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Research article

Overexpression of the *Galega orientalis* gibberellin receptor improves biomass production in transgenic tobacco

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ABSTRACT

Gibberellins (GAs) are well-known phytohormones that contribute to a wide range of plant growth and development functions including stem elongation and leaf expansion. GA receptors perceive GA and transmit signals to activate GA-regulated reactions. In this study, a GA receptor gene with homology to other leguminous plants was isolated from *Galega orientalis* and termed *GoGID*. The 1732-bp full-length *GoGID* gene included an open reading frame of 1035 bp encoding a peptide of 344 amino acids. Sequence analysis indicated that GoGID shares conserved HGGS motif and active amino acid sites (Ser-Asp-Val/Ile) that are essential for maintaining it GA-binding activity. *GoGID* mRNA expression was more abundant in leaves than in roots or stems and could be up-regulated by the exogenous hormones. Overexpression of *GoGID* in transgenic tobacco plants promoted plant elongation and improved biomass production. These results suggested that *GoGID* functions as a GA receptor to alter GA-mediated signaling. *GoGID* may have a role in genetic engineering for the improvement of forage crops.

1. Introduction

The demand for more plant-derived products has been increasing spectacularly because of the continuously growing human population and the increased consumption of petroleum fuel alternatives [1,2]. Since the Green Revolution of the 1960s, which contributed to increase food production [2], genetic engineering has assumed an important role in improving the quantity and quality of biomass [3,4]. Plant hormones, especially brassinosteroids, auxins, gibberellins (GA), and cytokinins, regulate plant growth processes and play pivotal roles in biomass production [2]. GA is a well-known phytohormone that influences a wide range of plant growth and developmental processes that are crucial for biomass yield, such as stem elongation and leaf expansion [5,6]. Genes involved in GA signaling have been isolated and extensively characterized in rice and *Arabidopsis* [7,8].

GA receptor protein(s) are the primary factors mediating GA perception in mono- and dicotyledonous plants [9]. The first GA receptor of the GID1 (GIBBERELLIN INSENSITIVE DWARF1) gene was identified in the rice, *Oryza sativa* (*OsGID1*) [10]. In *Arabidopsis*, the GA receptors *AtGID1a*, *AtGID1b*, and *AtGID1c*, were identified [11]. GA receptors subsequently were described in cotton [12], barley [13], and aspen [14]. The GA signal is perceived by the GA receptor, then the GA-GID1 complex interacts with DELLA proteins that negatively regulate GA signaling and restrict plant growth presumably by transcriptional re-programming [15–18]. The E3 ubiquitin ligase complex ($SCF^{SLY1/GID2}$) recognizes the GA-GID1–DELLA complex, and GA-GID1–DELLA subsequently is degraded by the 26S proteasome. This process enables the de-repression and transcription of GA-responsive genes, thereby regulating plant growth [5,6,19].

Overproducing OsGID1 in transgenic rice was associated with longer light-green leaves compared with control plants; this is considered the GA-overdose phenotype [10]. Overexpression of *PttGIDs* enhanced GA signaling in *Arabidopsis thaliana* and aspen [14]. Transgenic *Arabidopsis* plants have taller stems and larger rosettes. The transgenic hybrid aspen grew faster than the wild-type and had longer internodes, leaves, and petioles [14]. GID1 also might play a critical role in mesocotyl elongation in maize [20]. These findings support that GID plays an important role in plant architecture via the GA signal. In *Arabidopsis*, three members of the GID family have been described [9,11,21]. Although many functional redundancies among GIDs are not unusual, each GID has a distinct role during plant development in *Arabidopsis*. AtGID1b is necessary

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for normal stem growth, and AtGID1c affects the heights of stamens [21]. Regarding seed germination, the three *GID1* genes of *Arabidopsis* show different levels of importance. The strong germination inhibition of the *gid1agid1c* mutant compared with the delayed germination of the *gid1c* mutant indicated that AtGID1c was more important than AtGID1a, whereas AtGID1b appeared to play a minor role [22].

Galega orientalis is a perennial nitrogen-fixing forage legume with applicability to forage production and soil improvement because of its strong root system and effective spread via underground stolons [23,24]. In recent years, this forage legume has been introduced widely into northwest China. In the present study, we describe the identification of the GA receptor gene, *GoGID*, from *G. orientalis*. Our findings demonstrate that *GoGID* responds to hormones, promotes plant elongation, and improves biomass production when ectopically expressed in tobacco.

2. Results

2.1. Cloning and sequence analysis of the GoGID gene from G. orientalis

Based on a 707-bp EST, a 354-bp fragment was isolated using 3′ RACE, and a 1604 bp fragment was obtained using 5′ RACE. By combining the three fragments, the full-length cDNA of 1732 bp was obtained. This species contained a 1035-bp ORF encoding a protein of 344 amino acids. The molecular mass of the putative protein was approximately 39.08 kDa, and the pI was approximately 6.34. A blastx query reported a high identity between this putative protein and other GID family genes. GIDs show sequence similarity to a family of HSLs (hormone-sensitive lipases). However, GIDs have special structural motifs and amino acid sequences that differ from those in HSLs [10,25]. A multiple alignment indicated that GoGID contained the HGGS motif, which is conserved among GID family genes. In addition, the His active site in the catalytic triad of HSLs (Ser-Asp-His) was substituted to Val in OsGID1 or to Ile in AtGIDs (Ser-Asp-Val/Ile), this difference was conserved in GoGID (Fig. 1). Because the conserved amino acids and motifs were present at the expected positions in the deduced GoGID sequence, the identified gene was tentatively named *GoGID* (Genbank accession no. HM989010). A phylogenetic tree was rooted using other GIDs and was divided into two clades as reported previously [22]: one belonging to monocotyledonous species and the other belonging to dicotyledonous species (Fig. 2). GoGID and MtGID-b were closest to each other, and both were categorized into the eudicot GID1b subfamily. The GoGID sequence showed 92% identity to MtGID-b, 89%% to GmGID1B, 83% to PtGID, 74% to AtGID1b, and 60% to OsGID1.

2.2. Expression pattern and subcellular localization of GoGID

Semi-quantitative RT-PCR was performed to analyze the expression pattern of GoGID in different organs or under different hormone treatments. GoGID transcripts were abundant in leaves compared with roots and stems (Fig. 3**A**). Compared to controls, the expression levels of GoGID in leaves increased substantially after GA3 and IAA treatments, and significant increases were observed after 4 h (Fig. 3**B**, **C**). These results strongly suggested that GoGID may be involved in the GA and IAA signaling pathways.

To examine the subcellular localization of GoGID, a GoGID—GFP fusion was constructed. The recombinant plasmid was transformed into onion epidermal cells using a gene gun. In cells expressing pCAMBIA1302-GoGID fusion proteins, fluorescence was detected primarily in the nucleus and plasma membrane with a faint cytosolic signal (Fig. 3**D**, **E**, **F**).

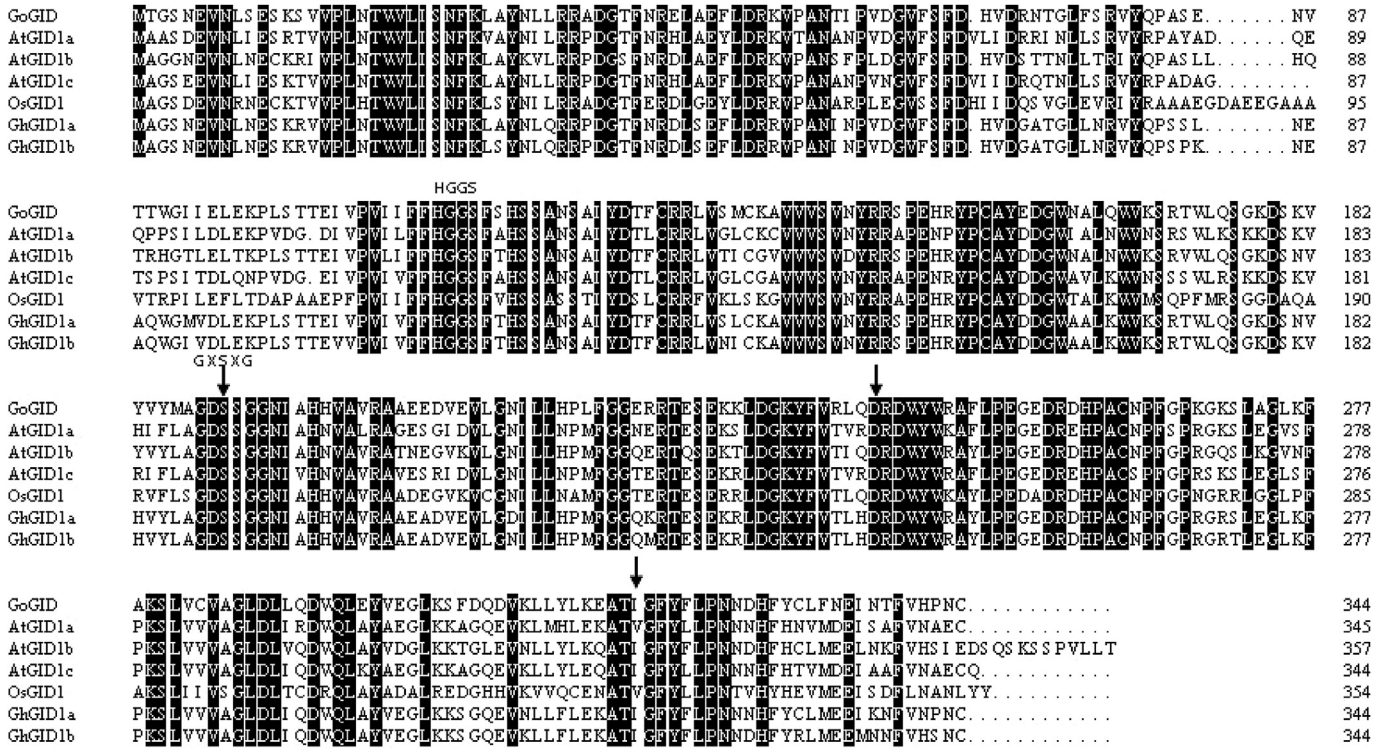

Fig. 1. The amino acid sequences of GoGID, OsGID1, AtGID1s, and GhGID1s were aligned using DNAMAN v.6.0 with default parameters, followed by manual alignment. The following sequences were used: GoGID (ADO22685), AtGID1a (Q9MAA7), AtGID1b (Q9LYC1), AtGID1c (Q940G6), OsGID1 (Q6L545), GhGID1a (ABG89394), and GhGID1b (ABQ96123). Dark shading represents 100% conserved regions. The HGGS and GXSXG motifs were conserved in GIDs. The three amino acid residues essential for maintaining GA-binding activity (arrows) also were conserved in GoGID. Numbers indicate the position from the start codon.

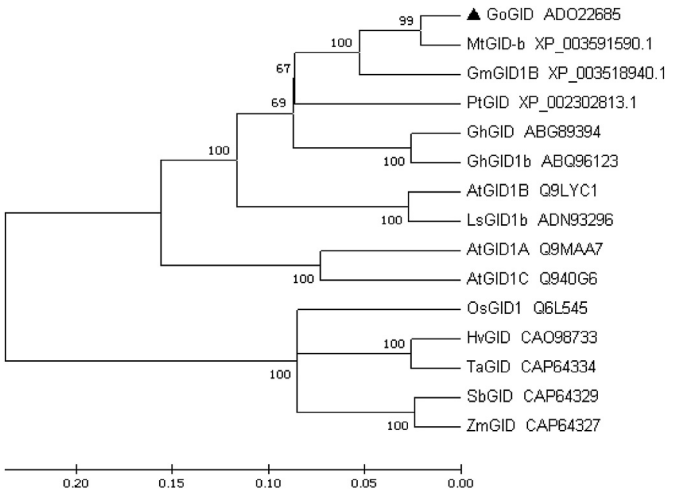

Fig. 2. Phylogenetic tree analysis of GoGID and several other GID proteins. Fifteen-residue sequences from the following species were aligned into a phylogenetic tree using the neighbor-joining method: *G. orientalis* (GoGID), *Medicago truncatula* (MtGID-b), *Glycine max* (GmGID1B), *Populus trichocarpa* (PtGID), *Gossypium hirsutum* (GhGID, GhGID1b), *A. thaliana* (AtGID1A, AtGID1B, AtGID1C), *Lepidium sativum* (LsGID), *O. sativa* subsp. *japonica* (OsGID1), *Hordeum vulgare* (HvGID), *Triticum aestivum* (TaGID), *Sorghum bicolor* (SbGID), and *Zea mays* (ZmGID). The Genbank accession numbers used in phylogenetic tree analysis are indicated. The reliability of the tree was measured by bootstrap analysis.

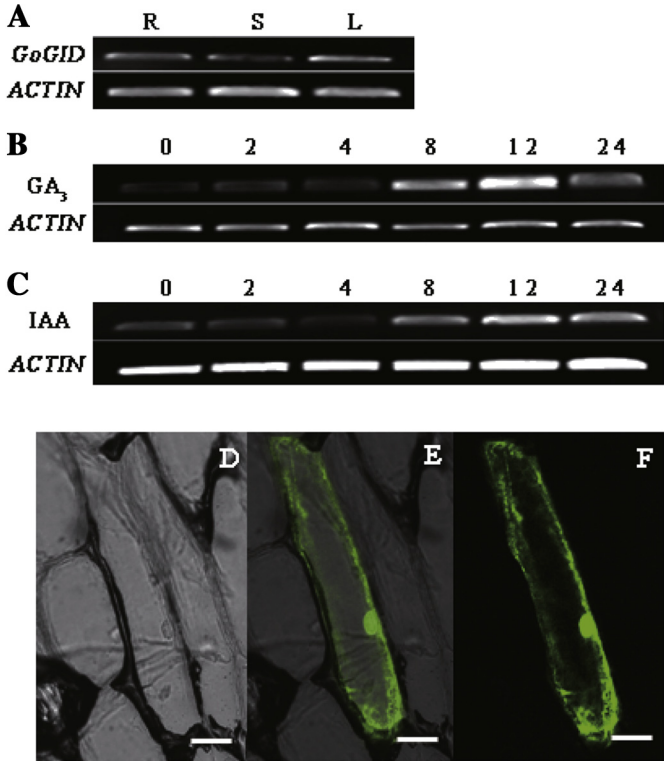

Fig. 3. (**A**) Expression patterns of *GoGID* in various organs under normal growth conditions. (**B**, **C**) Expression patterns of *GoGID* under GA_3 and IAA treatments, respectively. R: root, S: stem, L: leaf. The *GoACTIN* gene was used as a loading control. (**D**) Subcellular localization of GoGID in onion epidermal cells. The *G. orientalis* pCAMBIA1302-GoGID fusion plasmid was transformed into onion epidermal cells using a gene gun, and fluorescence signals were examined using confocal laser scanning microscopy. The photographs were taken in bright light vision. (**E**) Bright and dark vision. (**F**) Dark-field vision. Bar represents 100 μm.

2.3. Expression of GoGID in transgenic tobacco

To investigate *GoGID* function *in vivo*, a pBI121-*GoGID* vector was constructed and introduced into tobacco plants by *Agrobacterium tumefaciens*-mediated transformation. Eight T_0-independent lines were obtained by kanamycin-resistance selection and were confirmed by PCR analysis. RT-PCR was used to identify transgenic lines, and eight transgenic plants were confirmed to express the *GoGID* gene, whereas the wild-type and empty-vector controls did not (Fig. 4**A**). Three transgenic lines were chosen randomly, seeds were harvested after maturation, and the progenies were used for further analysis. To investigate whether over-expression of the *GoGID* gene in tobacco alters plant phenotypes, the height, fresh weight, dry weight, and seed weight were measured in three transgenic lines and in the non-transgenic lines.

No differences were observed between empty-vector control (CTRL) and wild-type plants regarding these four parameters. The selected transgenic lines presented similar phenotypic traits, with increased heights, larger leaves, and improved vigor when grown in a greenhouse, compared with wild-type plants (Fig. 4**B**). The heights of transgenic lines 1, 2, and 3 were 22.4%, 30.0%, and 42.4% higher ($P < 0.05$), respectively, than that of the wild-type plant (Fig. 5**A**). In accordance with the effects on plant growth, biomass production also increased in transgenic plants. The fresh weights from transgenic lines 1, 2, and 3 were 15%, 52%, and 94% higher than the non-transgenic plants respectively. The transgenic lines exhibited 1.4- to 2.1-fold increases in dry weight compared with controls (Fig. 5**B**, **C**). In contrast, the transgenic lines exhibited a reduction of 22−27% in seed weight (Fig. 5**D**).

3. Discussion

Food security remains a major challenge worldwide due to continuing population growth [26]. Forage crops are critical to the livestock industry and to sustainable agriculture worldwide. In addition, some forage species are highly productive and have the potential to be used as bioenergy crops [27]. Dry matter biomass yield is a major objective for forage crops and bioenergy crops [28,29]. Biomass yield is a highly complex trait, and various approaches have been applied to improve this trait. In the past 10 years, several genes influencing biomass have been identified, but the associated molecular mechanism remains poorly understood, especially in non-model plants [2,30]. GA plays an important role in plant development. *GID1*-over-expressing aspen have been shown to grow faster than wild-type [14]. However, evidence that *GID* genes mediate plant development is limited.

In the present study, the full-length *GoGID* cDNA was isolated from *G. orientalis*, with 74% identity to the *Arabidopsis AtGID1b* gene. Sequence analysis indicated that the deduced GoGID protein contained the HGGS motif and three conserved amino acids (Ser-Asp-Val/Ile); these characteristics are conserved among plant GID1s and are important for GA binding [25]. Phylogenetic analysis suggested that *GoGID* is closest in homology to GIDs from leguminous plants and belongs to the GID1b subfamily. These results supported that *GoGID* is a novel GID gene. Additionally, we observed that *GoGID* has a higher transcript abundance in leaves than in roots and stems and was significantly induced by GA hormone.

As one of the most important phytohormones, the effects of GA often interact with other plant hormones. Auxin is well-known to be involved in virtually every aspect of plant growth and development [31]. Crosstalk between GA and auxin have been reported previously in several physiological studies [32,33]. Auxin can induce the transcription of various GA biosynthesis genes in a range of plant species [34−36], particularly GA 20-oxidases, GA 3-oxidases, and GA 2-oxidases. Previous research suggests that

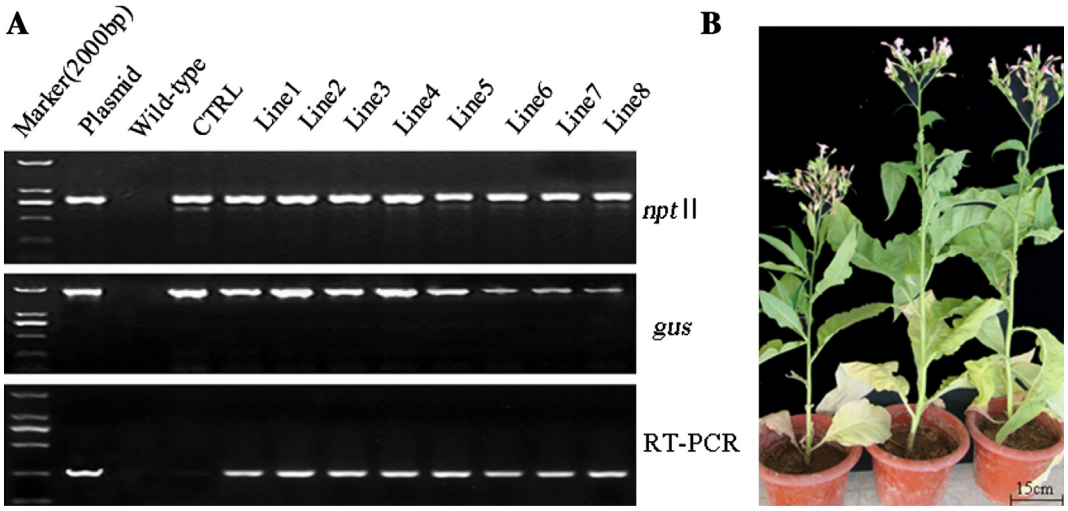

Fig. 4. (**A**) Molecular characterization of transgenic lines. PCR analysis of regenerated tobacco plants, the positive control (pBI121-*GoGID* plasmid), and the negative control (wild-type). The sizes of the DNA fragments were 795 bp for *NPTII* (upper) and 1812 bp for *GUS* (middle). The RT-PCR fragment size was 240 bp (lower). (**B**) Gross morphology of transgenic lines and control plants. Scale bar, 15 cm.

auxin promotes *Arabidopsis* growth by modulating the GA response [37]. To interpret the transcriptional regulation of the GA receptor by auxin, we examined the abundance of *GoGID* under IAA treatment. The expression level of *GoGID* was increased substantially in the presence of IAA. Therefore, the regulation of IAA for plant growth by modulating the GA response may be initiated at a much earlier stage (*e.g.*, the GA signaling reception stage) of the GA signaling pathway.

The function of *GIDs* has been investigated in only a few plants, particularly model plants and some important crops [9,10,12,14]. Overexpression of *GID* resulted in a GA-hypersensitive phenotype in rice [10]. In transgenic rice or *Arabidopsis* studies, biomass characteristics were often unevaluated because of a lack of practical value, so the relationship between GIDs and biomass remained obscure. For forage or bioenergy crops, however, biomass is the primary trait for plant utility. Consistent with previous studies

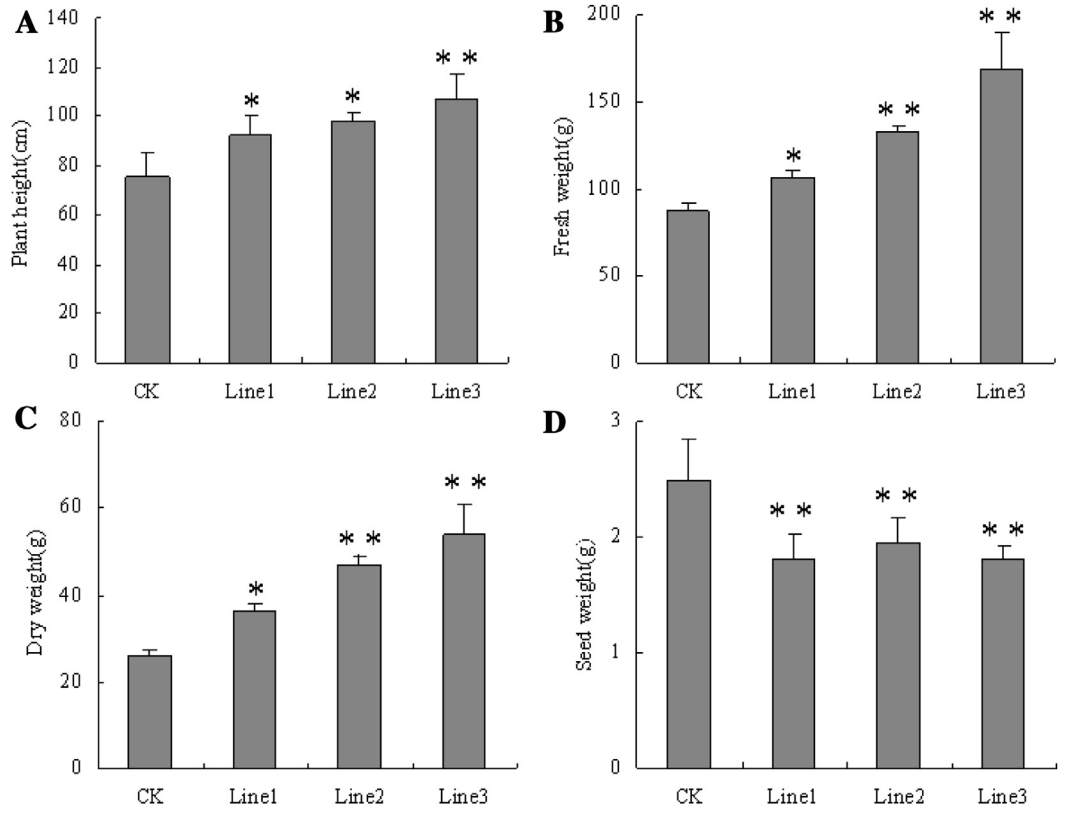

Fig. 5. (**A**) Plant heights, (**B**, **C**) biomass yields, and (**D**) seed weights of transgenic tobacco plants. The transgenic and control plants were harvested after 5-months growth in the greenhouse. Values are means ± SE ($n = 3$). One or two asterisks indicate significance corresponding to $P < 0.05$ (*) or 0.01 (**) (one-way ANOVA, Dunnett's test).

[10,14], our *GoGID* transgenic lines were taller than wild-type plants. The seed weights of transgenic lines were decreased compared with controls, which also has been reported in rice overexpressing *OsGID* [10]. The phenotypes of taller height and poorer fertility in the transgenic lines were consistent with a GA-overdose phenotype [10], suggesting that *GoGID* likely altered GA signaling as a GA receptor. The transgenic plants exhibited 1.4- to 2.1-fold increases in dry biomass yield, 0.2- to 0.4-fold increases in plant height, and larger biomasses than controls, perhaps because the endogenous GID levels were rate-limiting for GA reception at some stage, and ectopic over-expression of *GoGID* increased the GA pathway flux.

In conclusion, a GA receptor-encoding gene was isolated from *G. orientalis* and could be induced by IAA and GA_3. Ectopic expression of *GoGID* in tobacco improved biomass yields, indicating that this gene alters plant growth and morphology and contributes to the regulation of the GA signaling pathway. This study also illustrated the efficacy of over-expressing a single gene to increase biomass, which is applicable to breeding programs to generate new germplasm in forage and bioenergy crops.

4. Materials and methods

4.1. Plant materials and growth conditions

Surface-sterilized seeds of *G. orientalis* were germinated in petri dishes at 24 °C under 12 h light/12 h dark conditions. After 2–3 d, seedlings were transplanted into pots with 3:1 (v/v) vermiculite:perlite and grown in a growth chamber at 28 °C under a 16 h light/8 h dark photoperiod cycle. Five-week old plants were used in experiments.

4.2. Isolation of full-length GoGID gene

Total RNA was isolated from leaf samples using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Based on a known expressed sequence tag (EST), rapid amplification of cDNA ends (RACE) was performed to amplify the uncharacterized 3′- and 5′-cDNA ends of *GoGID* according to the manufacturer's protocol (Clontech, USA). Primers GSP1 (5′-AGGTTGGGCTATGCTCAAAGTTCC-3′) and GSP2 (5′-GGACAGTGCTACTTCGTGTTCTATGCG-3′) were used in a polymerase chain reaction (PCR) to amplify the 5′ and 3′ cDNA ends, respectively. Generation of the first-strand cDNA and amplification of 3′ and 5′ cDNA ends were performed using a Smart RACE cDNA amplification kit (Clontech, USA). The amplified PCR fragment then was cloned into a pMD18-T vector and sequenced.

4.3. Sequence and structural analyses

Sequence homology analysis was performed using BLAST tools (http://blast.ncbi.nlm.nih.gov/). Multiple alignment of the deduced amino acid sequences was prepared using DNAMAN v.6.0 software. A search for open reading frames (ORF) and translation of the nucleotide sequences were performed using the ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). A phylogenetic tree was constructed using the neighbor-joining method with MEGA 5.0 software.

4.4. Semi-quantitative RT-PCR analysis of GoGID

Semi-quantitative reverse-transcription (RT)-PCR was performed to analyze the expression patterns of *GoGID* in *G. orientalis* treated with GA_3 or indole-3-acetic acid (IAA). Five-week-old *G. orientalis* seedlings were treated by foliage spraying with 2.5 μmol L^{-1} GA_3 or with 5 μmol L^{-1} IAA. Leaves were treated for 0, 2, 4, 8, 12 or 24 h, and total RNA subsequently was extracted for semi-quantitative PCR. Real-time quantitative RT-PCR was used to characterize *GoGID* using primers GoGID-F (5′-GCAAAGTCCCAGCCAATACAA-3′) and GoGID-R (5′-CGGCAGAAAGTGTCGTAAATAGC-3′). The *G. orientalis ACTIN* gene was amplified using primers GoACT-F (5′-GGACAAGTTATCACCATCGG-3′) and GoACT-R (5′-TCAGCAATACCTGGAAACATAG-3′). RT-PCR products were electrophoresed through 2% agarose gels and were visualized with ethidium bromide under UV light. Each experiment was performed independently at least twice. Gene expression levels were normalized to the density of the housekeeping gene actin.

4.5. Subcellular localization of GoGID protein

To observe the subcellular localization of GoGID, a *GoGID–GFP* fusion plasmid was constructed. The following specific primers were designed that included *Spe*I restriction sites (underlined) and that targeted the coding sequence of *GoGID*: GFP-F (5′-CGGACTAGTATGACTGGAAGTAATGAAGTCAAC-3′) and GFP-R (5′-CGGACTAGTACAGTTAGGGTGCACAAAGGT-3′) were designed to amplify the coding sequence of *GoGID*. The PCR product was cloned into a pMD18-T vector to generate the recombinant plasmid, pMD18-T-*GoGID*. This plasmid was combined with the vector, pCAMBIA1302, and both of two were digested with *Spe*I restriction endonucleases. Digested vector and the *GoGID* gene fragment were ligated using T4 DNA ligase, and the correct *GoGID–GFP* fusion construct was obtained by sequencing. The empty vector control, pCAMBIA1302, and the *GoGID–GFP* fusion construct were transformed into living onion epidermal cells using a Model PDS-1000/He Biolistic Particle Delivery System according to the manufacturer's protocol (Bio-Rad, USA). Cells were incubated for 1 d at 25 °C and then were observed for transient expression of the GoGID–GFP fusion protein using confocal laser scanning microscopy (Olympus FV500, Japan).

4.6. Generation of transgenic tobacco plants

To express *GoGID* in tobacco, a fragment containing the entire coding region of *GoGID* was amplified using the primers GoGID-121-F (5′-GCTCTAGAATGACTGGAAGTAATGAAGTCAACC-3′) and GoGID-121-R (5′-CGGGATCCACAGTTAGGGTGCACAAAG-3′) which contained *Xba*I and *Bam*HI restriction sites (underlined), respectively, at their 5′ ends. The amplicon was digested with *Xba*I and *Bam*HI and was ligated with the co-digested binary vector, pBI121 (Clontech, Japan). The construct was transferred into *A. tumefaciens* strain LBA4404 using the freezing/heat shock method. Tobacco plants were transformed using a previously described *A. tumefaciens* (LBA4404)-mediated method [38]. The explants were selected on MS medium containing 40 μg ml^{-1} kanamycin and 500 μg ml^{-1} carbenicillin. Tobacco cultures were regenerated into plantlets in plastic jars and then were potted in soil when roots had formed.

The transferred gene was detected in regenerated transgenic tobacco plants using PCR. DNA was isolated from transgenic and non-transgenic plants using the CTAB method [39]. The vector sequences, *NPT*II and *GUS*, were examined by PCR. The primers F1 (5′-ATGATTGAACAAGATGGATTGCACGCAG-3′) and R1 (5′-TCAGAAGAACTCGTCAAGAAGGCGA-3′) were designed to amplify the *NPT*II fragment. The primers F2 (5′-ATGTTACGTCCTGTAGAAACCCCAACC-3′) and R2 (5′-TCATTGTTTGCCTCCCTGCTGC-3′) were designed to amplify the *GUS* fragment. Positive lines were verified using RT-PCR with GoGID-F/R primers.

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