Constitutive overexpression of GsIMaT2 gene from wild soybean enhances rhizobia interaction and increase nodulation in soybean (Glycine max)

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Abstract

Background: Since the root nodules formation is regulated by specific and complex interactions of legume and rhizobial genes, there are still too many questions to be answered about the role of the genes involved in the regulation of the nodulation signaling pathway.

Results: The genetic and biological roles of the isoflavone-7-O-beta-glucoside 6″-O-malonyltransferase gene GsIMaT2 from wild soybean (Glycine soja) in the regulation of nodule and root growth in soybean (Glycine max) were examined in this work. The effect of overexpressing GsIMaT2 from G. soja on the soybean nodulation signaling system and strigolactone production was investigated. We discovered that the GsIMaT2 increased nodule numbers, fresh nodule weight, root weight, and root length by boosting strigolactone formation. Furthermore, we examined the isoflavone concentration of transgenic G. max hairy roots 10 and 20 days after rhizobial inoculation. Malonyldaidzin, malonylgenistin, daidzein, and glycitein levels were considerably higher in GsMaT2-OE hairy roots after 10- and 20-days of Bradyrhizobium japonicum infection compared to the control. These findings suggest that isoflavones and their biosynthetic genes play unique functions in the nodulation signaling system in G. max.

Conclusions: Finally, our results indicate the potential effects of the GsIMaT2 gene on soybean root growth and nodulation. This study provides novel insights for understanding the epistatic relationship between isoflavones, root development, and nodulation in soybean.

Highlights: * Cloning and Characterization of 7-O-beta-glucoside 6″-O-malonyltransferase (GsIMaT2) gene from wild soybean (G. soja).
* The role of GsIMaT2 gene in the regulation of root nodule development.
* Overexpression of GsMaT2 gene increases the accumulation of isoflavonoid in transgenic soybean hairy roots.

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Background
The symbiotic interaction between soybean roots and B. japonicum bacteria leads to the formation of unique structures known as root nodules. Hosted inside the root nodule, rhizobia can transform the molecular nitrogen gas (N₂) from the atmosphere into ammonia (NH₃), which will be readily available to the plant, and for this exchange of benefits deal, rhizobia is amended with plant carbohydrates [1, 2]. Various factors regulate root nodule formation, such as certain plant hormones, some metabolic enzymes, and definite transcription factors from the approach of the nodulation signal to nodule initiation, development, and maturation [3, 4]. Furthermore, several genes related to secondary metabolism (e.g., Phenylpropanoids and isoflavonoids biosyntheses) were identified by microarray analysis from Lotus japonicus nodule with higher frequency in nodule parenchyma (NP) and nodule vascular bundle (NC) compared with un-nodulated root [5]. Previously, we found that the overexpression of GmIMaT2 from soybean and MtMaT3 genes from Medicago sativa led to a dramatic increase in isoflavonoid malonates, which consider signals to straightforward the symbiotic interaction of legume plants and rhizobia [6, 7].

Isoflavone components are considered the largest ecophysiological active secondary metabolites, with various structures almost exclusively represented in legumes [8]. Legumes are one of the most vital food staff worldwide; leguminous species (e.g., soybean, snow pea, lentil, lupine mung bean, hairy vetch, alfalfa, medicago, white clover, and red clover) produce various isoflavones compounds, which play role(s) as auxin transport regulators, plant defense, plant growth, acting as signals to regulate the symbiotic interaction of legume plants and rhizobia [6, 9–15]. The genome of some Legumes plants, such as soybean and Medicago has several MaT homologues; some genes (e.g., MtMaT1, MtMaT4, MtMaT5, and MtMaT6) were characterized from M. truncatula and other genes (e.g., GmlMaT1, GmlMaT3, GmMT7, and GmlF7MaT) were characterized from G. max [6, 16–19]. These previous genes use malonyl-CoA as the only acyl donor to convert glycitin, genistin, and daidzin compounds to glycitin 6-o-malonates, genistin, and daidzin [6, 16–19]. In an endeavor to understand the ability of soybean plants to generate various types and amounts of isoflavone, we found many characterized flavonoid malonyltransferase genes in the soybean genome that different studies tried to characterize some of them [6, 16–19].

We here report a new malonyltransferase gene GsIMaT2 from wild soybean (G. soja). Various studies have shown that the isoflavonoid malonates stimulate the expression of Nod factor-encoding genes that are involved in nodulation signaling, such as GmNRF1α, GmNRF5α, GmNSP1α, GmNSP2α, GmDMI2α and GmDMI3β in domesticated soybean [16–19]. Moreover, strigolactones (SLs) are a class of hormones widely present in most plant species such as Arabidopsis, Pea, Rice, Petunia, and soybean [20–24]. Strigolactones (SLs) have different physiological roles correlated to root growth and development, branching of the shoot, and mycorrhiza and root nodules in legumes [24–26]. Earlier studies have illustrated that strigolactone genes detected in the root of soybean and alfalfa seedlings enhance nodulation by inducing the expression of Nod genes in rhizobial bacteria [4, 24, 27, 28].

Over the years, the transformation of cultivated soybean (G. max) hairy roots using Agrobacterium rhizogenes has become a powerful way to characterize proteins-encoding genes involved in root biological roles such as plant-microbe communication, nutrient uptake, and hormone transport [29]. We have successfully used this system to clarify the role of two More Axillary Growth genes (GmMAX1α and GmMAX4α) in soybean nodulation [28]. This work offers a functional characterization of the wild-type G. soja isoflavone malonyltransferase 2-encoding gene GsIMaT2. The results disclosed its association with flavonoid and isoflavonoid biosynthesis, and rhizobial nodulation in domesticated G. max. The inclusion methodologies that were employed to reach this goal are the following: (i) overexpression of (GsIMaT2) gene in the domesticated soybean hairy roots; (ii) inspecting nodulation and root growth characters after inoculation with B. japonicum. (iii) Profiling isoflavonoids in transgenic G. max hairy roots by HPLC (iv) Monitoring the transcription of genes implicated in nodulation signaling and strigolactones biosynthesis by qRT-PCR. Interestingly, our findings support the significance of the GsIMaT2 in rhizobial infection by elucidating the links between nodulation-signaling genes, strigolactone-biosynthesizing genes, and GsIMaT2.

Results
Identification of GsIMaT2 gene from G. soja plant genomics
The GsIMaT2 was retrieved from the wild soybean genome by managing a BLASTP search against the G. soja genome using other isoflavone-7-O-beta-glucoside
6″-O-malonyltransferase proteins from *G. max*, *L. albus*, and *M. truncatula* as queries to authenticate authen-
tic homology. This attitude recognized various proteins
firmly correlated to GsIMaT2. The obtained sequences
were put forward for phylogenetic analysis (Additional
file 1: Fig. S1). Our retrieved GsIMaT2 product is
clustered within a monophyletic group with only other
isoflavone malonyltransferases from either *G. max* or *G.
soja*. Besides, the phylogenetic analysis ratifies the close
evolutionary relationship between GsIMaT2 and GmIM-
MaT2 (Additional file 1: Fig. S1). From multiple sequence
alignment analyses by CLUSTALW (https://www.
genome.jp/tools-bin/clustalw), and from the prediction of
protein secondary structure, we found that the GsIMaT2
has two amino acid differences from GmIMaT2 at Q75L
and D192Y (Additional files 2 and 3: Fig. S2 and S3). The
putative expression analyses of the GsIMaT2 were done
as of its orthologous Glyma.18G258000 from *G. max*
across twenty-eight soybean tissues after inoculation and
fertilization (Root Hair 12HAI, Roothair_12HAlmock,
Root Hair 24 HAI, Roothair_24HAlmock, Root Hair 48
HAI, Roothair_48HAlmock, Root Hair 48 HAI Stripped,
SAM, Flower, Green_Pods, Leaves, Nodule, Root, Root_
tip, Young Leaf, Flower, One CM Pod, Pod Shell (10-13
DAF), Pod Shell (14 - 17 DAF), Nodule, Root, Seed 10 -
13 DAF, Seed 14 - 17 DAF, Seed 21 DAF, Seed 25 DAF,
Seed 28 DAF, Seed 35 DAF, and Seed 42 DAF) using the
e-Plant Soybean database (http://bar.utoronto.ca/eplant_ soybean/). Remarkably, the highest expression levels
of Glyma.18G258000 were found in the root tip. Root
Hair 48 HAI, Root hair_48HAlmock, and Root Hair 24
HAI. Also, the highest expression level of our target gene
was observed at Root, Seed 35 DAF, Seed 42 DAF, and
Seed 28 DAF (Additional file 4: Fig. S4A and B). These
results agree with [6, 16, 24, 30–34], which indicated
fections that higher expression levels of isoflavonoid genes such as, GmMT7, GmIMaT1, and GmIMaT3 were
detected in roots and seeds. In plants, the isoflavone-7-O-beta-
glucoside 6″-O-malonyltransferase (GsIMaT2: EC:
2.3.1.115) gene plays essential roles in two important
pathways isoflavonoid biosynthesis (KEGG:map 00943;
https://www.kegg.jp/pathway/map=map00943&keywo-
dr=2.3.1.115) and Flavone and flavonol biosynthesis
(KEGG:map 00944; https://www.kegg.jp/pathway/map=
map00944&keyword=2.3.1.115) which are responsible for generating several structures from iso-
flavonoids, Flavone and flavonol [31, 35]. In these two
pathways, the isoflavone malonyl transferase enzyme
can use both malonyl-CoA (CPD:C00083) and biocha-
nin A 7-O-beta-D-glucoside (CPD:C05376) as substrates
for generating various isoflavonoid components such as
Medicarpin 3-O-glucoside-6′-malonate (CPD:C16231);
Formononetin 7-O-glucoside-6″-O-
malonate (CPD:C16222); Malonyldaidzin (CPD:C16191);
Malonylglycitin (CPD:C16197); Biochanin A 7-O-beta-
D-glucoside 6″-O-malonate (CPD:C12625); Malonyl-
geninin (CPD:C16192) and Malonylapiin (CPD:C05622)
[31, 35]. These previous isoflavones compounds also play a
key role in plant–bacteria interactions by intermediat-
ing the symbiosis between legumes plants and *N2*-fixing
bacteria [6, 8]. In relevance to plant-microbe interaction,
we can classify the Rhizobium genes into two groups,
the first group which related to the synthesis of bacterial cell
surfaces such as β-1,2-glucans (*nvd* genes), lipopolysac-
charides (*ips* genes), capsular polysaccharides of *K antii-
gens, and exopolysaccharides (*exo* genes) [6, 8, 9]. While
the second group comprises nodulation (nod) genes. Iso-
flavonoids from thelegumes act as a key factor in induc-
ing the activation of rhizobial nodulation genes through
two steps; in the first step the flavonoids released from
plant roots form a complex with the NodD protein to
induce the transcription of bacterial nod genes.

On the other hand, in the second step, a Rhizobium
soil bacterium produces Nod factors (lipooligosaccharide
signals) that promote the root responses through various
structural nod genes [6, 8, 9, 13, 16, 24]. Consequently,
the subcellular localization of the GsIMaT2 products
was predicted using the Cell eFP browsers (http://bar.
utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) from its closest
orthologous protein in Arabidopsis. From this analysis,
the GsIMaT2 localizes chiefly in the cytosol, followed by
the endoplasmic reticulum, mitochondria, nucleus, plas-
tids, and Golgi (Additional file 4: Fig. S4C).

**Effect of GsIMaT2 gene over-expression on soybean
nodulation after *B. japonicum* (USDA110) infection**

To evaluate the effect of GsIMaT2 gene in transgenic
*G. max* hairy roots, the GsMaT2 gene from *G. soja*
was cloned and over-expressed on soybean nodulation upon
10- and 20-days after *B. japonicum* (USDA110) infection.
Chimeric *G. max* plants were grown in vermiculite soil
then the hairy roots were inoculated with *B. japonicum*
to examine the impacts of GsIMaT2 on soybean
odulation and root phenotypes after 10- and 20-days after
rhizobial infection (DAI) as shown in Fig. 1A-D. The
following root and nodule characteristics were investi-
gated: root length (cm), fresh root weight (gram), nodule
number, and fresh nodule weight (gram), as indicated in
Fig. 2A. 10- and 20-days after inoculation, qRT-PCR was
used to confirm the expression level of GsIMaT2, which
showed considerable overexpression as compared to the
GUS control. (Fig. 2B). Our results reveal that GsIMaT2
overexpression improved root length and fresh
root weight (Fig. 2A). Furthermore, as compared to GUS
lines, overexpression of the GsMaT2 gene also resulted
in higher nodule numbers and significantly raised nodule fresh weight for a given amount of root (Fig. 2A). As a brief outcome, overexpression of the \( \text{GsIMaT2} \) gene may alter soybean nodulation 10- and 20-days after rhizobia infection. (Fig. 1A-D and Fig. 2A). Our findings suggest that the \( \text{GsmIMaT2} \) gene may play a sustained role in the \( \text{B. japonicum} \)-induced soybean nodulation signaling pathway.

\textbf{GsMaT2 overexpression changed isoflavone profiles in transgenic soybean hairy roots}

To explore the consequence of the wild-type isoflavone malonyltransferase \( \text{GsmMaT2} \) on isoflavone malonylation, quantitative HPLC was performed to analyze various isoflavones in transgenic \( \text{G. max} \) hairy roots (Fig. 2C). The analysis revealed that malonyldaizdizin, malonylgenistin, daidzein and glycinein levels were significantly increased in \( \text{GsMaT2-OE} \) hairy roots after 10- and 20-days of \( \text{B. japonicum} \) infection compared with the control (Fig. 2C). While, glycitin, fenistin and genistein were significantly decreased in \( \text{GsMaT2-OE} \) hairy roots after 10- and 20-days after \( \text{B. japonicum} \) infection as compared with the control (Fig. 2C). On the other hand, daidzin was increased with a few levels after 10-days from infection, and exhibited an \( \sim 1.5 \)-fold increase after 20-days of infection in comparison with the control (Fig. 2C). These findings results are in accordance with [6, 16] they revealing that the overexpression of \( \text{GmlMaT1}, \text{GmlMaT2}, \) and \( \text{GmlMaT3} \) increased the concentrations of malonyldaizdizin, malonylgenistin, daidzein and glycinein in transgenic \( \text{G. max} \) hairy roots.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Effect of \( \text{GsIMaT2} \) gene overexpression on soybean root nodulation. Roots and nodules were examined on the 10th and 20th days after rhizobia were inoculated with \( \text{B. japonicum} \) strain USDA110. Composite plants were generated by transformation with the KS99 vector harboring overexpression cassettes for GUS (control) and \( \text{GsIMaT2} \). Roots were inoculated with rhizobia. \textbf{A} Root and shoot phenotypes of 10 and 20-d-old \( \text{G. max} \) plants. \textbf{B} Locations where nodules formed on hairy roots overexpressing 10 and 20-days after rhizobial inoculation. \textbf{C} Nodules developed on secondary roots. \textbf{D} Cross-sections of \( \text{G. max} \) nodules. Photographs in \textbf{C} and \textbf{D} were taken with a DP-73 microscope camera set (Olympus, Tokyo, Japan). Scale bars in \textbf{C} and \textbf{D} = 500 \text{μm}.}
\end{figure}
Fig. 2  Effects of GsIMaT2 gene overexpression on root growth and nodule development at 10 and 20 days of rhizobia inoculation. A In vivo root length (cm), fresh root weight (gram), nodule numbers, and fresh nodule weight (gram) were examined (n = 10-12). The blue and red columns represent the effect of gene overexpression 10- and 20-days after rhizobial inoculation. Data are presented as means ± SD, and statistical significance is based on Student’s t-test (*P < 0.05; **P < 0.01) with GUS-overexpressing hairy roots as the control. B Quantitative RT-PCR for in vivo hairy roots after 10- and 20-days from B. japonicum (USDA110) infection. The error bars indicate the SD of three qRT-PCR biological replicates. C HPLC analysis for profiling the isoflavonoids in the transgenic G. max hairy roots after 10 and 20 days post-inoculation.
Overexpression of GsIMA2 gene in transgenic soybean hairy roots altered the expression of nodulation and SL biosynthesis genes

The creation of root nodules takes place when legume roots and rhizobia recognize one another, resulting in the formation of symbiotic interactions known as infection foci [36].

In order to decide whether the GsIMA2 gene influences rhizobial infection and nodule formation, we analyzed the transcript levels of the induction signaling genes and SLs biosynthesis in soybean hairy roots after 10-day B. japonicum infection to have a better understanding of the role of GsIMA2 during symbiotic nitrogen fixation in legumes. For that purpose, we analyzed the expression levels of 19 selected genes, including early nodulation signaling genes such as GmDMI2a, GmDMI2B, GmDMI3a, GmDMI3b, GmNSP1a, GmNSP1b, GmNSP2a, GmNSP2b, GmNINA, GmNINB, GmNRF1, GmNRF5, and GmEndo40, and SL synthetic genes such as GmMAX1a, GmMAX1B, GmMAX2, GmMAX3, GmMAX4a, and GmMAX4B.

Our outcomes manifested that the chosen nineteen SLs biosynthesis and signaling pathway genes were divergently expressed in the hairy roots 10 DAF (Fig. 3). Expression levels of GmDMI2a, GmDMI2B, GmDMI3a, GmDMI3b, GmNSP1a, GmNSP1b, GmNSP2a, GmNSP2b, GmNINA, GmNINB, GmNRF1, GmNRF5, and GmEndo40 were the highest in nodules overexpressing GsIMA2. While the GmMAX1a gene was at the lowest expression level in hairy roots overexpressing GsIMA2. The expression patterns of nodulation signaling and SLs biosynthesis genes in hairy roots overexpressing GsIMA2 gene during the first 10 days of root nodulation suggested that, overall, GsIMA2 play important roles during nodulation signaling and the early stages of nodule development.

Overexpressing GsIMA2 gene changed the expression of nodulation and SL biosynthesis genes in soybean nodules upon B. japonicum (USDA110) infection

To determine if GsIMA2 overexpression plays a functional role during rhizobial infection at the early stages of nodule formation and development, we studied the effect of GsIMA2 overexpression on the expression of signaling genes and SLs biosynthesis in nodulation 10 DAI by B. japonicum. As a result, the expression levels of the same nineteen previously identified genes, early nodulation signaling, and SL biosynthesis genes were evaluated and analyzed. These findings illustrated that at 10 DAI, the previously mentioned genes were differentially activated in nodules (Fig. 4). Intriguingly, the expression levels of GmDMI2a, GmDMI2B, GmDMI3a, GmDMI3b, GmNSP1a, GmNSP2a, GmNINA, GmNINB, GmNRF1, GmNRF5, GmEnod40, GmMAX2, GmMAX4a, and GmMAX4B genes were highest in nodules overexpressing GsIMA2. Conversely, GmNSP1B, GmNSP2B, GmMAX1a, GmMAX1B, and GmMAX3 transcription levels were markedly declined in nodules by overexpressing GsIMA2 (Fig. 4). This result indicates that GsIMA2 has a censorious symbiotic role during nodulation signaling and the early stages of nodule formation. To investigate the sustained effects of GsIMA2 gene overexpression on root and nodule development, we looked at the expression of nodulation signaling and SLs biosynthesis genes in hairy roots and nodules of soybean at 20 DAI. Moreover, qRT-PCR was used to examine the expression levels of the same collection of nodulation signaling and SLs biosynthesis genes. A 20 DAI, the prior genes were differentially induced in hairy roots and nodules, according to the findings (Figs. 5 and 6). As shown in the result (Fig. 5), the expression of GmDMI2a, GmDMI2B, GmDMI3a, GmNSP1a, GmNSP2a, GmNINA, GmNINB, GmNRF1, GmNRF5, and GmEndo40 were the highest in nodules overexpressing GsIMA2 compared with GUS as control at 20 DAI. However, the expression levels of GmDMI3B, GmNSP1B, GmNSP2B, GmNINB, and GmMAX4B genes were decreased in hairy roots overexpressing GsIMA2 (Fig. 5), signifying the role of GsIMA2 at a late stage of rhizobial infection. In addition, the expression of GmDMI2a, GmDMI2B, GmDMI3a, GmNSP1a, GmNSP2a, GmNRF1, GmEnod40, GmMAX2, GmMAX3, GmMAX4a, and GmMAX4B genes were the highest in nodules overexpressing GsIMA2 compared with GUS as control at 20 DAI.

While, the expression levels of GmDMI3B, GmNSP1B, GmNSP2B, GmNINA, GmNINB, GmNRF5, GmMAX1a, and GmMAX1B genes were the lowest in nodules overexpressing GsIMA2 (Fig. 6), suggesting diverse roles of this GsIMA2 gene during hairy soybean roots and nodules development. Consequently, these data indicate that GsIMA2 expression orchestrates nodulation signaling and SL biosynthesis genes in hairy roots and nodules at 20 DAI. Furthermore, these findings show that GsIMA2 has a long-term effect on root development, nodule formation, and nodule development, even in mature root systems undergoing active symbiotic nitrogen fixation.

Discussion

Characterization, putative expression patterns, and putative subcellular localization of GsIMA2 from G. soja plant

Wild and cultivated soybean (G. soja [Sieb. and Zucc.] and G. max [L.] Merr) are considered one of the oldest polyploid (pa leopolyploid) plants and one of the most vital food crops worldwide. Soybean isoflavonoids were
Fig. 3 Expression profiles of nodulation and SL biosynthesis genes under the effect of GsIMaT2 gene overexpression in soybean transgenic hairy roots after 10 days of rhizobia inoculation. Gene expression was analyzed using quantitative real-time PCR compared to GUS as a control. The housekeeping GmB-ACTIN gene was used as an internal reference gene for expression normalization. The error bars indicate the SD of three qRT-PCR biological replicates.
Fig. 4  Expression profiles of nodulation and SL biosynthesis genes under the effect of GsIMaT2 gene overexpression in soybean nodules after 10 days of rhizobia inoculation. Gene expression was analyzed using quantitative real-time PCR compared to GUS as a control. The housekeeping GmB-ACTIN gene was used as an internal reference gene for expression normalization. The error bars indicate the SD of three qRT-PCR biological replicates.
analyzed several decades ago and were found to contain several of aglycon and glucoside isoflavonoids components, such as daidzein, genistein, glycitein, aglycones, malonyldaidzin, malonylegenisin, and malonylglycitin [6, 37, 38]. Despite this, only a limited number of recent reports describe the role and function of isoflavonoid genes in soybean root growth and nodulation [8, 19, 30, 39]. In this study, we identified the isoflavone-7-O-beta-glucoside 6'-O-malonyltransferase (GslMaT2) gene in the wild soybean genome by BLAST search using the putative isoflavone-7-O-beta-glucoside 6'-O-malonyltransferase gene from G. max, L. albus, and M. truncatula. Phylogenetic analysis showed that the close homology to GslMaT2 from G. soja is GmlMaT2 from G. max (Additional file 1: Fig. S1). To recognize GslMaT2 physiological roles, its expression patterns in twenty-eight different tissues following inoculation and fertilization based on their increased resemblance to Glyma.18G258000 gene from G. max were identified. This GslMaT2 gene was detected in all the tissues and predominantly expressed after inoculation in (Root_tip, Root Hair 48 HAI, Root hair_48HAImock, and Root Hair 24 HAI), and after fertilization in (Root, Seed 35 DAF, Seed 42 DAF, and Seed 28 DAF), which are nearly similar to these homologous genes GmMT7, GmMaT1, GmMaT2, GmMaT3 and GmMaT4 from soybean (Additional file 4: Fig. S4 A and B) [6, 16, 30–34]. Moreover, putative subcellular localization studies based on Arabidopsis protein localization for recognized synthesis sites from the Cell eFP database (http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) revealed that the GslMaT2 presents mainly in the cytosol and endoplasmic reticulum (Additional file 4: Fig. S4C). These in silico results align with earlier studies that exhibited cytosol and endoplasmic reticulum as the main loci for isoflavonoids [6, 7, 33, 40–42]. The putative expression patterns and putative subcellular localization of GslMaT2 underscore the possible roles of (iso) flavonoids in yielding flavonoids found at infection sites and during infection to attract rhizobia and establish nodulation [39]. Therefore, cloning the full-length cDNA of GslMaT2 and examining its role in soybean root and nodule development through overexpressing in hairy root systems is crucial to proving this hypothesis (Fig. 1A-D). The results demonstrated that this gene plays a significant role in increasing root length, fresh root weight, nodule number, and fresh nodule weight, compared to the GUS control in transgenic G. max hairy roots (Fig. 2A). Isoflavonoids and their derivatives are reported to affect legume nodulation, and well-documented to function in M. truncatula and G. max root nodule.

**Overexpression of GsMaT2 gene changed accumulation of isoflavonoid in transgenic soybean hairy roots**

Exhilaratingly, we revealed that the overexpression of GsMaT2 enhanced isoflavonoid amassing in transgenic soybean hairy roots (Fig. 2C). From our results, we found that the malonyldaidzin, malonylegenisin, daidzein and glycitein were significantly increased in GsMaT2-OE hairy roots after 10- and 20-days from B. japonicum infection compared with the GUS control (Fig. 2C). Particularly, isoflavonoid compounds such as glyceollins, daidzein, malonyldaidzin, genistein, and malonylegenisin were reported to affect plant growth, nodule formation and interaction with other microbial communities [6, 7, 12, 14, 15]. However, plenty of evidence showed the role of isoflavonoid like daidzein and genistein that are secreted from roots in root–bacteria symbiotic interaction, which starts from secreting and transporting to the plasma membrane of root cells [43]. Then inducing the expression of Nod genes in rhizobial bacteria to form infection threads and nodules formation in root cortical [2, 17]. Likewise, isoflavonoid compounds have been shown to affect nodule formation and root hair patterning in soybean and Medicago. For example, isoflavonoid genes such as MtMaT1, MtMaT4, MtMaT5, MtMaT6, GmlMaT1, GmMaT2, and GmMaT3 can affect nodule and root development in soybean and medicago transgenic roots, likely through modulating the accumulation of isoflavonoid [6, 16, 41, 42]. Therefore, it is not surprising that the overexpression of the GsMaT2 gene in transgenic soybean hairy roots showed similar effects on the isoflavonoid accumulation and nodules formation.

**Effect of GsMaT2 overexpression in hairy roots growth and soybean nodulation**

To shed light on the role of GsMaT2 overexpression in controlling hairy roots growth and nodulation, we investigated the effect of GsMaT2 gene overexpression on the expression levels of nodule signaling and SLs biosynthesis genes in hairy roots and nodules of transgenic soybean upon 10 and 20-days after B. japonicum inoculation. Quantitative real-time (qRT) PCR was used to determine the expression levels of nineteen selected genes (GmDMI2a, GmDMI2B, GmDMI3a, GmDMI3b, GmNSP1a, GmNSP1B, GmNSP2a, GmNSP2B, GmNINa,

(See figure on next page.)

**Fig. 5** Expression profiles of nodulation and SL biosynthesis genes under the effect of GslMaT2 gene overexpression in soybean transgenic hairy roots after 20 days of rhizobia inoculation. Gene expression was analyzed using quantitative real-time PCR compared to GUS as a control. The housekeeping GmB-ACTIN gene was used as an internal reference gene for expression normalization. The error bars indicate the SD of three qRT-PCR biological replicates.
Fig. 5 (See legend on previous page.)
Fig. 6 Expression profiles of nodulation and SL biosynthesis genes under the effect of GsIMaT2 gene overexpression in soybean nodules after 20 days of rhizobia inoculation. Gene expression was analyzed using quantitative real-time PCR compared to GUS as a control. The housekeeping GmB-ACTIN gene was used as an internal reference gene for expression normalization. The error bars indicate the SD of three qRT-PCR biological replicates.
**GsMaT2** gene in the legume that specifically recognizes and binds to compatible, species-specific Nod factors produced by rhizobia [44–48]. These results are supported by [3, 16, 24, 49–52] that findings highlight the importance of Nod factor receptors (NFRs) genes from *M. truncatula, L. japonicus*, and *G. max* for the interaction with root-rhizobia, activating early nodulin gene expression and nodule organogenesis. Furthermore, Nodule Inception genes (*GmNINA* and *GmNINb*) are early key regulators of nodule organogenesis and infection thread formation [53–55]. Moreover, the transcriptional regulators’ Nodulation Signaling Pathway1 (*NSP1*) and *NSP2* are essential for inducing and activating the expression of Nodule Inception (*NIN*), Early Nodulin coding genes (*ENOD11* and *ENOD40*), and Ethylene Response Factor Required for Nodulation1 coding gene (*ERN1*). In general, the overexpression of *GsMaT2* in the *G. max* hairy roots system enhances the formation of early nodulation genes such as *DM12a*, *DMI3a*, *NSP2β*, *NSP1α*, *NFR5α*, and *NFR1α* but compromised in *GmMaT2* knockdown compared with the control. So, we propose that both hormones (such as strigolactones or brassinosteroids) are likely to act linked with the autoregulation of the nodulation (AON) system, having a role in the promotion of nodule formation and maintenance of meristematic activity during nodule development [25, 26, 28, 54, 59, 60]. In general, the overexpression of the *GsMaT2* gene from wild soybean led to increased transcription of nodulation signaling and SL biosynthesis genes. This finding points to isoflavone-7-O-beta-glucoside 6″-O-malonyltransferase playing critical roles in root development and nodulation in soybean. Our results demonstrate that the overexpression of the *GsMaT2* gene studied here significantly increased nodulation and root growth compared to control hairy roots of soybean overexpressing GUS. Finally, the main findings are summarised in Fig. 7.

### Conclusions

In summary, this study focuses on cloning *GsMaT2* from wild soybean (*G. soja*), encoding isoflavone-7-O-beta-glucoside 6″-O-malonyltransferase, over-expressed in hairy root systems of the cultivated soybean (*G. max*), and assessing the root architecture, growth, and nodulation. Substantial differences in root growth, root nodulation, expression levels of nodulation signaling, and SL biosynthesis genes were observed. We further used bioinformatics and the putative expression analysis as tools to predict the role of *GsMaT2* in root and nodule development. Our data suggest that the *GsMaT2* gene promotes root development and nodulation signaling by activating nodulation signaling and SL synthetic genes. These findings prove that the overexpression of the *GsMaT2* gene could affect the nodulation signaling pathway and nodulation. Our study clarifies our understanding of the exact function of the *GsMaT2* gene in root and nodulation, including the role of increased *GsMaT2* expression in both root and nodules, in conjunction with the nodulation signaling and SL synthetic genes that are crucial for legume nodulation production.

### Material and methods

#### Phylogenetic analysis

The full-length cDNA of *GsIMaT2* was retrieved from GenBank ID: XM_028358532.1 and Phytozone ID: GlysoPI483463.18G200800.. Additionally, the *GsMaT2* product was identified as (A0A445FY23) from the UniProt database [62], which was then used as a query in a blastp search with a cutoff e-value <1e−04 to retrieve authentic homologous proteins from Glycine soja, Glycine max, Nicotiana tabacum, Arabidopsis thaliana, Salvia splendens and Amborella trichopoda [63]. All retrieved protein sequences were aligned by Decipher [64]. The ProtTest v3.4.2 was used to select the best-fitted amino acid substitution model based on the lowest Bayesian information criterion (BIC) score [65]. Afterwards, the Bayesian phylogenetic tree was constructed via MrBayes v3.2.6. with the Jones–Taylor–Thornton (JTT) amino acid substitution model with invariant sites, discrete gamma model, and (+ F) for the empirical equilibrium of amino acid frequencies [66]. Finally, the phylogenetic tree was introduced to the iTOL (Interactive Tree of Life) web tool for visualization [67].

#### In-silico differential gene expression analysis

Tissue-specific expression data from twenty-eight soybean tissues after inoculation and fertilization (e.g., Root Hair 12HAI, Root Hair 24 HAI, Root Hair 48 HAI) were used for visualization.
SAM, Flower, Green_Pods, Leaves, Nodule, Root, Root_tip, Young Leaf, Flower, One CM Pod, Pod Shell (10-13 DAF), Pod Shell (14-17 DAF), Nodule, Root, Seed 10-13 DAF, Seed 14-17 DAF, Seed 21 DAF, Seed 25 DAF, Seed 28 DAF, Seed 35 DAF and Seed 42 DAF) were extracted from public RNA-Seq Atlas of soybean (http://bar.utoronto.ca/eplant_soysbean/). Additionally, GsIMA2T2 predicted subcellular localization was inferred from its Arabiposis homologous genes as retrieved from the Arabidopsis Information Resource (https://phytozome.jgi.doe.gov/pz/portal.html#info?alias=Org_Athaliana). Ultimately, the image that showed the subcellular localization.
localization was conducted using Cell Electronic Fluorescent Pictograph Browsers (Cell eFP: http://bar.utoronto.ca/cell_efp/cgi-bin/cell_efp.cgi) [68, 69].

RNA extraction and cDNA synthesis
Total RNA from three biological replicates of *G. soja* was extracted from roots using TRIzol reagent (Invitrogen, CA, United States) according to the manufacturer’s methods and instructions. Also, total RNA was extracted from twelve biological replicates of *G. max* hairy roots and nodulated roots upon 10 and 20 days of rhizobial inoculation. Total RNA samples were treated with DNase I (Takara, China). RNA quality was examined on 1.2% Agarose gels, and the purity and concentration were analyzed using a Nano-Photometer spectrophotometer (IMPLEN, CA, USA). cDNA synthesis for gene cloning and qPCR was performed with a 10μg total RNA pool produced by mixing equal volumes of the three RNA replicates in a tube using a commercial reverse transcription kit (M-MLV, China) according to the manufacturer’s protocol [24, 68, 70, 71].

Cloning of full-length isoflavone-7-O-beta-glucoside 6″-O-malonyltransferase (*GsIMaT2*) gene
The *GsIMaT2* full-length cDNAs (GenBank ID: ON520655.1) with a length of 1536bp was obtained by PCR amplification using short and long gene-specific primers designed based on the transcriptome sequencing of *G. soja* from soybean database (https://phytozome.jgi.doe.gov/pz/portal.html). Root cDNA was used as a template for the first PCR, which was performed with short primers, such as *GsIMaT2* forward 5′-ATGGCA GTGAAAAATTCAAAAGTC − 3′ and reverse 5′- TCA CTCATCTTTCAGTCTCCATG − 3′ with the KOD-Plus DNA polymerase (Toyobo, Japan) with the following cycling conditions: an initial step of 4 min at 95 °C followed by 34 cycles of denaturation for 10s at 98 °C; 30s at 60 °C and an extension for 2 min at 68 °C, and a final extension step for 11 min at 68 °C. The first PCR products were used as templates for PCR cloning using long primers, such as *GsIMaT2* forward 5′-GGGGACAAG TTTGTACAAAAAAGCAGGCTTCAGGGCAAGTGGGA AAAATTC-3′ and reverse 5′-GGGGAC CACTTGTGA CAAGAAGCTTGGGGTACACTCATCTTCTAGCC-3′ with the and KOD-Plus DNA polymerase. The amplified PCR products were purified using (QIAEX II Gel Extraction Kit, China) and cloned into the Gateway entry vector pDONR221 using BP Clonase (Gateway™ BP Clonase™ II Enzyme mix, Invitrogen) [24, 68, 70, 71]. The resulting pDONR221 construct harbouring target gene was sequenced, and the LR Clonase (Gateway™ LR Clonase™ Enzyme mix, Invitrogen) was used for recombination into the destination vector pB2GW7 for *G. max* hairy root transformation to produce composite soybean plants. Sanger sequencing confirmed that all final constructs contained *GsIMaT2* cDNAs. The construct was introduced into *Agrobacterium rhizogenes* strain “K599” by direct electroporation.

Soybean hairy root transformation and rhizobial inoculation
Seeds of soybean cv. “Tianlong 1” were surface sterilized by placing 150 seeds in 15 × 100 mm Petri dishes in a single layer. The plates were placed inside a 1000-mL beaker with 200 mL of commercial bleach. Ten microliters of concentrated (12N) HCl were applied dropwise to the beaker’s internal wall; the container was sealed with a plastic cover and kept overnight (16 hours). The following morning, the sterilized seeds were germinated in sterile vermiculite in a growth chamber (12-h photoperiod, 28 °C day/25 °C night, and 70% humidity) for a few days until hairy root transformation.

Recombinant *A. rhizogenes* were grown for two days at 28 °C on solid LB media supplemented with 50 μg/mL of each streptomycin and spectinomycin. An individual colony of each construct was inoculated into 1 mL of liquid LB medium with the same antibiotics and grown at 28 °C under 200 rpm agitation overnight. After 24h, the liquid cultures were transferred into a 250-mL conical flask containing 50 mL of LB media supplemented with the same antibiotics and grown in a shaker at 28 °C until an optical density (OD<sub>600</sub>) of 0.6–8.0 was reached. Overnight cell cultures were harvested by centrifugation at 5000rpm for 10 min at 4 °C, and the pellet was re-suspended to an OD of half-strength B5 medium containing 3% sucrose. Healthy and vigorous seedlings with unfolded green cotyledons were inoculated with *A. rhizogenes* strain K599 harboring the binary vectors by injecting the hypocotyls proximal to the cotyledon with the bacterial suspension. The infected seedlings were then transplanted into 10 cm x10 cm × 8.5 cm pots with vermiculite with the infection site buried, and each pot was covered with a transparent plastic bag to retain humidity.

The rhizobial inoculations of hairy roots were carried out with 15-day-old plants (10 days after root transformation). A culture of *B. japonicum* strain “USDA-110” was cultured on yeast extract mannitol agar (YMA) at 28 °C. After ten days of hairy root emergence, the optical density (OD<sub>600</sub>) of a rhizobium liquid YM culture was adjusted to 0.08-1.0, and about 50 mL were applied to each pot. After ten and twenty days of rhizobial inoculation, the plants (n = 10–12) with well-developed hairy roots and nodules were photographed and harvested for measurements and RNA isolation to assess gene expression. Hairy root systems from each plant were considered independent transformation events [24, 28].
Quantitative real-time PCR analyses

Quantitative real-time PCR (qRT-PCR) was performed using an iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad) with SYBR Green fluorescence (and ROX as a passive reference dye; Newbio Industry, China) in a total reaction volume of 20 μL, as described previously [24, 68, 70, 71]. Gene-specific primers for GmActin as a reference gene and GsIMaT2 were used. Primers were designed with the IDT dna tool (https://eu.idtdna.com/scitools/Applications/RealTimePCR/), and their sequences are listed in Additional file 5: Table S1. Additionally, gene-specific primers for nineteen G. max genes involved in early nodulation signaling genes such as GmDM12a, GmDM12B, GmDM13a, GmDM13b, GmNSP1a, GmNSP1B, GmNSP2a, GmNSP2b, GmNINa, GmNINa, GmNSP2a, GmNSP2b, GmNSP3a, and SL synthetic genes such as GmMAX1a, GmMAX1b, GmMAX2, GmMAX3, GmMAX4a, and GmMAX4b are listed in Additional file 5: Table S1. The amplicon sizes were designed between 145 and 160 bp. The quantitative RT-PCR standard conditions were: 95 °C for 3 min, 34 amplification cycles (95°C for 10 s, 58°C or 60°C for 30 s, and 72°C for 20 s), followed by 65°C for 5 s and 95°C for 5 s. The relative expression levels were calculated by comparing the target genes’ cycle thresholds (CTs) with the reference gene GmActin. Data quantification was carried out with the Bio-Rad IQ™5 Multicolor Real-Time Manager software using the 2-ΔΔCt method [71–73] and GmActin as a reference housekeeping gene for normalisation. Values are presented as means ± SE of three different RNA pool replicates.

Statistical analyses

The Student's t-test analyzed Soybean hairy root measurements to estimate the effects of gene overexpression and time on the number of nodules, nodule fresh weight (gram), fresh root weight (gram), and root length (cm) compared to the control roots (GUS-overexpressing hairy roots). Each column represents the mean±SD of the parameter, and statistical significance was based on the Student's t-test (*P<0.05; ** P<0.01) with GUS-overexpressing hairy roots as control.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-022-03811-6.
Acknowledgments
The authors thank Prof. Osama Ezzat Elsayed for proofreading the manuscript. We owe thanks to Dr. Ahmed Ali for giving us the G. soja and G. max seeds. The authors would also like to acknowledge Dr. Mohamed Hamdy Amar and Dr. Wael Moussa for their constructive comments. We recognize all members of the Molecular Genetic Lab for their encouragement and assistance with experiments. M.A. would like to thank the Desert Research Center (DRC) for moral support.

Authors’ contributions
M.A conceived and designed the study. M. A and A.M.A. performed in vivo transgenic soybean hairy roots. D.B.D, M. Z, M.A and A. N performed the qRT-PCR analyses. M. A wrote the manuscript. M. Z, and A.N. revised the manuscript. All authors discussed the results, commented on the manuscript, and participated in the analysis of the data.

Funding
Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. GenBank accession number: Glycine soja isoflavone-7-O-glucoconjugate 6′-O-malonyltransferase (GmMaT2, GenBank: ON520655.1) https://www.ncbi.nlm.nih.gov/nuccore/ON520655.1.

Declarations
Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 14 May 2022 Accepted: 25 August 2022 Published online: 09 September 2022

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