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CLONING AND CHARACTERIZATION OF TWO GIBBERELLIN RECEPTOR GENES IN YAM (*DIOSCOREA OPPOSITA*)

WEN-HONG LONG[1], SHENG-SHENG XU[1*], JIN-GUI MENG[1], JUN-XIA YANG[1,2], RONG-PING YANG[1], YAN-BI DUAN[3], XUE-MEI ZHANG[4] AND CHUN-HUA MA[1*]

[1]*College of Horticulture and Landscape, Yunnan Agricultural University，Kunming, 650201, Yunnan, People's Republic of China*
[2]*Longchuan Vocational High School, Mangshi, 678700, Yunnan, People's Republic of China*
[3]*College of Biotechnology and Agronomy, Yunnan Agricultural University，Kunming, 650201, Yunnan, People's Republic of China*
[4]*Experiment Teaching Center for Agriculture Specialty，Yunnan Agricultural University，Kunming, 650201, Yunnan, People's Republic of China*
**Corresponding authors' e-mail: 45334273@qq.com; 563139036@qq.com*

Abstract

GID1 (gibberellin insensitive dwarf1) is a gibberellin receptor and plays an important role in the function of gibberellin. The full-length cDNA sequences of gibberellin receptor genes *DoGID1B* (GenBank No. MK253679) and *DoGID1B2* (GenBank No. MK301538) were cloned by RT-PCR and RACE from yam (*Dioscorea opposita*). The expression of genes *DoGID1B* and *DoGID1B2* in yam bulbils in dormant and sprouting stage and its response to paclobutrazol were studied by RT-qPCR. Amino acid composition, physicochemical properties, hydrophilicity, signal peptide, transmembrane structure, curled spiral composition and domain of the two deduced protein DoGID1B and DoGID1B2 were highly similar to GID1 proteins of *Arabidopsis thaliana* (GenBank No. NM_116166.5) and rice (*Oryza sativa Japonic*a, GenBank No. XM_015784475.2). Both DoGID1B and DoGID1B2 proteins contain binding sites for hormones GA, repressor protein DELLA and hormone-sensitive lipase. Multiple sequence alignment indicated that the deduced DoGID1B and DoGID1B2 proteins shared high sequence identity with GID1s from other species. Phylogenetic analysis showed that DoGID1B and DoGID1B2 proteins were grouped in the clade of monocotyledon GID1, but the two proteins were clustered in different sub-clades. RT-qPCR studies showed that the expression of *DoGID1B* and *DoGID1B2* genes was up-regulated when yam bulbils sprouting, and the up-regulation of *DoGID1B* was higher than that of *DoGID1B2*. In addition, the up-regulation could be shifted earlier by paclobutrazol. These findings help us to reveal the molecular mechanism of gibberellin transduction and to regulate the growth and sprouting of yam.

Key words: *Dioscorea opposita*, Gibberellin receptor, GID1, Gene expression.

Introduction

Gibberellins (GAs) are a kind of biguanide compound widely present in plants. As important plant hormones, they can regulate various physiological processes in plant growth and development, such as stem elongation, leaf extension, flower bud differentiation and dormancy breaking, etc. (Olszewski *et al*., 2002). Recent studies have found that endogenous gibberellin affect fruit bearing and embryo development (Kafkas *et al.*, 2010) and improve the quality cotton fiber (Chen *et al*., 2017). Gibberellins act on plants by a special transduction pathway to produce their effect. This process is implemented in a complex and ingenious signal transduction pathway. GID1 (gibberellin insensitive dwarf1) is a soluble protein that transduces the signal of gibberellins to downstream components. The triple mutant lacking the gibberellin receptor does not respond to gibberellin (Griffiths *et al*., 2006). The GID1 protein is derived from hormone-sensitive lipase (HSL) and retains the HSL family α/β folding and catalytic triplet structure (Ueguchi-Tanaka *et al*., 2010). First, the gibberellin receptor GID1 binds to gibberellin to form a dimer, which then forms a trimer with DELLA. The formation of trimers reduces the inhibitory effect of DELLA protein on plant growth and promotes the binding of DELLA to a specific ubiquitin E3 ligase complex ($SCF^{SLY1/GID2}$), by which DELLA protein is polyubiquitinated and subsequently degraded by 26S protease (Sun, 2010). Therefore, the inhibition of plant growth is relieved and gibberellin effect on plants occurs (Dill *et al.*, 2004; Fu *et al*., 2004; Griffiths *et al*., 2006). In this process, GID1 plays an important role as a key component of signal transduction, which binds to gibberellin, then induces the degradation of DELLA protein, an important negative regulatory element in plant growth and development.

The gibberellin receptor gene *GID1* was firstly isolated from GA-insensitive mutants of rice (*Oryza sativa*). The *GID1* gene encodes an unknown protein with similarity to the hormone-sensitive lipases (Ueguchi-Tanaka *et al*., 2005). Then three gibberellin receptor genes (*AtGID1a*, *AtGID1b* and *AtGID1c*), each an ortholog of the rice GA receptor gene (*OsGID1*), were cloned from *Arabidopsis* (Nakajima *et al*., 2006). The gibberellin receptor genes of various species were subsequently cloned. But the full-length sequence of the *GID1* gene has not been cloned from yam (*Dioscorea opposita*).

Yam belongs to genus of *Dioscorea* in Dioscoreaceae. Its underground tubers are popular vegetables for Chinese, Japanese, etc., and are also used in traditional Chinese medicine system. Yam bulbils are edible, and their value as a reproductive organ is more prominent. Yam is an asexual reproduction plant. When the stem segments are used for propagation, production cost is high, reproductive coefficient is low and promotion of new variants is slow. If the bulbils are used for propagation, these problems can be

solved. However, there is a long period of dormancy after the bulbils are harvested, which limits the timely planting in production. The current consensus is that plant dormancy and germination are associated with endogenous hormones abscisic acid (ABA) and GA; ABA induces dormancy, and gibberellin induces germination (Gubler *et al*., 2005; Kucera *et al*., 2005; Finkelstein *et al*., 2008). Previous studies showed that the endogenous hormone GA_3 content is high during the growth period of bulbils, and it remains high even bulbils becoming dormancy (Long *et al*., 2011a), then declines during dormancy (Long *et al*., 2013). Bulbils are promoted to sprout by paclobutrazol solution (Long *et al*., 2011b). An interesting question unanswered is whether paclobutrazol promotes bulbils sprout by simply reducing GA_3 content or by regulating GA signal transduction pathway. Two *GID1* genes of yam were cloned and characterized. Expression changes of the two genes in bulbils at different stage and their response to paclobutrazol were detected using reverse transcription quantitative real-time PCR (RT-qPCR). Our findings lay the foundation for further study on sprouting mechanism of yam bulbils.

Materials and Methods

Plant material and RNA extraction: The local cultivar 'Niuwei' yam (*Dioscorea opposita*) in Lufeng County, Yunnan Province, southwest of China was used as material. The epidermis of sprouting bulbils was frozen in liquid nitrogen then stored in a refrigerator at -80°C for *GID1* gene cloning. The extraction of RNA was carried out using a plant RNA extraction kit for polysaccharide polyphenol samples (Beijing Huayueyang Biotechnology Co., Ltd.) according to the specific instructions.

Cloning of intermediate fragments of genes: The synthesis of cDNA was performed using PrimeScriptTM II 1st strand cDNA synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Primers (Table 1) were designed with Primer5.0 Software according to sequences of four *GID1* genes (GenBank Nos. BQMI01000019.1, BBQWI01003574.1, BDMK 01000697.1, and BDML01002103.1) in transcriptome information of *Dioscorea rotundata* registered in NCBI. The total volume of the reaction system was 25μL, including 0.5μL of cDNA, 0.5μL of upstream primer, 0.5μL of downstream primer, 0.25μL of LATaq, 2μL of dNTP, 2.5μL of buffer, and 18.75μL of dH_2O. Reaction procedure: pre-denatured at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 50 s, with a final extension at 72°C for 5 min. The PCR products were electrophoretically separated on a 1.2% agarose gel. The target gene fragments in agarose gel were recovered, purified, linked to the vector PMD18-T, and transformed into *Escherichia coli* for culture. After *E. coli* culture, positive colony extraction, enzyme digestion, and double enzyme digestion were performed, and the successfully cloned products were sent to Kunming Shuoqing Biotechnology Co., Ltd. for sequencing.

RACE-PCR and gene assembly: RACE-PCR primers (Table 1) were designed according to linker instructions of 3'-terminus and 5'-terminus and information of intermediate fragment sequences. PCR amplification, purification, transformation, restriction enzyme digestion and sequencing of the cDNA were carried out as described above. The obtained sequences of intermediate, 3'-terminus and 5'-terminus were spliced to full sequences of cDNA with DNAMAN8.0.

Analysis of genes and their deduced protein: Homology alignment analysis of genes was performed using BLAST of the NCBI database. Open reading frames (ORF) of genes were detected and translated into amino acid sequences with DNAMAN8.0. Predictions of physicochemical properties of the deduced amino acid sequences were performed using ProtParam (http://web.expasy.org/protparam/). Signal peptides of the deduced amino acids were predicted with SignalP 4.1(http://www.cbs.dtu.dk/services/SignalP).

The Hydrophobicity analysis was performed using ProtScale (http://web.expasy.org/protscale/). The secondary structures of the deduced amino acids were determined by SOPMA (http://npsa-pbil. ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). The transmembrane domains were predicted by TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The conserved domains were predicted with NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Structural and functional domains were identified by SMART tool (http://smart.embl-heidelberg.de/). Putative amino acids of GID1 genes of yam were compared with those of other species by DNAMAN8.0 tool. For phylogenetic analysis, a maximum-likelihood tree was constructed with default parameters using MEGA7.0, and default parameters.

Table 1. Primers for RT-PCR and RT-qPCR.

Sequence (5'-3')	Function	Sequence (5'-3')	Function
GAGTGAAGAAATCAACGCCA AGGAAGCAAGTAGAAGCCAA	RT-PCR of intermediate fragments of *DoGID1B*	GCTCATCTCCAACTTCAAAC CCACCACAACGATACTTCTC	RT-PCR of intermediate fragments *of DoGID1B2*
GGTGCCGATTTCAGGGTTAC TTGTGTTCAGAGAGAGTGCT GGCTTCTACTTGCTTCCTAA	RT-PCR of 3'-terminus of *DoGID1B*	CCAATGGGAATGATCTCAAA CCGAGAAGTATCGTTGTGGT GGTTCTACTTGCTGCCCAAC	RT-PCR of 3'-terminus of *DoGID1B2*
ATTGGATGGGAAGTAAAGCC TAGGAGTAGAGTTTGCGGGG	RT-PCR of 5'-terminus of *DoGID1B*	ATCTCCACCTTCCGCACCTC AAGGTGGCGGTTGAAGGTG	RT-PCR of 5'-terminus of *DoGID1B2*
GTGCTTACGATGACGGTTGG CGGTGTCCTACATTGCCCTC	RT-qPCR of *DoGID1B*	GGTGCTCATCTCCAACTTCAAAC CAGTATCAGGAAAGGGCGTG	RT-qPCR of *DoGID1B*
GGTGAACTGTATGAGGCGG GCATCCACACAAGCAAAGCA	RT-PCR of *CHC*		

Note: Clathrin Heavy Chain gene (*CHC*) was used as an internal control

Expression of genes in yam bulbils at different periods and their responses to paclobutrazol: At the time of harvest of yam bulbils, 30d after harvest, and sprouting (shoots were near 0.5 cm, in the next year spring), five bulbils were sampled each time, and the epidermis of the bulbils near the sprouting part was cut. The epidermis was frozen in liquid nitrogen and stored in a -80°C refrigerator for further use.

Bulbils being dormant for 60d were immersed in the solution of 5 mg/L, 255 mg/L, 45 mg/L, 65 mg/L or 85 mg/L paclobutrazol (purchased from Beijing Zhenxiang Technology Co., Ltd.) for 24 hours, and water was used as the control. Treated bulbils were washed with water and placed in a moist vermiculite matrix in an incubator to sprout at 22°C. Bulbils were sampled before treatment, 14d, 28d, 42d, and 56d days after treatment. Five bulbils were sampled at each time point for each treatment. The epidermis of the bulbils near the sprouting part was cut, which was then frozen in liquid nitrogen and stored in a -80°C refrigerator for further use.

Clathrin heavy chain (*CHC*) gene, which is relatively stable during sprouting of yam bulbils in previous studies, was used as an internal control for normalization. The primers for RT-qPCR analysis are listed in table 1. The quantitative reaction was performed in a 20μL of ABI step one plus Real-Time PCR System (Applied Biosystems, USA), including 10μL qPCR Master Mix, 0.4 μL each primer, 4μL cDNA and ddH_2O 5.2μL. PCR amplification was performed under the following conditions: 90 s at 95°C, followed by 40 cycles of 95°C for 5 s, 60°C for 15 s, and 70°C for 20 s. Three technical replications were performed for all quantitative PCRs. The relative changes in gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Results

Cloning and analysis of *DoGID1B* and *DoGID1B2*: Using the primers for intermediate fragments, two nucleotide fragments (948bp and 817bp, respectively) were obtained by RT-PCR. Each nucleotide sequence was highly homologous to *GID1* nucleotide of other species according to BlastX alignment in NCBI. Then the two obtained nucleotide sequences were used to design primers for RT-PCR of 5' and 3'-termini. Two nucleotide sequences (311bp and 438bp, respectively) of 5'-terminus and two nucleotide sequences (230bp and 264bp, respectively) of 3'-terminus were obtained by RACE-PCR. All of the four nucleotide sequences were highly homologous to *GID1* nucleotide of other species. The full-length cDNAs of the two genes (1285 bp and 1606 bp, respectively) were assembled with intermidiate sequences by DNAMAN8.0 tool. The similarity of two full-length sequences was only 49.13%, which indicated that these sequences were different. The two nucleotide sequences were most homologous to *Musa acuminata* (XM_009394833) and *Phoenix dactylifera* (XM_017846166), with a similarity of 67.56% and 77.55%, respectively. Both nucleotide sequences were most homologous to *AtGID1B* (NM_116166) (with a similarity of 65.65% and 67.61%, respectively) among the three *GID1* genes *of Arabidopsis thaliana*. Therefore, the two nucleotide sequences were different genes in the *GID1* family. Then they were named *DoGID1B* and *DoGID1B2*, respectively. Both *DoGID1B* and *DoGID1B2* were deposited in the GenBank database (accession number: MK256679 and MK301538). The cDNA sequence of *DoGID1B* (with an ORF of 1023 bp) encoded a protein of 340 amino acids, and the cDNA sequence of *DoGID1B2* (with an ORF of 1065 bp) encoded a protein of 354 amino acids. GID1b was distinct from GID1a and GID1c in both species (*Lepidium sativum*) and (*Arabidopsis thaliana*) in transcript expression patterns (Voegele *et al.*, 2011). The acquisition of full-length sequence of *DoGID1B* and *DoGID1B2*, homologs of *AtGID1B*, will lay the foundation for sudies on some special growth and development.

Analysis of predicted protein sequence: Bioinformatics of proteins DoGID1B and DoGID1B2 was compared with that of OsGID1 (GID1 of *Oryza sativa*, XM_015784475.2) and AtGID1B (GID1 of *Arabidopsis thaliana*, NM_116166.5). Physicochemical properties of proteins were predicted using Protparam online software (Table 2). Molecular weight, theoretic ioelectric point and atomic composition of DoGID1B and DoGID1B2 were similar to those of OsGID1 and AtGID1B. The content of leucine (Leu) was the highest in both DoGID1B and DoGID1B2, which was the same as the composition of AtGID1B. The protein instability indexes of the two yam proteins were 56.63 and 43.33, respectively, indicating that these two yam proteins were unstable, which were similar to OsGID1 and AtGID1B.

Coiled-coil of GID1 proteins was also analyzed using SOPMA online software (Table 2). The DoGID1B protein was similar in structure to the DoGID1B2 protein, with the most random coils (45.88% and 44.35%), followed by alpha-helix (32.35% and 32.77%), extended strands (17.65% and 18.36%), and the least beta-turn (4.12% and 4.52%). The four proteins DoGID1B, DoGID1B2, OsGID1 and AtGID1B were all of the same composition, i.e., random coil > alpha-helix > extended chain > beta-turn.

Transmembrane domains and signal peptides of proteins were predicted by TMHMM and SignalP 4.1 online software, respectively. None of the four proteins had a transmembrane structure or signal peptide (Table 2).

The hydrophilicity of proteins was predicted using Protscale online tool. Both the N-terminus and C-terminus of the DoGID1B protein were hydrophilic, while the DoGID1B2 protein was hydrophobic in both termini. Both termini of DoGID1B protein were identical to those of OsGID1, and both termini of DoGID1B2 protein were identical to those of AtGID1B. Although the DoGID1B2 protein was hydrophobic in both termini, its grand average of hydropathicity was negative. The minimum and maximum of hydropathicities of amino acid residues in four proteins were similar, and the amino acid residues with the extreme hydropathicity were similar in position except OsGID1. Four proteins were all hydrophilic (Table 2).

Table 2. Bioinformatics analysis of GID1 proteins.

	DoGID1B	DoGID1B2	OsGID1	AtGID1B
Molecular weight (kDa)	38.03	39.54	39.46	40.30
Theoretic isoelectric point	7.27	6.71	5.66	6.86
Highest amino acid/content (%)	Leu (L)/11.2	Leu (L)/9.3	Ala (A) / 9.6%	Leu (L) /11.2
Atomic composition	$C_{1724}H_{2617}N_{475}O_{480}S_{11}$	$C_{1779}H_{2730}N_{484}O_{520}S_{10}$	$C_{1756}H_{2691}N_{495}O_{521}S_{12}$	$C_{1819}H_{2794}N_{496}O_{525}S_{9}$
Instability index	56.63	43.33	53.52	45.42
Random coil (%)	45.88	44.35	45.20	50.28
α-helix (%)	32.35	32.77	32.49	27.65
extended chain(%)	17.65	18.36	17.51	18.16
β-turn (%)	4.12	4.52	4.80	3.91
Transmembrane region	non	non	non	non
Signal peptide	non	non	non	non
N-terminus	hydrophilic	hydrophobic	hydrophilic	hydrophobic
C-terminus	hydrophilic	hydrophobic	hydrophilic	hydrophobic
Minimum hydropathicity/position	-3.044/143aa	-2.833/159aa	-2.911/235aa	-3.044/150aa
Maximum hydropathicity/ position	2.467/280aa.	2.511/295aa	2.211/293aa	2.989/139aa
Grand average of hydropathicity	-0.094	-0.236	-0.244	-0.237
Domain/position	Abhydrolase super family/101-316aa	Abhydrolase superfamily/118-331aa	Abhydrolase super family/116-329aa	Abhydrolase super family/109-322aa

Note: OsGID1 is GID1 of *Oryza sativa* (GenBank No. XM_015784475), AtGID1B is GID1B of *Arabidopsis thaliana* (GenBank No.NM_116166.5)

Table 3. Confidently predicted domains.

Protein	Name	Start	End	E-value
DoGID1B	Pfm:COesterase	71	144	0.000036
	Pfm:Abhydrolase_3	101	317	2.9e-50
DoGID1B2	Pfm:COesterase	74	161	3.5e-7
	Pfm:Abhydrolase_3	118	332	6.6e-59
OsGID1	low complexity	83	95	N/A
	Pfm:COesterase	101	217	8.6e-9
	Pfm:Abhydrolase_3	116	330	4.7e-58
AtGID1B	Pfm:COesterase	64	212	9.4e-10
	Pfm:Abhydrolase_3	109	323	1e-10

Note: OsGID1 is GID1 of *Oryza sativa* (GenBank No. XM_015784475), AtGID1B is GID1B of *Arabidopsis thaliana* (GenBank No.NM_116166.5)

The conserved domains were predicted with NCBI online software (Table 2). The results showed that both conserved domains of DoGID1B and DoGID1B2 belonged to abhydrolase family, with conserved interval of 101-316aa and 118-331aa, respectively. Further prediction of protein structure and functional regions using SMART online tools showed there were domains of carboxylesterase and abhydrolase (alpha/beta hydrolase fold) in DoGID1B and DoGID1B2, and carboxylesterase was close to the N-terminus while abhydrolase was close to the C-terminus (Table 3). The overall structure of the Gid1 protein is alpha/beta hydrolase fold (Ueguchi-Tanaka *et al*., 2010), and the overall structure of GID1-GA complexes shows an α/β-hydrolase fold similar to that of HSLs except for an N-terminal lid (Shimada *et al*., 2008). Here we saw the basic structure of alpha/beta hydrolase fold in all GID1 proteins, so we further believed that the two genes from yam encoded the gibberellin receptor GID1.

Analysis of multiple sequence alignment of DoGID1B, DoGID1B2 and GID1 proteins from other species was performed with DNAMAN8.0 software (Fig. 1). It was found the overall identity of 18 proteins was 64.58%. Many binding sites with protein DELLA or hormone GA were present in GID1 protein sequence (Hirano *et al*., 2007). These sites in sequences of DoGID1B and DoGID1B2 were TWVLIS, DR, FFHGGSF, HS, IYD, YRR, DGW, GDSSGGNI, GNI, MY, LDGKYF, DWY and GFY from the N-terminus to the C-terminus. Most of the sites were conserved, except that amino acid V in the site TWVLIS in DoGID1B protein was substituted by I, and amino acid Y in LDGKYF site in DoGID1B was replaced by F. This result indicated that DoGID1B and DoGID1B2 proteins are likely to be involved in GA signal recognition and transduction. The *GID1* gene encodes a protein similar to a hormone-sensitive lipase, and it contains domains HGG and GXSXG relating to hormone sensitive lipase (HSL) (Ueguchi-Tanaka *et al*., 2005; Hirano *et al*., 2008). In DoGID1B and DoGID1B2 proteins, these two domains were also present, and the domain GXSXG was clearly found in all 18 GID1 proteins. Among the three amino acids S, D and H relating to HSL catalysis (Hirano *et al*., 2007), amino acids S and D were conserved while H was replaced by I in DoGID1B and DoGID1B2 proteins. All of the three amino acids, G^{169}, G^{196} and R^{251}, which are related to the function of GID1in spontaneous mutant of rice (Ueguchi-Tanaka *et al*., 2007), were also conserved in DoGID1B and DoGID1B2.

Phylogenetic analysis: To evaluate the phylogenetic relationship between GID1 proteins in yam and other plants, a phylogenetic tree was conducted (Fig. 2). The phylogenetic tree grouped GID1 proteins from Monocotyledoneae into a clade, and GID1 proteins from Dicotyledoneae into another clade. Two GID1 proteins in yam, which is a monocotyledon plant, were grouped into the first clade (clade of Monocotyledoneae). In the first group, *Agapanthus praecox* and *Asparagus officinalis*, which are all plants of Liliaceae, were closest. *Elaeis guineensis* and *Phoenix dactylifera*, both of which belong to the same family of Palmae, were also very close. In the second clade (clade of Dicotyledoneae), *Arabidopsis thaliana* and *Eutrema salsugineum*, both of which are Brassicaceae plants, were most homologous. The relationship of *Nicotiana sylvestris* and *Solanum*

tuberosum, which are all Solanaceae plants, was closer. In conclusion, the evolutionary relationship is consistent with the species classification, and the similarity of GID1 protein sequences of plants with a closer kinship was higher. Interestingly, of the two GID1s of yam, which belong to Dioscoreaceae family of Dioscoreales order, DoGID1B was closely related to GID1 of rice (*Oryza sativa Japonic*a, a plant of Gramineae of Poales), while DoGID1B was closely related to GID1 of other plants such as *Phalaenopsis equestris* (a plant of Orchidaceae of Asparagales). Previous study indicated that the evolution of GID1 is earlier than that of plants of Brassicaceae of Brassicales and plants of Malvaceae of Malvales (Dong *et al*., 2009). GID1s in Monocotyledon plants are separated from dicotyledon GID1s (Voegele *et al*., 2011). These results indicated that the evolution of GID1 was earlier than that of plants in different orders, but later than that of plants in different classes.

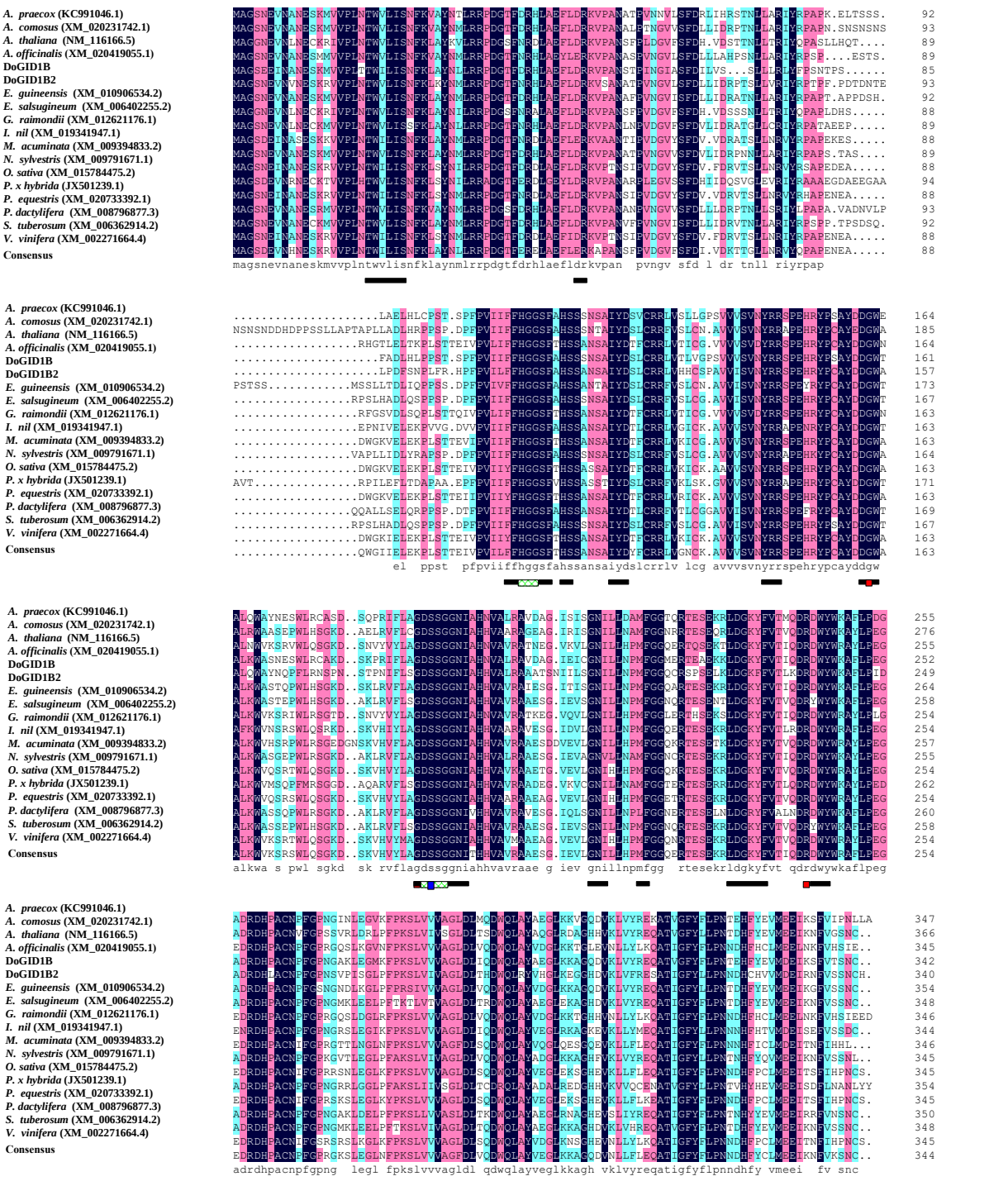

Fig. 1. Multiple sequence alignment of DoGID1B, DoGID1B2 and GID1 proteins from other species.

Note: Black background, red background, blue background and white background represent homology of 100%, 75%~99%, 50%~74% and 0~33%, respectively. The black bars indicate the amino acid residues binding to the DELLA protein or GA hormone. Green symbols mean binding sites of hormone sensitive lipase. The red squares indicate three amino acids (G^{169}, G^{196} and R^{251}) relating to function of GID1 protein in spontaneous mutant of rice. The blue squares show amino acids (S, D and H) relating to HSL catalysis.

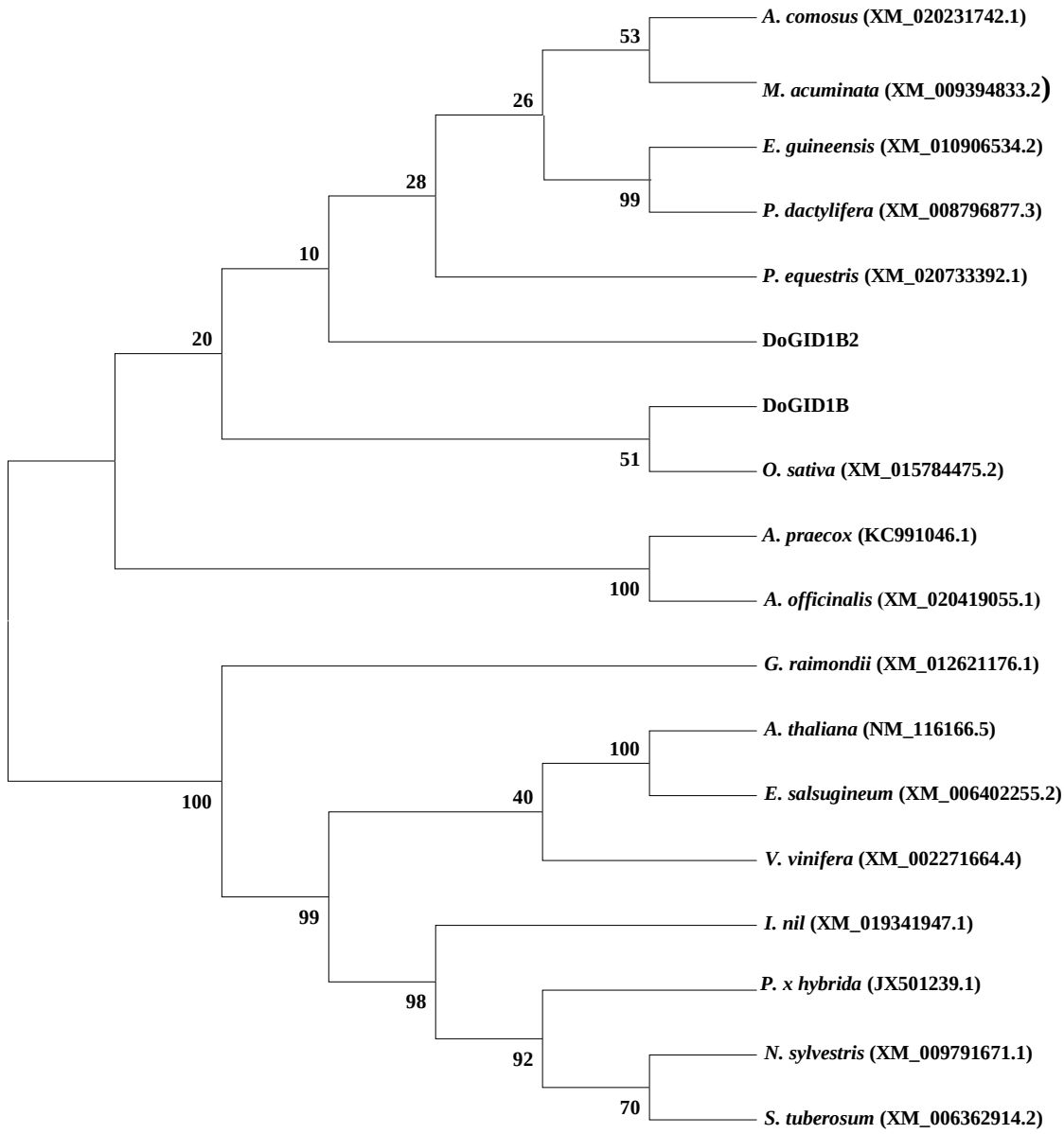

Fig. 2. Phylogenetic tree of predicted proteins of DoGID1B, DoGID1B2 and other plants GID1s.
The species and GenBank accession numbers of the sequences are as follows: *Agapanthus praecox* (KC991046.1), *Ananas comosus*(XM_020231742.1), *Arabidopsis thaliana* (NM_116166.5), *Asparagus officinalis*(XM_020419055.1), *Elaeis guineensis* (XM_010906534.2), *Eutrema salsugineum* (XM_006402255.2), *Gossypium raimondii* (XM_012621176.1), *Ipomoea nil* (XM_019341947.1), *Musa acuminata* (XM_009394833.2), *Nicotiana sylvestris* (XM_009791671.1), *Oryza sativa Japonica*(XM_015784475.2), P*etunia x hybrida* (JX501239.1), *Phalaenopsis equestris* (XM_020733392.1), *Phoenix dactylifera* (XM_008796877.3), *Solanum tuberosum* (XM_006362914.2) and *Vitis vinifera* (XM_002271664.4).

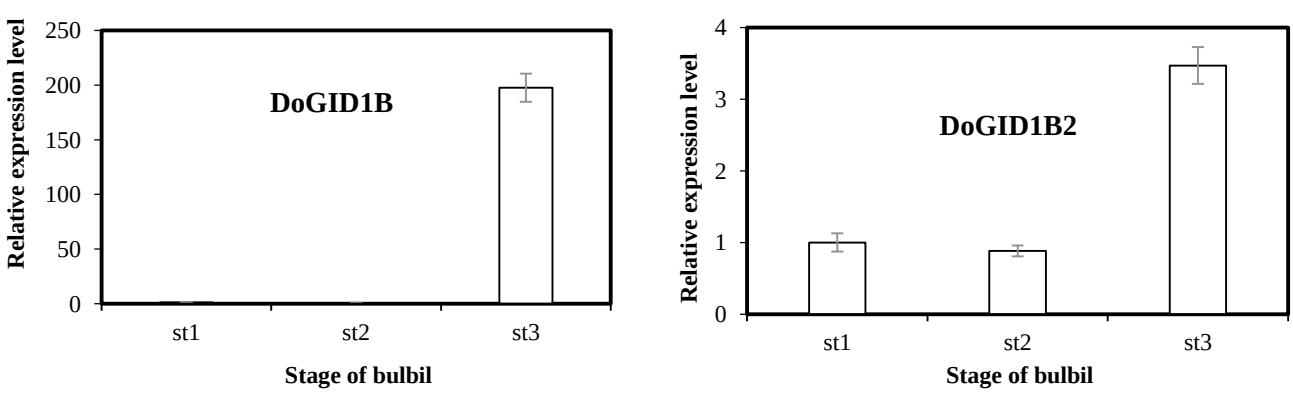

Fig. 3. Relative expression level of *DoGID1B* and *DoGID1B2* genes in bulbil in different stage. St1 is stage of becoming dormany, st2 is stage of semi-dormancy, st3 is stage of germination.

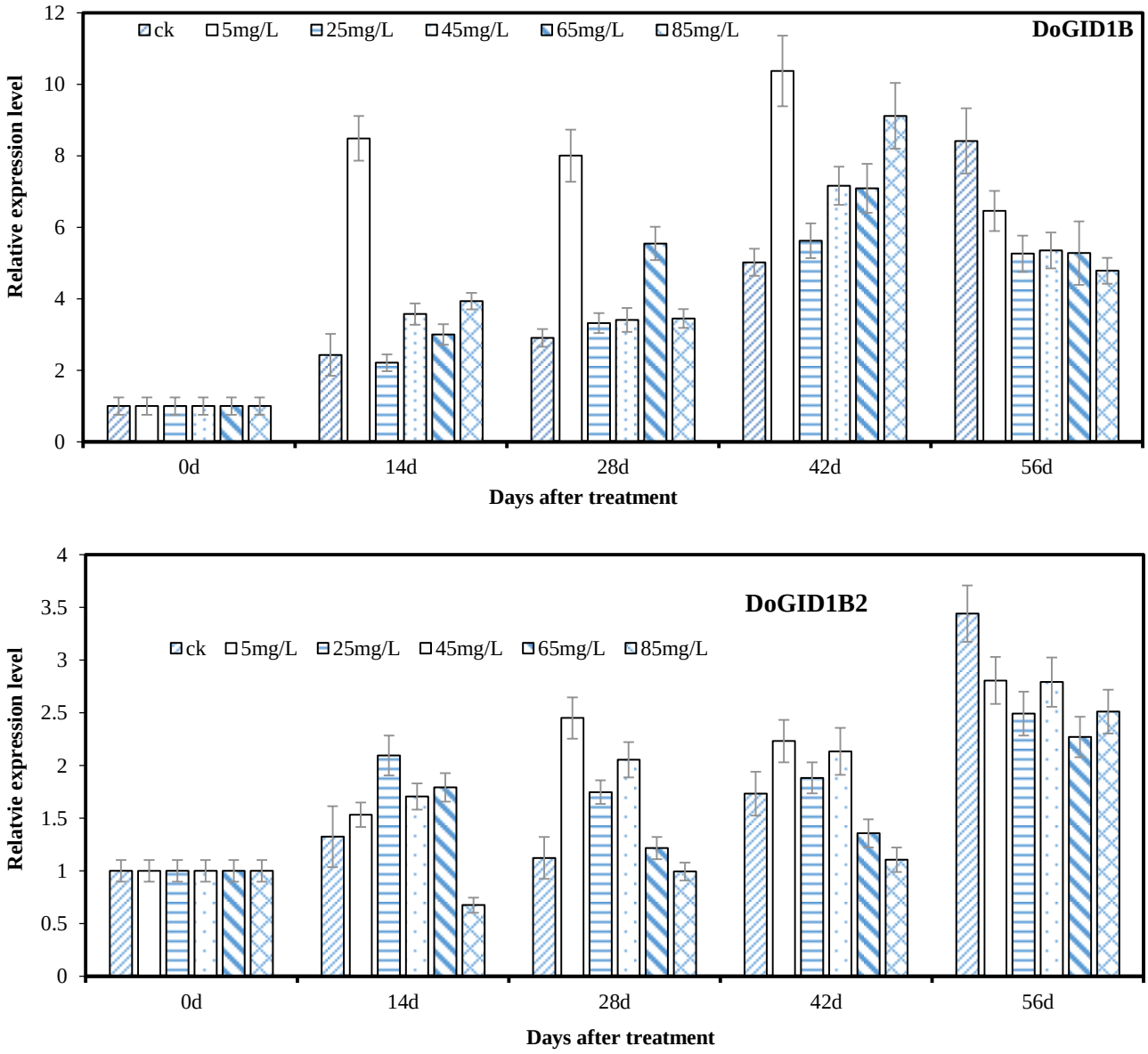

Fig. 4. Relative expression level of *DoGID1B* and *DoGID1B2* genes in bulbil treated with paclobutrazol.

Expression of genes *DoGID1B* and *DoGID1B2* in yam bulbils at different periods and their responses to paclobutrazol: Relative expression level of *DoGID1B* and *DoGID1B2* genes was tested in the absence of water supplementation at different stages (Fig. 3). It was shown from Fig. 3 that the relative expression level of *GID1B* and *GID1B2* genes was at its maximum when sprouting. The *GID1B* mRNA was about 197.5 times higher in sprouting stage than that in dormant stage, while *GID1B2* mRNA was only 3.5 times higher in sprouting stage than that in dormant stage, indicating that the upregulation was evident in *GID1B* than in *GID1B2* when the bulbils were sprouting.

Further studies on the sprouting of yam bulbils without treatment with paclobutrazol in a moist substrate revealed that the expression levels of *GID1B* and *GID1B2* also showed an overall upward trend. The expression level of *GID1B* by the 56th day, when the bulbils began to sprout, was 8.4 times of the baseline, while the expression level of *GID1B2* was 8.4 times of the baseline (Fig. 3). This trend could be seen from the change mode of genes expression in water control samples in Fig. 4.

The above results indicated that the expression of *DoGID1B* and *DoGID1B* genes was increased when the bulbils sprout in the presence or absence of water, and the up-regulation value of *DoGID1B* was higher. The double knockout mutant atgid1a atgid1c showed a dwarf phenotype, while other double mutants were of normal height compared with the wild-type ones. This result indicated that *AtGID1A* and *AtGID1C* are critical to stem growth of *Arabidopsis* (Iuchi *et al*., 2007). While *AtGID1B* is also shown to play an important role in root growth (Suzuki *et al*., 2009; Yamaguchi *et al*., 2016). We found *DoGID1B* was closely related to sprouting of yam bulbils, though *DoGID1B* was more homologous to *AtGID1B*. Therefore, it is reasonable to suppose that there may be different gibberellin signal transduction mechanisms in yam bulbils sprouting, and it is possible to clone new genes from yam in the future that may be more homologous to *AtGID1A* and *AtGID1C* and more closely related to sprouting of bulbils.

From 14 days to 42 days after treatment with paclobutrazol (5-85 mg/L), the expression of *GID1B* in the bulbils was up-regulated, and the overall expression in the treatment groups was higher than that in the control groups. For example, treatment with paclobutrazol at 5 mg/L resulted in an increase in the expression by 2.1-3.5 times of the controls during this period. By day 56, the expression level of the control was higher than that of the treatment groups. This conclusion was also true for the expression of *GID1B2* gene as shown in a parallel experiment. These results indicated that paclobutrazol could shift the up-regulation of *GID1B* and *GID1B2* earlier in the course of sprouting.

Studies showed that *GID1* genes are up-regulated or down-regulated during plant growth, organ sprouting or seed germinating. Expression level of *GID1* genes is decreased during shoot sprouting of *Camellia sinensis* and cormel sprouting of *Gladiolus hybridus* (Yue *et al.*, 2013; Luo *et al.*, 2016). While expression level of *GID1* genes is increased during yam bulbils sprouting according to our results. Response of expression level of GID1 genes to exogenous GA is different in different plants. Expression level of *GID1* genes in *Camellia sinensis* and *cotton* (*Gossypium hisutum*) is decreased by exogenous GA (Yue *et al.*, 2013; Dong *et al.*, 2009). Expression level of *GID1* gene in *Medicago sativa* is increased by exogenous GA (Chen *et al.*, 2016). Some of the *GID1* genes in *Vitis vinifera* are up-regulated and some others are down-regulated after treatment with exogenous GA (Ge *et al.*, 2011). Both *DoGID1B* and *DoGID1B2* were up-regulated in our study by paclobutrazol, an inhibitor of gibberellins. In general, plants are promoted to germinate, grow and sprout by GA. But the yam bulbils sprout earlier, after treatment with paclobutrazol (Long *et al.*, 2011b). The reason that paclobutrazol promotes the sprouting of the bulbils earlier is probably that it increases the expression of *DoGID1B* and *DoGID1B2*, leading to an accumulation of the gibberellin receptor GID1, thereby forming more GID1-GA-DELLA trimer, and ultimately removing the DELLA-mediated growth inhibition. In addition, studies on rice *gid1* mutants have shown that GID1 protein interacts with the rice DELLA protein even in the absence of GA (Yamamoto *et al.*, 2010). So it is possible that more GID1 proteins induced by paclobutrazol are able to interact with DELLA and subsequently promote yam bulbils sprout.

Previous studies showed that paclobutrazol increases expression level of some genes. Expression level of key enzyme genes *GA20-ox* and *GA3-ox* for gibberellin synthesis is increased by paclobutrazol in *Prunus avium* (Liu *et al.*, 2013). Expression level of flowering gene *FT* in *Amygdalus persica* is also increased by paclobutrazol (Peng *et al.*, 2014). Reports on the effects of paclobutrazol on *GID1* gene expression are rare. In this study, the effect of paclobutrazol on *DoGID1B* and *DoGID1B2* genes was tested, and the result should be of reference value for further study of *GID1* gene and understanding of paclobutrazol.

Conclusions

Two gibberellin receptor genes *DoGID1B* and *DoGID1B2* of yam have the basic characteristics of the GID1 gene of *Arabidopsis* and rice. The expression of both *DoGID1B* and *DoGID1B2* genes was increased in yam bulbils when sprouting, and the increased expression value of *DoGID1B* gene was higher than that of *DoGID1B2*. The expression levels of the two genes were further promoted by paclobutrazol. These results provide basic information for further study on the function of genes *DoGID1B* and *DoGID1B2*.

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