Knockdown of \textit{GmD53a} confers strigolactones mediated rhizobia interaction and promotes nodulation in soybean

Naveed Rehman\textsuperscript{1}, Fahim Ullah Khan\textsuperscript{3}, Muhammad Imran\textsuperscript{3}, Shahid Ali Rajput\textsuperscript{4}, Yiming Li\textsuperscript{1}, Ihteram Ullah\textsuperscript{5}, Rana waseem Akhtar\textsuperscript{4}, Muhammad Imran\textsuperscript{1}, Arwa Abdulkreem AL-Huqail\textsuperscript{6}, Ahmad El Askary\textsuperscript{7}, Amany Salah Khalifa\textsuperscript{8} and Muhammad Tehseen Azhar\textsuperscript{9,10}

\textsuperscript{1} State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangdong Provincial Key Laboratory of Plant Molecular Breeding, South China Agricultural University, Guangzhou, China
\textsuperscript{2} Department of Agriculture, Hazara University, Dodhial, Mansehra, Mansehra, Khyber Pakhtunkhwa, Pakistan
\textsuperscript{3} Department of Agriculture, South China Agricultural University, Guangzhou, Guangdong, China
\textsuperscript{4} Faculty of Veterinary and Animal Sciences, Muhammad Nawaz Shareef University of Agriculture, Multan, Punjab, Pakistan
\textsuperscript{5} Department of Plant Breeding & Genetics, Gomal University, Dera Ismail Khan, Khyber Pakhtunkhwa, Pakistan
\textsuperscript{6} Department of Biology, College of Science, Princess Nourah Bint Abdulrahman University, Riyadh, Saudi Arabia
\textsuperscript{7} Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, Taif University, Taif, Saudi Arabia
\textsuperscript{8} Department of Clinical Pathology and Pharmaceutics, College of Pharmacy, Taif University, Taif, Saudi Arabia
\textsuperscript{9} Institute of Molecular Biology and Biotechnology, Bahauddin Zakariya University, Multan, Punjab, Pakistan
\textsuperscript{10} School of Agriculture Sciences, Zhengzhou University, Zhengzhou, China

\textbf{ABSTRACT}

\textbf{Background.} Strigolactones (SLs) play a key role in modulating plant root growth, shoot branching, and plant-symbiont interaction. However, despite their significance, the components of SL biosynthesis and signaling in soybean and their role in soybean-rhizobia interaction is unknown.

\textbf{Methods.} In this study, we identified and functionally characterized the \textit{GmD53a} from soybean. The \textit{GmD53a} ORFs were amplified from root cDNA using primers for \textit{GmD53a} RNA interference. To induce transgenic hairy roots of soybean, electric shock was used to transform pB7WG1WG2 vectors containing \textit{GmD53a} knockdown and \textit{GUS} into \textit{K599} strains of \textit{Agrobacterium rhizogenes}. The hairy roots and nodules were collected and examined for root nodules ratio and RNA was extracted after 4 weeks of rhizobia inoculation.

\textbf{Results.} A tissue-specific expression assay showed that \textit{GmD53a} was differentially expressed in plant parts, predominantly in the stem and nodule. Furthermore, its expression was significantly up-regulated during rhizobia infection and varied with nodule formation. The \textit{GmD53a}-knockdown chimical plants were produced to further check its role in soybean nodulation in comparison with control \textit{GUS}. In knockdown lines, the \textit{GmD53a} (suppressor of strigolactone MAX2) has a higher...
number of nodules compared to control lines. Furthermore, the expression levels of several nodulation genes essential for initiation and formation of nodules were altered in *GmD53a*-knockdown lines.

**Conclusion.** The results revealed that SL biosynthesis and signaling are not conserved but also have close interaction between SL and legume rhizobia.

**Subjects** Agricultural Science, Bioinformatics, Biotechnology, Plant Science

**Keywords** *GmD53a*, Nitrogen fixation, Rhizobia positive interaction, Symbiotic, Biotic factor, Sustainability, Food security, Signaling, Soybean nodulation, Strigolactone

**INTRODUCTION**

Strigolactone (SLs), a new class of terpenoid lactones is generated from carotenoids, and it was identified as a component of root secretion for parasitic witchweed germination (*Xie, Yoneyama & Yoneyama, 2010*). SLs are required for the formation of symbiotic arbuscular-mycorrhizal fungi in plants which are effected by phosphorus deprivation (*Akiyama, Matsuzaki & Hayashi, 2005; Bouwmeester et al., 2003; Waldie, McCulloch & Leyser, 2014*), and has also been involved significantly in rhizobia-legume interaction (*Foo et al., 2013; Liu et al., 2013; Liu et al., 2011*). Rhizobia-legume interaction also activates defense responses and increases salt tolerance in soybean seedlings (*Qu et al., 2016*). In addition, under water deficit conditions, inoculation of rhizobia on soybean resulted in an increased number of nodules (*Kibido et al., 2020*).

The discovery of strigol supported the agricultural community in understanding and developing system to increase nitrogen fixation. Genetically, SLs are associated with numerous shoot mutants, such as *Ramosus* of pea (*Pisum sativum*) (*Beveridge, Symons & Turnbull, 2000*), More Axillary Growth (*MAX*) of *Arabidopsis thaliana* (*Booker et al., 2005*), Decreased Apical Dominance (*DAD*) of petunia (*Petunia hybrida*) (*Simons et al., 2007*) and Dwarf or High-Tillering Dwarf (*D/HTD*) of rice (*Oryza sativa*) (*Arite et al., 2007; Lin et al., 2009*). The SLs are found to be synthesized from carotenoid pathways employing a carotenoid biosynthesis inhibitor and carotenoid metabolism mutants (*Matusova et al., 2005*). In addition, rice mutants *d17* and *d10* in CCD7 and CCD8 were found to have defective mechanisms, and *ccd8/rms1* deficiency in SL synthesis was discovered in mutants of pea (*Gomez-Roldan et al., 2008; Umehara et al., 2008*). The roles of *CCD7* and *CCD8*, *MAX1*, and P450 cytochrome in the production of SLs have also been discovered. However, strong evidence is required to prove that SLs are made up of carotenoids (*Crawford et al., 2010*). *Lin et al. (2009)* also found the Fe-containing protein DWARF27 (D27) in the biosynthesis of SLs. SLs biosynthesis genes *D27*, *CCD7*, and *CCD8*; the three primary biosynthetic enzymes that complete the sequential processes and produce CL as a product (*Alder et al., 2012*). *D27* catalyzes the reversible isomerization of all-Trans-β-carotene at the C-9 position to create 9-cis-β-carotene. First *CCD7* utilizes all-Trans-β-carotene as a substrate and generated all-trans-β-10-carotenol, and then the *CCD7* cleaves the 9-cis-carotene and converts it to the 9-cis-apo-10-carotenol, which is consumed by *CCD8* to make CL (*Schwartz, Qin & Loewen, 2004*). A P450 mono-oxygenase, encoded
by Arabidopsis MAX1 and lotus LBO further converts carlactone into 5-deoxystrigol and other bioactive SLs (Abe et al., 2014; Breakspear et al., 2014). Furthermore, rice D14 or petunia DAD2, α/β-fold hydrolase that can hydrolyze SLs and act as an SL receptor. In the presence of SLs, MAX2/D3 group of F-box proteins binds with D14 for the production of D14/Skp1–Cullin–F-Box (SCF) E3 ubiquitin ligase complex D14-SCFD3/MAX2 (De Saint Germain et al., 2016; Hamiaux et al., 2012; Stirnberg, Furner & Ottoline Leyser, 2007; Yao et al., 2016). Notably, MAX2 and SCF (Skp1–Cullin–F-Box) play a critical role in SL-triggered protein degradation (Zhao et al., 2014). D53 proteins can create D53–D14–SCFD3 protein complexes by interacting with D14 and D3 proteins. These allow the proteasome system to preferentially degrade the ubiquitin D53 protein, and activate the expression of downstream target genes that lead to the regulation of tillering in cereal crops. This means that tillering ability can be influenced by modulating D53 expression, which can alter the SL signal transduction (Lv et al., 2019).

Although D53 protein belongs to the small group or family SMAX1-like (SMXL) and shares similarities with enzymes such as Ctp ATPase enzymes, little is known about how D53 protein suppresses SLs signaling (Stanga et al., 2013). SLs are generated in roots and stems and transported upwards through the xylem to higher regions of the plant or extruded into the extracellular spaces (Kohlen et al., 2011). An ATP-binding cassette (ABC) transporter Pleiotropic Drug Resistance1 (PDR1) was identified as a SL exporter (Kretzschmar et al., 2012). Despite significant advances in understanding SL production and signaling, many SL-related events, such as complicated cross-talks or interactions between SLs and other hormones remain unknown.

Recently, it has been found that SL biosynthesis and signal transduction may play a significant role in soybean nodulation (Ahmad et al., 2020; Bennett & Leyser, 2014; Bennett et al., 2016; Rehman et al., 2018). Because the D53 gene is important for tillering regulation, we used RNAi techniques to knock down the homologous soybean gene of D53 (GmD53a), and examined the physicochemical properties and structure of GmD53 protein using bioinformatics tools. Furthermore, the role of GmD53a protein in soybean root nodulation was demonstrated in this study.

**MATERIALS AND METHODS**

**Plant growth conditions**

Seeds of soybean were surface sterilized with sodium hypochlorite (NaClo) and hydrochloric acid (HCL) and grown in three-gallon pots containing vermiculite soils at National Key Laboratories, Huazhong Agricultural University, Wuhan. The seedlings were grown at 26/20 °C (day/night) temperature, photoperiod of 14/10 h, 800 µmol m⁻² s⁻¹ light intensity and 60% humidity was maintained in growth chamber. Seeds, stems, roots, flowers, leaves, of and pods from soybean cultivar (Tianlong no. 1) were harvested at different growth phases. After treatments, all selected tissues were sensibly removed and instantly put in liquid nitrogen and then stored at −80 °C. Then, RNA was extracted and later on cDNA was synthesized according the protocol of supplier.
Construction of vector
The GmD53a (Glyma.11G230700.1) ORFs were amplified from root cDNA by using primers for GmD53a RNA interference (Table S1). Following that, RNA was isolated with TRIzole reagent (Invitrogen) or RNA kit (Biotech, Beijing) from soybean roots, and 10 g of RNA was taken in the cDNA synthesis utilizing the first-strand synthesis technique (Invitrogen) and cDNA was used as a template for the amplification of GmD53a as previously described (Ahmad et al., 2020). The amplified fragments were used for directionally cloning in pDONR221 vector via BP clonase and then recombined into the pB7GWIWG2 destination vector via LR clonase (Life Technologies, Rockville, MD, USA) after ORFs were cloned into T-easy vector (Promega, Madison, WI, USA) and sequenced. For comparison, the GUS gene was also recombined into both vectors. Then the constructs were transformed into A. rhizogenes K599 by electroporation and were utilized to induce hairy roots.

Induction of transgenic hairy root and nodulation assay GmD53a
To induce transgenic hairy roots of soybean, electric shock was used to transform pB7WG1WG2 vectors containing GmD53a knockdown and GUS into K599 strains of Agrobacterium rhizogenes. A. rhizogenes strain K599 harboring pB7GWIWGII-Gm53a, cDNA fragments for knockdown constructs, or GUS were grown on LB-agar medium at 28 °C with spectinomycin and streptomycin as selection markers. The overnight Agrobacterium cultures were used for transformation of soybean cultivar. Seven days old seedlings of soybean were wounded at the hypocotyls region before being incubated in high humidity for 24 h carrying A. rhizogenes constructs (Ahmad et al., 2020). Hairy roots were emerged from the wounding sites after one week of infection, whereas non-transgenic roots were removed after one week. About 1 week after hairy root emergence when transgenic hairy roots were about to support the plants, the chimeric soybean plants were examined for transgene expression in hairy roots, and the main of non-transgenic roots are removed, before being inoculated with rhizobia strain USDA110 cultured in the YMA at 28 °C. About 25 mL rhizobia bacteria solution (OD 600 nm) was applied to each plant pot. The hairy roots and nodules were examined and collected for root nodules ratio, and RNA was extracted after 4 weeks of inoculation. Three independent experiments of transformation with 10 individual transgenic lines under equal treatments and growth conditions were carried out for each vector including GUS as a control. We determined the numbers of nodules from hairy roots per grams for normalization.

Rhizobia infection
For the rhizobia infection experiment, seedlings of about 14 days old were inoculated with "USDA-110" (O.D600 = 0.08–0.1) strain of Bradyrhizobium japonicum in the YMA medium. Roots of infected plants were collected at 0, 6, 12, 24, 36, and 48 h after inoculation. These frozen tissues were used the extraction of RNA to synthesize cDNA.

QRT-PCR analysis of gene expression
Total RNA was isolated from several tissues i.e., seeds, leaf, stem, flower, root, and nodules), or transgenic hairy roots by using TRIzol (Invitrogen, Carlsbad, CA, USA)
or RNA kit (Biotech, Beijing) according to instructions from the manufacturer. About 10 g of total RNA was digested with RNase-free DNaseI (Promega, Madison, WI, USA) to remove any genomic DNA contamination for each sample. A NanoDrop ND-2000 UV spectrophotometer (Thermo Scientific, Waltham, MA, USA) was used to measure the concentration of RNA. The first strand cDNA was prepared from 10 µg total RNA using the Superscript III first strand synthesis system (Invitrogen). All of cDNA samples were diluted with sterile water according to the requirements for qRT-PCR analysis where gene-specific primers were used (Table S1). The reaction mixture of 20 µL containing 2.5 µL SYBR Master Mix (Applied Biosystems), 1 µL primer mix (0.4 µL from each primer, 0.2 µL ddH2O), and 2 µL of 100 ng cDNA were put in 96-well plates (iQ5 Real Time PCR System; Bio-Rad) for all of selected tissues. The transcript levels were normalized by using GmACTIN1 as previously described (Ahmad et al., 2020).

Bioinformatics analysis
Protein sequences of SLs signaling genes reported from monocots and dicots were obtained from NCBI or phytozome database (phytozome.jgi.doe.gov/) for phylogenetic analysis and identification of SLs signaling genes. The phylogenetic tree was constructed by MEGA6, and Neighbor-joining was performed with 1,000 bootstraps (Tamura et al., 2013). Bioinformatics analyses on protein sequences of SL pathway genes were obtained from NCBI (https://www.ncbi.nlm.nih.gov/) and phytozome (phytozome.jgi.doe.gov/). The sequence similarities and identities were determined from Blast2 (http://pga2.mgh.harvard.edu:8080/rtpcrblast/wblast2_cs.html). The RNA Seq-Atlas data from nine soybean tissues were extracted from public databases (http://soybase.org/soyseq/) to acquire the tissue-specific expression and stages of nodule development.

Statistical analysis
The data were recorded from three independent experiments for each trait, and Student’s t-test was applied for analysis using Statistix 8.1 software. The confidence limit 95% represents the significant between two-tailed data (Rehman et al., 2018).

RESULTS
Identification of SL biosynthetic genes from soybean genome
The homologs of other organisms with 1 × 10^{-3} permissive E cut-off values were discovered in tBLASTp searches to assess the evolutionary relationship of GmD53 with other plant species. A neighbor-joining phylogenetic tree was generated by using the D53 proteins from 12 dicotyledons and three monocotyledons by using MEGA6.0 software to compare the evolutionary relationships between GmD53a and other plant D53 proteins (Fig. 1A). The phylogenetic analysis revealed that all D53 proteins were divided into two main groups, i.e., dicotyledonous D53 proteins and monocotyledonous D53 proteins, indicating that dicotyledon D53 proteins were highly homologous. This group included all of dicotyledon D53 proteins which were separated into three sub-groups. One sub-group had D53 proteins from Orchidaceae, Palmae, and Musaceae, while other sub-groups were composed of D53 proteins from Gramineae. Thus, D53 proteins from Gramineous are closely related, and
ScD53 protein was closely related to sorghum (*Sorghum bicolor*) D53 protein followed by maize (*Zea mays*) and millet (*Panicum miliaceum*). Furthermore, homology analysis by using Blastp program in NCBI showed that ScD53 had 93%, 82%, 80%, and 69% identity with D53 proteins of sorghum, maize, foxtail (*Setaria italic*), and rice (*Oryza sativa*), respectively. Besides, GmD53a was located on chromosome number 11, and exon/intron distribution analysis showed that GmD53a contains four exons and three
introns with varying lengths (Fig. 1B). Further analysis of GmD53a protein sequence alignment revealed that all the 17 members were delimited by CLP-R and ATPase-AAA domain (Fig. 1C), suggesting that GmD53a is evolutionarily conserved in plant species.

**Tissue-specific and altered expression in nodule stages of GmD53a**

The expression patterns of the strigolactone repressor *GmD53a* gene were assessed in various tissues and organs. We performed a BLAST search of the rice *D53* gene in Phytozome and obtained two close homologs *GmD53a* and *GmD53b*. We have compared gene expression patterns to public databases (http://soybase.org/soyseq/) *GmD53a* (Figs. 2: S1) to understand the role of these genes. Data on the expression levels of genes implicated in strigolactone signaling in nine different plant tissues (pod, root hair, root, nodule, stem, leaf, seed, shoot apical meristem (sam), stem, and flower) were studied. In soybean plants, we explored how the *GmD53a* gene was expressed in different tissues and organs. *GmD53a* was most abundant in the stem, pods, nodules, and leaves, while *GmD53b* was abundant in the stem, Shoot Apical Meristem (SAM), and leaves (Fig. S1). However, low expression was observed in the remaining tissues (Figs. 2, S1). *GmD53a* was more abundant in most of tissues and organs, particularly roots and nodules, than *GmD53b* (Fig. S1).

In addition, we also examined the expression of *GmD53a* gene, which is involved in SL signaling, throughout nodule development and senescence. We evaluated the RNA-Seq data from soybean nodules at various phases of development and validated it with qRT-PCR. The results revealed that *GmD53a* was differently expressed in nodules at various stages of development till nodule senescence (Fig. S2). The data were validated through qRT-PCR which was consistent with the RNA-Seq results. Interestingly, the transcript levels of
GmD53a increased significantly during growth stages of nodule formation, and it was maximum at stage 2, and then declined afterward (Fig. 2).

Effect of GmD53a-knockdown on soybean nodulation

The knockdown chimerical soybean plants known as GmD53a-KD were developed to investigate the function of SLs Dwarf gene GmD53a in soybean nodulation, (Fig. 3A). The plants were infected with GmD53a containing RNAi vector and were collected after four weeks of rhizobium inoculation. The ratio of hairy roots to nodules was examined, and it was found that GmD53a had substantially more nodules per gram of fresh-weight hairy roots than GUS control (Fig. 3B). GmD53a-KD plants produced 60% more nodules than GUS control plants \((P < 0.01)\). This change in number of nodules in GmD53a-KD plants was seen in three separate trials. However, the total size of mature root system did not differ among knockdown and GUS control plants, unlike the number of nodules on mature roots. The qRT-PCR was used to analyze the genetic background of GmD53a-KD, which revealed the considerable repression of GmD53a-KD plants \((P < 0.05)\) (Fig. 3C). The number of nodules on GmD53a-KD plants differed significantly from the control (GUS) plants (Fig. 3). As a result, GmD53a in SL signaling pathway acted as an antagonist in soybean nodulation.

Response to rhizobia infection of GmD53a-KD hairy roots

Apart from known activities in plant-mycorrhizal interactions, SLs have been reported to alter legume nodulation (Foo & Davies, 2011). The GmD53a was expressed after infection with B. japonicum (USDA110). To further explore how SL operates in soybean nodulation and expression of SL suppressor GmD53a in response to B. japonica infection, the GmD53a-KD and GUS control chimerical hairy roots were infected with rhizobia strain USDA110, and expression was assessed at various intervals after infection (Fig. 4). The results showed that GmD53a expression in hairy roots of GUS controls was reduced at 6 h and continued to decline significantly at 12 and 24 h (Fig. 4). Similarly, GmD53a expression was maximum at 36 h and it was declined at 48 h (Fig. 4). The expression level of GmD53a was increased considerably at every time point and peaked at 48 h as compared to GUS control (Fig. 4).

Effect of GmD53a-knockdown on nodulation signaling pathway genes

Strigolactones are essential signaling molecules in plant-arbuscular mycorrhizal fungi interactions (Akiyama, Matsuzaki & Hayashi, 2005). Additionally, strigolactone influenced the nodulation in Glycine max, Medicago sativa, Pisum sativum, and Lotus japonicas (Ahmad et al., 2020; Foo et al., 2013; Liu et al., 2013; Soto et al., 2010; Rehman et al., 2018). Several genes are involved in symbiotic infection and nodulation was analyzed for expression by using qRT-PCR to see how knockdown of GmD53a exerts the effects on nodulation. Surprisingly, GmD53a knockdown was shown to dramatically up-regulated all of identified nodulation genes (Fig. 5). Among them, the cathway genes; does not make infection (DMI2α, GLYMA12G28860) were down-regulated while the expression of DMI2β (GLYMA16G00500) was found to be higher. A similar expression was recorded for DMI3α (GLYMA15G35070) and DMI3β (GLYMA08G24361) (Fig. 5). Nod Factor Receptor 1α (NFR1α; GLYMA02G43860) expression was considerably up-regulated in
Figure 3 Effects of GmD53a-knockdown on soybean nodulation. Chimerical soybean plants were generated by transformation with K599 harboring, GmD53a-knockdown, or GUS vector. Plants with wild-type shoots and transgenic hairy roots were inoculated with Bradyrhizobium japonicum strain USDA110. Nodule numbers from the hairy roots were counted and the roots were sampled for gene expression analysis at 28 days post inoculation. (A) Chimerical soybean plants with wild-type shoots but transgenic hairy roots. GmD53a-KD plants developed less nodules as compared with GUS control. (B) Hairy root fresh weight (g) and nodule numbers ratio in GmD53a-KD and GUS control lines. (C) qRT-PCR confirmation of GmD53a-KD in transgenic hairy roots as compared to GUS control. Gene expression was determined by qRT-PCR with GmACTIN as an internal control. Data are expressed as means ± s.d. from at least 3 independent experiments with biological replicates. Differences were analyzed, *p < 0.05; **p < 0.01 in student’s t-test. Gm53a-KD (P0.05), whereas NFR1β (GLYMA14G05060) expression was significantly down-regulated in GmD53a-KD. The expression of Nod Factor Receptor 5α (NFR5α; GLYMA01G38560) was considerably increased (P < 0.01) in Gm53a-KD as compared to control GUS, while the expression of Nod Factor Receptor 5β (GLYMA11G06740) was dramatically decreased (P < 0.01) in Gm53a-KD (Fig. 5). In GmD53a-KD lines against the GUS, the Nodule Inception α (NINα, GLYMA04G00210) was marginally lowered, while the (NINβ, GLYMA02G48080) was down-regulated. Nodulation Signaling Pathway 1 (NSP1α, GLYMA16G01020; NSP1β, GLYMA07G04430), and Nodulation Signaling Pathway 2 genes (NSP2α, GLYMA06G11610; NSP2β GLYMA04G43090) were significantly up-regulated (P < 0.01) in Gm53a-KD compared to GUS control (Fig. 5).
Figure 4 Effect of Rhizobia inoculation on GmD53a-KD transgenic lines. Chimerical soybean plants were generated by transformation with K599 harboring, GmD53a-knockdown, or GUS vector. Plants with wild-type shoots and transgenic hairy roots were inoculated with Bradyrhizobium japonicum strain USDA110. Roots were collected at different time point after inoculation at 0 h, 6 h, 12 h, 24 h, 36 h and 48 h. Gene expression was determined by qRT-PCR with GmACTIN as an internal control. Data are expressed as means ± s.d. from at least 3 independent experiments with biological replicates. Differences were analyzed, *p < 0.05; **p < 0.01 in student’s t-test.

The Early Nodulation 40 (ENOD40, Glyma01g03470) was up-regulated in GmD53a-KD gene compared to GUS control. Most of the nodulation pathway genes were significantly up-regulated in GmD53a-KD hairy root lines, which were in line with the result of rhizobia infection which might explain why GmD53a-KD transgenic hairy roots had more nodules than the GUS control (Fig. 5).

DISCUSSION

Soybeans are one of the important economic crops, and popularity is increasingly due to their role in nitrogen-fixing capabilities in animal feeds, the food sector, and sustainable agriculture. Understanding the role of SLs in soybeans is critical for the development of new accessions of soybean because the cited gene plays crucial roles in controlling the plant architecture, shoot branching, root growth, plant-mycorrhization, and legume-rhizobium interactions. While the literature on studies on SLs in soybeans is scanty because these genes have not been investigated thoroughly by many researchers. Therefore, this research work was planned to investigate the role of SL signaling genes in the development, nodulation and architecture of soybean.

Unlike diploid model legumes such as Medicago and Lotus, which have only one copy of homolog of the SL biosynthesis gene, while the tetraploid soybean genome has many copies of each MAX or Dwarf gene. Orthologs from pea, Arabidopsis, and rice are similar to each other. SLs are typically synthesized in roots and stems and transported upward to shoots.
and leaves in other plants (Kohlen et al., 2011; Yoneyama et al., 2008). SLs biosynthesis genes have higher expression in roots and stems, but SL signaling genes can be expressed anywhere in plants (Kohlen et al., 2011; Yoneyama et al., 2008). Indeed, the expression of GmD53a in soybean confirms the conserved SL signaling components. In addition, rhizobia infections have significantly altered the expression of SL suppressor GmD53a. According to another study on pea, SLs play a key role in the development of infection threads after rhizobia infection by influencing early nodulation gene expression (McAdam et al., 2017).

SLs are linked to legume nodulation, in addition to their physiological effects on root growth, shoot branching, and mycorrhizal branching (Foo, 2013; Foo & Davies, 2011; Foo et al., 2013). The pea SLs-deficient mutant rms1/CCD8 produces 40% fewer nodules than the wild type, although synthetic SLs analog GR24 partially reversed the phenotype (Foo & Davies, 2011). SLs were reduced by 80% in Lotus japonicus LjCCD7-silenced plants, and nodules were reduced by 20% when compared to control plants (Liu et al., 2013; Liu et al., 2011). In GmD53a-KD soybean roots, the structural grounds for nodule initiation,
development, and rhizobia interaction were significantly changed. As previously stated, root hair development and immediate responses of root hairs to rhizobial Nod factors are crucial for nodulation (Oldroyd, 2013). We also found that knocking down of GmD53a, a SL suppressor, boosted soybean nodulation. GmD53a-KD hairy roots had a 60% (P < 0.01) increase in nodule quantity compared to GUS, implying that GmD53a-KD influenced nodulation in chimerical transgenic hairy roots compared to control. The gene expression analysis revealed that GmD53a knockdown hairy roots also altered the expression of several key genes which are involved in nodulation. Similar to CCD7 and CCD8 in pea and Medicago (Liu et al., 2013; Liu et al., 2011; Van Zeijl et al., 2015), over-expression of GmMAX1a, 2a, 3a, and 4a were also involved in nodulation initiation and development as evidenced by genetic and molecular data (Ahmad et al., 2020); ur (Rehman et al., 2018). On the other hand, soybean GmD53a-KD had other opposite effects on nodulation in soybean. It was previously established that increase in the expression of D10 in d53 mutant explains a feedback control of the SL pathway (Zhou et al., 2013). The same researchers have also reported that that in d53 mutant, the expression of an axillary bud inhibitor FINE CULM 1 (FC1), which is an orthologue of TEOSINTE BRANCHED1 (TB1) and Arabidopsis BRANCHED 1 (BRC1) was down-regulated, implying that D53 is involved in SL biosynthesis or signaling (Zhou et al., 2013). Likewise, the accumulation of SLs in the roots of d53 mutant was significantly higher than compared to WT (Zhou et al., 2013). The increase in number of nodules in GmD53a-KD describes that SLs level might be higher in roots of GmD53a-KD compared to GUS control.

In particular, the d53 mutant plants had more number of tillers than the wild type (Zhou et al., 2013). The expression level in transgenics was linked with severity of tillering phenotype. In the same way, over-expression of D53 gene resulted in a slight increase in tillering compared to control plants (Zhou et al., 2013). These findings strongly suggest that D53 protein functions as a suppressor of the SL-mediated branching inhibition pathway, and the more tillering phenotype of d53 mutant was generated by a gain of function mutation in d53. RNA interference (RNAi) was used to develop D53 knockdown transgenic plants; as expected, the number of tillers was reduced (Zhou et al., 2013). These findings support the hypothesis that d53 mutation increases the D53 activity in repressing SL signaling (Zhou et al., 2013). Notably, a decreased expression of D53 significantly reduced the tiller number of d3 and d14 mutant lines, respectively, indicating that the reduction of D53 expression could partially rescue the signaling defects of d3 and d14 and the result is consistent with its negative role in SL signaling (Jiang et al., 2013). It also validated the increased nodule number in GmD53a-KD lines compared to GUS control.

Downstream factors such as nodulin genes, and ENOD40 are connected to NFR1α genes which are involved in Nod factor perception and involved in Nod factor signal transductions, namely, DMI2α and DMI3β, NINα, and NSP2β (Oldroyd, Engstrom & Long, 2001; Oldroyd, 2013). Our findings showed that GmD53a knockdown affects the nodulation signaling pathway.

MtD27 expression in nodulation is influenced by several symbiotic signaling pathways, including MtDMI1, MtDMI2, and MtDMI3/MtCCaMK, in addition to NSP1 and NSP2 (van Zeijl et al., 2015). Nodulation, which is dependent on SL signaling, was disrupted
in the rms1/CCD8 pea SLs-deficient mutant (Foo & Davies, 2011). In Lotus japonicas LjCCD7-silenced plants, nodulation abnormalities were also found as compared to control plants (Liu et al., 2013; Liu et al., 2011). GmD53a-KD may affect nodulation in soybean by influencing nodule initiation genes (Fig. 5).

CONCLUSION

In this study, the soybean genome was exploited to find closely related homologs of SMXL6, 7, 8 from Arabidopsis, and D53 from rice to further figure out whether and how soybean SL signaling biosynthesis is involved in controlling the various growth and developmental phases in the soybean plant. The results showed that GmD53a is involved in the nodulation of soybean. This study not only demonstrated that SL signaling is conserved across the reported crop plants but also revealed that how GmD53a controls the developmental phases and legume-rhizobia interaction. However, further investigating the roles of these signal components may provide potential targets for directional improvement of crop traits, and further research that focuses on these aspects is highly desired.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding

This work was supported by Taif University Researchers Supporting Project number (TURSP-2020/123), Taif University, Taif, Saudi Arabia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures

The following grant information was disclosed by the authors:
Taif University Researchers Supporting Project number (TURSP-2020/123), Taif University, Taif, Saudi Arabia.

Competing Interests

The authors declare there are no competing interests.

Author Contributions

• Naveed Rehman conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.
• Fahim Ullah Khan conceived and designed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
• Muhammad Imran performed the experiments, prepared figures and/or tables, and approved the final draft.
• Shahid Ali Rajput conceived and designed the experiments, performed the experiments, authored or reviewed drafts of the paper, and approved the final draft.
• Yiming Li conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, and approved the final draft.
• Ihteram Ullah conceived and designed the experiments, performed the experiments, analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
• Rana Waseem Akhtar, Ahmad El Askary and Amany Salah Khalifa analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.
• Muhammad Imran analyzed the data, prepared figures and/or tables, and approved the final draft.
• Arwa Abdulkreem Al-Huqail and Muhammad Tehseen Azhar analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability
The following information was supplied regarding data availability:
The raw data is available in the Supplemental File.

Supplemental Information
Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.12815#supplemental-information.

REFERENCES

Abe S, Sado A, Tanaka K, Kisugi T, Asami K, Ota S, Kim HI, Yoneyama K, Xie X, Ohnishi T. 2014. Carlactone is converted to carlactonoic acid by MAX1 in Arabidopsis and its methyl ester can directly interact with AtD14 in vitro. Proceedings of the National Academy of Sciences of the United States of America 111:18084–18089 DOI 10.1073/pnas.1410801111.

Ahmad MZ, Rehman NU, Yu S, Zhou Y, Bu Haq, Wang J, Li P, Zeng Z, Zhao J. 2020. GmMAX2–D14 and–KAI interaction-mediated SL and KAR signaling play essential roles in soybean root nodulation. The Plant Journal 101:334–351 DOI 10.1111/tpj.14545.

Akiyama K, Matsuzaki K-i, Hayashi H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. Nature 435:824–827 DOI 10.1038/nature03608.

Alder A, Jamil M, Marzorati M, Bruno M, Vermathen M, Bigler P, Ghisla S, Bouwmeester H, Beyer P, Al-Babili S. 2012. The path from β-carotene to carlactone, a strigolactone-like plant hormone. Science 335:1348–1351 DOI 10.1126/science.1218094.

Arite T, Iwata H, Ohshima K, Maekawa M, Nakajima M, Kojima M, Sakakibara H, KyoZuka J. 2007. DWARF10, an RMS1/MAX4/DAD1 ortholog, controls lateral bud outgrowth in rice. The Plant Journal 51:1019–1029 DOI 10.1111/j.1365-313X.2007.03210.x.

Bennett T, Leyser O. 2014. Strigolactone signalling: standing on the shoulders of DWARFs. Current Opinion in Plant Biology 22:7–13 DOI 10.1016/j.pbi.2014.08.001.
Strigolactone regulates shoot development through a core signalling pathway. *Biology Open* 5:1806–1820 DOI 10.1242/bio.021402.

Beveridge CA, Symons GM, Turnbull CG. 2000. Auxin inhibition of decapitation-induced branching is dependent on graft-transmissible signals regulated by genes Rms1 and Rms2. *Plant Physiology* 123:689–698 DOI 10.1104/pp.123.2.689.

Booker J, Sieberer T, Wright W, Williamson L, Willett B, Stirnberg P, Turnbull C, Srinivasan M, Goddard P, Leyser O. 2005. MAX1 encodes a cytochrome P450 family member that acts downstream of MAX3/4 to produce a carotenoid-derived branch-inhibiting hormone. *Developmental Cell* 8:443–449 DOI 10.1016/j.devcel.2005.01.009.

Bouwmeester HJ, Matusova R, Zhongkui S, Beale MH. 2003. Secondary metabolite signalling in host–parasitic plant interactions. *Current Opinion in Plant Biology* 6:358–364 DOI 10.1016/S1369-5266(03)00065-7.

Breakspear A, Liu C, Roy S, Stacey N, Rogers C, Trick M, Morieri G, Mysore KS, Wen J, Oldroyd GE. 2014. The root hair infectome of Medicago truncatula uncovers changes in cell cycle genes and reveals a requirement for auxin signaling in rhizobial infection. *The Plant Cell* 26:4680–4701 DOI 10.1105/tpc.114.133496.

Crawford S, Shinohara N, Sieberer T, Williamson L, George G, Hepworth J, Müller D, Domagalska MA, Leyser O. 2010. Strigolactones enhance competition between shoot branches by dampening auxin transport. *Development* 137:2905–2913 DOI 10.1242/dev.051987.

De Saint Germain A, Clavé G, Badet-Denisot M-A, Pillot J-P, Cornu D, Le Caer J-P, Burger M, Pelissier F, Retailleau P, Turnbull C. 2016. An histidine covalent receptor and butenolide complex mediates strigolactone perception. *Nature Chemical Biology* 12:787–794 DOI 10.1038/nchembio.2147.

Foo E. 2013. Auxin influences strigolactones in pea mycorrhizal symbiosis. *Journal of Plant Physiology* 170:523–528 DOI 10.1016/j.jplph.2012.11.002.

Foo E, Davies NW. 2011. Strigolactones promote nodulation in pea. *Planta* 234:1073–1081 DOI 10.1007/s00425-011-1516-7.

Foo E, Yoneyama K, Hugill CJ, Quittenden LJ, Reid JB. 2013. Strigolactones and the regulation of pea symbioses in response to nitrate and phosphate deficiency. *Molecular Plant* 6:76–87 DOI 10.1093/mp/sss115.

Gomez-Roldan V, Fermas S, Brewer PB, Puech-Pagès V, Dun EA, Pilott J-P, Letisse F, Matusova R, Danoun S, Portais J-C. 2008. Strigolactone inhibition of shoot branching. *Nature* 455:189–194 DOI 10.1038/nature07271.

Hamiaux C, Drummond RS, Janssen BJ, Ledger SE, Cooney JM, Newcomb RD, Snowden KC. 2012. DAD2 is an α/β hydrolase likely to be involved in the perception of the plant branching hormone, strigolactone. *Current Biology* 22:2032–2036 DOI 10.1016/j.cub.2012.08.007.

Jiang L, Liu X, Xiong G, Liu H, Chen F, Wang L, Meng X, Liu G, Yu H, Yuan Y. 2013. DWARF 53 acts as a repressor of strigolactone signalling in rice. *Nature* 504:401–405 DOI 10.1038/nature12870.
Kibido T, Kunert K, Makgopa M, Greve M, Vorster J. 2020. Improvement of rhizobium-soybean symbiosis and nitrogen fixation under drought. *Food and Energy Security* 9:e177.

Kohlen W, Charnikhova T, Liu Q, Bours R, Domagalska MA, Beguerie S, Verstappen F, Leyser O, Bouwmeester H, Ruyter-Spira C. 2011. Strigolactones are transported through the xylem and play a key role in shoot architectural response to phosphate deficiency in nonarbuscular mycorrhizal host *Arabidopsis*. *Plant Physiology* 155:974–987 DOI 10.1104/pp.110.164640.

Kretzschmar T, Kohlen W, Sasse J, Borghi L, Schlegel M, Bachelier JB, Reinhardt D, Bours R, Bouwmeester HJ, Martinoia E. 2012. A petunia ABC protein controls strigolactone-dependent symbiotic signalling and branching. *Nature* 483:341–344 DOI 10.1038/nature10873.

Lin H, Wang R, Qian Q, Yan M, Meng X, Fu Z, Yan C, Jiang B, Su Z, Li J. 2009. DWARF27, an iron-containing protein required for the biosynthesis of strigolactones, regulates rice tiller bud outgrowth. *The Plant Cell* 21:1512–1525 DOI 10.1105/tpc.109.065987.

Liu W, Kohlen W, Lillo A, Opden Camp R, Ivanov S, Hartog M, Limpens E, Jamil M, Smaczniak C, Kaufmann K. 2011. Strigolactone biosynthesis in *Medicago truncatula* and rice requires the symbiotic GRAS-type transcription factors NSP1 and NSP2. *The Plant Cell* 23:3853–3865 DOI 10.1105/tpc.111.089771.

Liu J, Novero M, Charnikhova T, Ferrandino A, Schubert A, Ruyter-Spira C, Bonfante P, Lovisolo C, Bouwmeester HJ, Cardinale F. 2013. Carotenoid cleavage dioxygenase 7 modulates plant growth, reproduction, senescence, and determinate nodulation in the model legume *Lotus japonicus*. *Journal of Experimental Botany* 64:1967–1981 DOI 10.1093/jxb/ert056.

Lv A-L, Li X-J, Li C-J, Liu H-B, Zi Q-Y, Lin X-Q, Liu X-L. 2019. Cloning and expression analysis of the ScD53 gene from sugarcane. *Sugar Tech* 21:898–908 DOI 10.1007/s12355-019-00730-z.

Matusova R, Rani K, Verstappen FW, Franssen MC, Beale MH, Bouwmeester HJ. 2005. The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobanche* spp. are derived from the carotenoid pathway. *Plant Physiology* 139:920–934 DOI 10.1104/pp.105.061382.

McAdam EL, Hugill C, Fort S, Samain E, Cottaz S, Davies NW, Reid JB, Foo E. 2017. Determining the site of action of strigolactones during nodulation. *Plant Physiology* 175:529–542 DOI 10.1104/pp.17.00741.

Oldroyd GED. 2013. Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. *Nature Reviews Microbiology* 11:252–263 DOI 10.1038/nrmicro2990.

Oldroyd GE, Engstrom EM, Long SR. 2001. Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. *The Plant Cell* 13:1835–1849 DOI 10.1105/TPC.010193.
Qu L, Huang Y, Zhu C, Zeng H, Shen C, Liu C, Zhao Y, Pi E. 2016. Rhizobia-inoculation enhances the soybean’s tolerance to salt stress. Plant and Soil 400:209–222 DOI 10.1007/s11104-015-2728-6.

Rehman Nur, Ali M, Ahmad MZ, Liang G, Zhao J. 2018. Strigolactones promote rhizobia interaction and increase nodulation in soybean (Glycine max). Microbial Pathogenesis 114:420–430 DOI 10.1016/j.micpath.2017.11.049.

Schwartz SH, Qin X, Loewen MC. 2004. The biochemical characterization of two carotenoid cleavage enzymes from Arabidopsis indicates that a carotenoid-derived compound inhibits lateral branching. Journal of Biological Chemistry 279:46940–46945 DOI 10.1074/jbc.M409004200.

Simons JL, Napoli CA, Janssen BJ, Plummer KM, Snowden KC. 2007. Analysis of the DECREASED APICAL DOMINANCE genes of petunia in the control of axillary branching. Plant Physiology 143:697–706 DOI 10.1104/pp.106.087957.

Soto MJ, Fernández-Aparicio M, Castellanos-Morales V, García-Garrido JM, Ocampo JA, Delgado MJ, Vierheilig H. 2010. First indications for the involvement of strigolactones on nodule formation in alfalfa (Medicago sativa). Soil Biology and Biochemistry 42:383–385 DOI 10.1016/j.soilbio.2009.11.007.

Stanga JP, Smith SM, Briggs WR, Nelson DC. 2013. Suppressor of more axillary growth2 controls seed germination and seedling development in Arabidopsis. Plant Physiology 163:318–330 DOI 10.1104/pp.113.221259.

Stirnberg P, Furner IJ, Ottoline Leyser H. 2007. MAX2 participates in an SCF complex which acts locally at the node to suppress shoot branching. The Plant Journal 50:80–94 DOI 10.1111/j.1365-313X.2007.03032.x.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. Molecular Biology and Evolution 30:2725–2729 DOI 10.1093/molbev/mst197.

Umehara M, Hanada A, Yoshida S, Akiyama K, Arite T, Takeda–Kamiya N, Magome H, Kamiya Y, Shirasu K, Yoneyama K. 2008. Inhibition of shoot branching by new terpenoid plant hormones. Nature 455:195–200 DOI 10.1038/nature07272.

Van Zeijl A, Liu W, Xiao TT, Kohlen W, Yang W-C, Bisseling T, Geurts R. 2015. The strigolactone biosynthesis gene DWARF27 is co-opted in rhizobium symbiosis. BMC Plant Biology 15:1–15 DOI 10.1186/s12870-014-0410-4.

Waldie T, McCulloch H, Leyser O. 2014. Strigolactones and the control of plant development: lessons from shoot branching. The Plant Journal 79:607–622 DOI 10.1111/tpj.12488.

Xie X, Yoneyama K, Yoneyama K. 2010. The strigolactone story. Annual Review of Phytopathology 48:93–117 DOI 10.1146/annurev-phyto-073009-114453.

Yao R, Ming Z, Yan L, Li S, Wang F, Ma S, Yu C, Yang M, Chen L, Chen L. 2016. DWARF14 is a non-canonical hormone receptor for strigolactone. Nature 536:469–473 DOI 10.1038/nature19073.

Yoneyama K, Xie X, Sekimoto H, Takeuchi Y, Ogasawara S, Akiyama K, Hayashi H, Yoneyama K. 2008. Strigolactones, host recognition signals for root parasitic plants and arbuscular mycorrhizal fungi, from Fabaceae plants. New Phytologist 179:484–494 DOI 10.1111/j.1469-8137.2008.02462.x.
Zhao J, Wang T, Wang M, Liu Y, Yuan S, Gao Y, Yin L, Sun W, Peng L, Zhang W. 2014. DWARF3 participates in an SCF complex and associates with DWARF14 to suppress rice shoot branching. *Plant and Cell Physiology* **55**:1096–1109 DOI 10.1093/pcp/pcu045.

Zhou F, Lin Q, Zhu L, Ren Y, Zhou K, Shabek N, Wu F, Mao H, Dong W, Gan L. 2013. D14–SCF D3-dependent degradation of D53 regulates strigolactone signalling. *Nature* **504**:406–410 DOI 10.1038/nature12878.