker for the MYC2 branch of the JA pathway, occurs on infection by the SCN Heterodera glycines in both Arabidopsis and soybean roots (Afzal et al., 2009; Puthoff et al., 2003); this up-regulation is due to damage-triggered immunity responses induced by the intracellular migration of cyst nematodes before the establishment of a permanent feeding site. In our study, the expression of Rhg1-GmAAT induced the transcription of GmMPK3 (Glyma.12G073000 and Glyma.U021800) and GmPDF1.2, a marker of the ERF branch of the JA pathway, but suppressed the expression of GmVSP2 (Table S2). These results imply that Rhg1-GmAAT might regulate the biosynthesis of JA via the MPK3 and MPK6 pathways, and that the ERF branch of the JA pathway might play a role in the resistance to SCN.

However, our metabolite profiling results were opposite to the established notion that JA induces other specialized metabolites. Most components were suppressed, not induced. This might be related to the up-regulation of the JAZ genes, repressors of JA signalling, in Rhg1-GmAAT-OX plants (Table S2). These results suggest that Rhg1-GmAAT could mediate JA signalling. Moreover, the fine regulation of the Rhg1-GmAAT gene on the JA biosynthesis and signalling pathway may contribute to the small number of induced compounds. Another reason might be that the method we used to conduct the metabolite profiling was not capable of detecting all kinds of metabolites, especially some specialized secondary metabolites in plants. Therefore, some JA-induced metabolites were unable to be detected, which caused the small number of JA-induced metabolites in this study. More specific mechanisms need to be further studied in the future.

At Rhg1, three tightly linked genes have been shown to contribute to resistance to SCN (Cook et al., 2012). These include an amino acid transporter gene (Glyma.18G022400), an α-SNAP gene (Glyma.18G022500) and a WI12 gene (Glyma.18G022700) (Cook et al., 2012). These three genes must be simultaneously overexpressed to provide resistance to SCN (Cook et al., 2012). Our results also showed that overexpression of Rhg1-GmAAT alone does not influence resistance to SCN (Fig. 6b). Until now, these three genes coordinate function to provide resistance to SCN has remained unknown. By analysis of the function of each gene individually, scientists have attempted to answer this question. The function of the α-SNAPs in Rhg1-mediated resistance has been elucidated (Bayless et al., 2016; Liu et al., 2017). The Rhg1 resistance-type α-SNAPs interrupt NSF function and vesicle trafficking, which results in cytotoxicity at sedentary feeding sites (Bayless et al., 2016). Our results showed that, by activating the JA pathway, Rhg1-GmAAT might be involved in the transport of glutamate in vivo and play a role in the resistance to SCN. No studies on the function of Rhg1-GmWI12 in the resistance to SCN have been conducted. The WI12 gene in ice plants (Mesembryanthemum crystallinum) is involved in cell wall modification at wound sites and is induced by JA (Yen et al., 2001). Our RNA-Seq results showed that overexpression of Rhg1-GmAAT increased the transcription of Rhg1-GmWI12 by 1.99-fold (Table S2). In addition, Rhg1-GmWI12 could also be induced by JA in soybean (Fig. S10, see Supporting Information). We speculated that Rhg1-GmWI12 might play a role in the intracellular migration of cyst nematodes, where the responses of plants to wounding mainly occurred. The three responsible genes at Rhg1 might have different or interrelated functions during the processes of nematode invasion and colonization. Once WI12 is elucidated, the coordination mechanism of these three genes with respect to resistance to SCN needs to be investigated to clarify the whole process concerning Rhg1-mediated resistance to SCN in soybean.

In summary, our results showed that Rhg1-GmAAT might be involved in the transport of glutamate in vivo. Overexpression of Rhg1-GmAAT increased the transportation of glutamate from the shoots to the roots, resulting in both the accumulation of free Glu in the roots and the up-regulation of the JA pathway, which contributed to resistance to SCN in an Rhg1-containing resistant germplasm. Our results could be helpful for an understanding of the whole Rhg1-mediated mechanism of resistance to SCN in soybean.

EXPERIMENTAL DETAILS

Plant material and growth conditions

Plants of the soybean cultivar Tianlong 1, PI88788, transgenic lines and NILs were obtained from the Oil Crops Research Institute, Chinese Academy of Agricultural Sciences, Wuhan, China. The NILs (NIL-R and NIL-S) were derived from a cross of PI437654 and Williams 82 through marker-assisted selection. The resistant soybean accession PI437654, which contains low copies of Rhg1-a and Rhg4, was backcrossed four times to the susceptible accession Williams 82 to produce the BC4F2 progenies. The BC4F2 plants, which were homozygous at Rhg4 but heterozygous at Rhg1, were self-pollinated to produce the BC4F2:3 individuals. From these individuals, the NILs of Rhg1 were developed, whose phenotypes were also confirmed in the glasshouse by evaluation.
and cloned into a pGWC entry vector (Chen et al., 2006). The Glu analogue), 50 mM was cloned into a pEGAD-GFP vector using the Age I restriction ligation-free method using a ClonExpress II One Step Cloning Kit (Vazyme, China).

To overexpress Rhg1-GmAAT-OX and NIL (NIL-R and NIL-S) plants were cultured in quarter-strength Murashige and Skoog medium supplemented with excess amounts of various amino acids [2 mM Tyr or Lys; 4 mM Leu or Met; 6 mM Val; 10 mM Phe, Ile, Ser, Thr or Trp; 20 mM His; 25 mM cysteine (Cys) or Gly; 50 mM asparagine (Asn) or Asp; and 100 mM Gln, Ala, proline (Pro), Arg or Glu] and grown for 21 days.

Dna constructs

To overexpress Rhg1-GmAAT (Glyma.18g022400), the full-length coding sequence of soybean Williams 82 cDNA was amplified and cloned into a pGWC entry vector (Chen et al., 2006). The constructed entry vector was recombined into a pB2GW7 plant transformation vector (Karimi et al., 2002) using the LR recombination reaction (Invitrogen, USA). To visualize GFP in tobacco epidermal cells and soybean hairy roots, Rhg1-GmAAT cDNA was cloned into a pEGAD-GFP vector using the AgeI restriction site, generating P35S-GmAAT-GFP. To generate Pgmaaat-GUS chimeric genes, the 1.6-kb promoter region of Rhg1-GmAAT from soybean Williams 82 genomic DNA was amplified and cloned upstream of the GUS reporter gene within the plant binary vector pCX-GUS-P (Chen et al., 2009). Unless otherwise specified, all constructs were generated by a sequence-independent, ligation-free method using a ClonExpress II One Step Cloning Kit (Vazyme, China).

The plant transformation constructs were introduced into Agrobacterium tumefaciens GV3101 and EHA105 strains by the freeze–thaw method. The GV3101 strains were used to transform Arabidopsis Col-0 plants by the floral dip method (Clough and Bent, 1998). The EHA105 strains were used to transform soybean cultivar Tianlong 1 plants via the Agrobacterium-mediated transformation method (Paz et al., 2006). Transformed lines were selected on soil by spraying with a 1:1000 dilution of Basta (Bayer CropScience, Germany).

Amino acid quantification

Samples were ground to a powder in liquid N2 and subsequently dissolved in ultrapure water. Derivatization was performed with an MSLAB-45+AA kit (MSLAB, China). HPLC-MS/MS analyses were performed using an Ultimate 3000 HPLC system (Dian, USA) connected to an API 3200Q TRAP liquid chromatograph coupled to a tandem mass spectrometry (LC/MS/MS) system (Applied Biosystems, USA), in accordance with the procedures described by Spitzner et al. (2008), by MSLAB. Estimation was performed in positive mode by multiple reaction monitoring (MRM) and a scan time of 20 min. The first transition was used for quantification; the other was used for confirmation. An equation developed from a five-point calibration was used as a standard.

Leaf phloem exudate and root xylem exudate collection

Plants were cultured in quarter-strength Murashige and Skoog medium for 3 weeks. Leaf phloem exudates were collected from the second and third leaflets from the top of nine 5-week-old plants, and placed into a tube containing 0.5 mL of 10 mM ethylenediaminetetraacetic acid (EDTA) for 24 h (Urquhart and Joy, 1981). The exudates from three plants were pooled as one sample. Phloem exudation rates were determined as nanomoles of amino acid exudate per milligram of leaf tissue [dry weight
The soybean seedlings were cultured in quarter-strength Murashige and Skoog medium for 4 weeks. The roots were harvested, rinsed briefly in phosphate-buffered solution, immediately frozen in liquid nitrogen and ground into a powder, after which they were extracted with methanol–water (8 : 2) at 4 °C. Endogenous jasmonates (JA, MeJA and JA-Ile) were quantified using HPLC-MS/MS as described previously (Fang et al., 2016). The nematode resistance allele at the rhg1 locus alters the proteome and primary metabolism of soybean roots. Plant Physiol. 151, 1264–1280.

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**Chemical treatments**

The treatment with nPG was designed on the basis of a previous report (Kinkema and Gresshoff, 2008). nPG, an inhibitor of JA biosynthesis, was dissolved in ethanol to make a 0.5 μm stock solution. Prior to infection, 12-day-old soybean seedlings were immersed in quarter-strength Murashige and Skoog medium and treated with either water containing 0.02% ethanol (control) or a 100 μm nPG solution daily for 3 days. These seedlings were then used to perform SCN assays.

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**AUTHOR CONTRIBUTIONS**

W.G. and Y.J. designed the research. W.G., F.Z., Q.Y., J.C., X.Z., X.S. and Y.J. performed the nematode assays. Z.L. and J.C. performed the chemical treatments. A.B. transformed the soybean plants. Z.L. and J.C. performed the nematode assays. W.Z. and W.G. analysed the data. W.G. and Y.J. wrote the paper. Y.J. coordinated the study.

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epidermal cells (a) and soybean hairy roots (b). In (a), from left to right: 4,6-diamidino-2-phenylindole (DAPI)-stained nuclear DNA, green fluorescent protein (GFP) fluorescence, bright-field and overlay panels. In (b), the fluorescence (left) and bright-field (middle) images are overlaid on the right side (scale bar, 20 µm). Nucleus (N) and plasma membrane (P) are indicated by white and yellow arrows, respectively.

Fig. S3 Expression of Rhg1-GmAAT. (a) The expression of Rhg1-GmAAT was determined by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in ecotype Colombia-0 (Col-0) and the transgenic Rhg1-GmAAT-OX (Rhg1-GmAAT-overexpressing) lines (at-1 and at-3). The level of ATACT7 transcript served as a loading control. (b–d) Expression of Rhg1-GmAAT determined by qRT-PCR in the wild-type (cultivar Tianlong 1) and transgenic Rhg1-GmAAT-OX lines (gm-2 and gm-3) (b), two soybean near-isogenic lines (NILs; NIL-S and NIL-R) (c), and Williams 82 and PI87988 (d). The expression levels of all samples were normalized to those of SKIP16. The values are the means ± standard deviations (SDs) (n = 3). WT, wild-type (cultivar Tianlong 1).

Fig. S4 Growth of Arabidopsis seedlings subjected to excess amounts of amino acids. Seedlings were grown on half-strength Murashige and Skoog medium supplemented with 2 mM tyrosine (Tyr) or lysine (Lys), 4 mM leucine (Leu) or methionine (Met), 6 mM valine (Val), 10 mM phenylalanine (Phe), isoleucine (Ile), serine (Ser), threonine (Thr) or tryptophan (Trp), 20 µM histidine (His), 25 µM cysteine (Cys) or glycine (Gly), 50 µM asparagine (Asp) or aspartic acid (Asp), and 100 mM glutamine (Gln), alanine (Ala), proline (Pro), arginine (Arg) or glutamic acid (Glu). As controls, Col-0 plants were grown on half-strength Murashige and Skoog medium only. Images were taken at 21 days after treatment. Scale bar, 1 cm.

Fig. S5 Growth of soybean seedlings subjected to excess amounts of amino acids. Twenty-one-day-old wild-type (cultivar Tianlong 1) and transgenic Rhg1-GmAAT-OX (Rhg1-GmAAT-overexpressing) line gm-3 were inoculated in quarter-strength Murashige and Skoog medium containing 50 mM aspartic acid (Asp) and glutamic acid (Glu), 75 mM glutamine (Gln), and 25 mM glycine (Gly). As controls, the wild-type (cultivar Tianlong 1) plants were grown on quarter-strength Murashige and Skoog medium only. Images were taken at 3 days after treatment. Scale bar, 5 cm. WT, wild-type; Ox, Rhg1-GmAAT-OX line gm-3.

Fig. S6 Responses of a pair of near-isogenic lines (NILs) to soybean cyst nematodes (SCNs). NIL-S, NIL-R and Hutcheson soybean seedlings were transplanted into sterilized sand, after which each plant was inoculated with 2000 I2 nematodes. Female cysts were quantified after 30 days. The experiments were repeated at least three times, each producing similar results. The values are the means ± standard deviations (SDs)
(n = 6). Asterisks indicate a statistically significant difference of NIL-R compared with Hutcheson. **P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test).

Fig. S7 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment scatter diagram of up-regulated differentially expressed genes (DEGs). Only the 20 most enriched pathways are displayed in the diagram. The degree of KEGG pathway enrichment is represented by the rich factor, Q-value and the number of unigenes enriched in a particular KEGG pathway. The rich factor is the ratio of differentially expressed unigenes enriched in a pathway to the total number of annotated unigenes in that pathway. The greater the rich factor, the greater the degree of enrichment. The Q-value indicates the corrected P value and ranges from zero to unity; a Q-value closer to zero indicates more enrichment.

Fig. S8 Jasmonic acid (JA) content in soybean roots. (a) JA content in a bulk (Bulk-S) of susceptible soybean varieties (Hutcheson, Magellan and Williams 82) and a bulk (Bulk-R) of naturally resistant soybean varieties (PI437655, PI495017C, PI209332, PI438503A and PI467312). (b) JA content in the roots of susceptible soybean variety Hutcheson and resistant soybean variety PI88788. Seedlings were cultured in quarter-strength Murashige and Skoog medium for 4 weeks, after which roots were harvested. Endogenous JA was quantified by following metabolic profiling procedures. The values are the means ± standard deviations (SDs) (n = 6). Asterisks indicate a statistically significant difference of resistant soybean varieties compared with susceptible soybean varieties. **P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test). CPS, counts per second.

Fig. S9 Expression of jasmonic acid (JA) biosynthesis genes expressed in n-propyl galate (nPG)-treated PI88788 roots. Twelve-day-old PI88788 seedlings were inoculated with either water containing 0.02% ethanol (mock) or a 100 μm nPG solution for 3 days. The total RNA was extracted from the roots. The expression levels of the genes of interest were assayed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The expression levels of all samples were normalized to SKIP16. The values are the means ± standard deviations (SDs) (n = 3). Asterisks indicate a statistically significant difference of nPG-treated roots compared with mock-treated roots. *0.01 < P < 0.05, **P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test).

Fig. S10 Expression of Rhg1-GmWI12 (Glyma.18G022700) induced by jasmonic acid (JA). Twelve-day-old Williams 82 soybean seedlings were cultured in quarter-strength Murashige and Skoog medium that contained either 50 μM JA or water containing 0.02% ethanol (as a control). The roots were sampled after 8 h, after which their total RNA was extracted. The expression of the genes of interest was assayed by real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The expression levels of all samples were normalized to SK1P16. The values are the means ± standard deviations (SDs) (n = 3). Asterisks indicate a statistically significant difference of JA-treated roots compared with control roots. *0.01 < P < 0.05, **P < 0.01 (multiple t-test followed by the Holm–Sidak post hoc test).

Table S1 Free amino acid contents in the roots and leaves of 21-day-old wild-type (cultivar Tianlong 1) and Rhg1-GmAAT-overexpressing (Rh1-GmAAT-OX) line gm-3. Amino acid contents were expressed as nanomoles per milligram. Root and leaf measurements were calculated using the dry weight (DW). Four plants constituted a sample, and three samples per line were used for the experiments. The values are the means ± standard deviations (SDs) (n = 3). The values shown in bold are significantly (P < 0.05) higher than those of the wild-type (multiple t-test followed by the Holm–Sidak post hoc test). This table contains the data presented in Fig. 4c,d, as well as additional information about the amino acids not presented in Fig. 4c,d. WT, wild-type (cultivar Tianlong 1); OX, Rhg1-GmAAT-OX line gm-3.

Table S2 RNA-sequencing (RNA-Seq) results regarding changes in the expression of jasmonic acid (JA)-related genes and Rhg1 genes on Rhg1-GmAAT overexpression. The values are the means ± standard deviations (SDs) (n = 3). FDR, false discovery rate.

Table S3 Metabolite analysis results regarding changes in hormone contents on Rhg1-GmAAT overexpression. The values are the means ± standard deviations (SDs) (n = 3). CPS, counts per second; VIP, variable importance in projection.

Table S4 Oligonucleotide primers used in this study.

Methods S1 Multiple alignments.

Methods S2 DNA construct.

Methods S3 RNA-sequencing (RNA-Seq): sample preparation and data analysis.

Methods S4 Metabolic profiling.