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OsGLU1, a putative membrane-bound endo-1,4-β-D-glucanase from rice, affects plant internode elongation

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Abstract

A dwarf mutant *glu* was identified from screening of T-DNA tagged rice population. Genetic analysis of the T1 generation of *glu* revealed that a segregation ratio of wild-type:dwarf phenotype was 3:1, suggesting that the mutated phenotype was controlled by a single recessive nuclear locus. The mutated gene *OsGLU1*, identified by Tail-PCR, encodes a putative membrane-bound endo-1,4-β-D-glucanase, which is highly conserved between mono- and dicotyledonous plants. Mutation of OsGLU1 resulted in a reduction in cell elongation, and a decrease in cellulose content but an increase in pectin content, suggesting that *OsGLU1* affects the internode elongation and cell wall components of rice plants. Transgenic *glu* mutants harboring the *OsGLU1* gene complemented the mutation and displayed the wild-type phenotype. In addition, *OsGLU1* RNAi plants showed similar phenotype as the glu mutant has. These results indicate that OsGLU1 plays important roles in plant cell growth. Gibberellins and brassinosteroids induced *OsGLU1* expression. In rice genome, endo-1,4-β-D-glucanases form a multiple gene family with 15 members, and each may have a distinct expression pattern in different organs. These results indicate that endo-1, 4-β-D-glucanases may play diverse roles in growth and developmental process of rice plants.

Introduction

Plant dwarfism is an important agronomic trait that significantly influences both the crop yield and stress tolerance ability. Various dwarf mutants have been isolated and characterized. Based on the molecular genetic studies with dwarf mutants in both Arabidopsis and rice, four main reasons associated with these dwarf phenotypes were found, that is, disruption of gibberellins (GA) and/or brassinosteroid (BR) related pathway, abnormal cell wall formation, and abnormal cell elongation (Ashikari *et al.*, 1999).

GA and BR are two major plant growth regulators that determine the plant height. Many GA-related dwarf mutants, for example, *GAI* in Arabidopsis (Peng *et al.*, 1997), and *slr1* in rice (Ikeda *et al.*, 2001), have been isolated and characterized (Olszewski *et al.*, 2002). The BR-deficient and BR-insensitive dwarf mutants have also been isolated (Bishop *et al.*, 2002). It has been shown that mutations in genes coding for BR biosynthetic enzymes and signal transduction components, for example, *dwf* (Choe *et al.*, 1998), *ddwf* (Kang *et al.*, 2001) and *bri1* (Clouse *et al.*, 1996) in Arabidopsis, *d2* and *d61* in rice, also resulted in dwarf phenotype.

Plant cell wall is a complex and dynamic structure because cell wall not only is sufficiently rigid to resist the high-osmotic pressure of the

protoplast and to provide mechanical support for the whole plant but also is sufficiently plastic to allow cell to grow (Roberts, 1990; Albersheim *et al*., 1994; McCann and Roberts, 1994). The primary cell wall of the higher plants is composed of a network of cellulose microfibrils embedded in gel-like matrix containing polysaccharides (hemicellulose and pectin) and proteins (Carpita and Gibeaut, 1993; Cosgrove, 1997). Loosening of the cell wall rather than an increase in turgor is essential for changes in cell shape and cell growth (Cosgrove, 1993). Some enzymes in the cell wall may contribute to a highly regulated coordination of the synthesis, assembly, deposition and remodeling of cell wall matrix components (Knox *et al*., 1991; Reiter, 2002). These wall modifications may lead in turn to changes in the pore size or viscosity of the wall matrix. Many mutants defective in cell wall have been isolated and characterized. For example, *RSW1* of Arabidopsis encodes the catalytic subunit of cellulose synthase. Temperature-sensitive mutation in this locus caused a reduction in cellulose content in cell wall, and resulted in a dwarf phenotype (Arioli *et al*., 1998). Cellulose content defects have been observed in the Arabidopsis *tbr* (Potikha and Delmer, 1995), *prc1* (Fagard *et al*., 2000), *irx* (Turner and Somerville, 1997) and *kob1* (Pagant *et al*., 2002). The *prc1* and *kob1* mutants showed cell expansion defects and dwarf phenotype, suggesting that cellulose synthesis actively contributes to the control of cell elongation and dwarf phenotype.

KOR, a membrane-bound endo-β-1,4-glucanase in Arabidopsis is important for normal cellulose synthesis. The dwarf mutant *kor1-1* and *kor1-2* have been obtained from a T-DNA-tagged population of Arabidopsis (Nicol *et al*., 1998; Zuo *et al*., 2000). Because T-DNA was inserted in the promoter of the *kor* gene in mutant *kor1-1* and *kor1-2*, weak expression and no expression of *kor* gene were observed in mutant *kor1-1* and *kor1-2,* respectively. Characterization of weak *kor1-1* showed that KOR1 was located predominantly on the plasma membrane and linked with cell expansion (Nicol *et al*., 1998). However, analysis of stronger *kor1-2* revealed that KOR1 was located to the cell plate and linked to cytokinesis (Zuo *et al*., 2000). The temperature-sensitive dwarf mutant *rsw2* and *acw1* with amino acid substitutions in the coding region of KOR showed abnormal cytokinesis and cell expansion defects (Lane *et al*., 2001; Sato *et al*., 2001). These results demonstrated that KOR was important for cellulose synthesis, cell cytokinesis, and expansion. In contrast, the relationship between a membrane-bound endo-β-1,4-glucanase and dwarfism in monocot plants has not been elucidated.

In this study, we identified a rice dwarf mutant *glu* by screening the T-DNA tagged rice population. The T-DNA inserted gene *OsGLU1* was cloned by Tail-PCR and further characterized. *OsGLU1* encodes a new member of the putative membrane-bound endo-β-1,4-glucanase family. Over expression and RNAi analysis of the *OsGLU1* gene in rice plants demonstrated its role in promotion of the internode elongation. Other homologous genes were also identified from rice and their expression pattern was examined. The possible functions of these genes were also discussed.

Experimental procedures

Construction of T-DNA tagging vector

In this experiment, the multifunctional vector, pCAS04, which contains the promoterless reporter β-glucuronidase gene *(gusA)* near to the right border and the rice actin1 promoter near to the left border, was derived from the binary vector pEP200 that carries a spectinomycin resistant gene expressed in *Agrobacterium* or *Escherichia coli*. To this end, the portion containing promoterless *gusA* gene and CaMV terminator was initially assembled into a pBluescript SK(-) vector, and cut by *Hin*dIII and *Bam*HI and inserted in pEP200, resulting in the plasmid pEP200-GUS. The complete cassette of maize ubiquitin promoter:*nptII:*CaMV terminator from plasmid pJFNPTII (a kind gift from J. Xu, IPK, Germany) was cut by restriction enzymes *Bst*XV and *Kpn*I and then inserted into the pEP200-GUS to form a plasmid pCAS03. To achieve the activation purpose, the rice actin 1 promoter was amplified with two primers (5′-aaggtaccccatggggattgaacaagatggattg-3′ and 5′-aaggtaccaagaactcgtcaagaaggcgatagaa-3′) both containing a *Kpn*I site, and cloned into pGEMT vector for sequencing. After sequence confirmation, the actin 1 promoter was inserted into the binary vector pCAS03 and the

final plasmid pCAS04 was made after examination of the insert orientation by PCR.

To make the construct for complementary experiment, the *OsGLU1* cDNA clone including the entire coding region and the 5′- and 3′-flanking regions, was inserted as a 2.2-kb fragment between the *Xba*I and *Sac*I sites of the hygromycin-resistant binary vector pZH01 containing the 35S promoter and the *NOS* terminator. The expression vector pZFGLU1 harboring the *OsGLU1* gene was thus resulted. The pZFGLU1 and the control vector pZH01 were introduced into the *glu* rice mutant as described by Hiei *et al.* (1994).

For the construction of the RNAi vector pZAGLU1, a 500-bp fragment of the *OsGLU1* gene was amplified using specific primers RNAiF containing *Xba*I and *Sac*I sites (5′-AAC TCT AGA GAG CTCGCAGGTGGGGAGCGGTG-CAAC-3′) and RNAiR containing *Sal*I and *Kpn*I sites (5′-GTAGTCGACGGTACCACCTGTGC ACCAGTAAGCTTATTG-3′). The sense and antisense fragments were assembled via respective restriction sites into vector pZH01. The RNAi vector pZAGLU1 contains the 35S promoter, the *NOS* terminator, and a fragment of *GUS* gene between the two insertional 500-bp fragments of the *OsGLU1* gene.

Plant materials and mutant screening

The rice variety *Oryza sativa* L. cv. Nipponbare and Lanshen was used for construction of a pool of T-DNA lines in rice. The *Agrobacterium tumefaciens* strain AGL1 carrying the binary plasmid pCAS04 was used for transformation experiments. The scutellum-driven embryonic calli were induced from Lanshen seeds. After one or two momths, the calli were incubated with *A. tumefaciens* at room temperature for 30 min, and then co-cultured on NB medium containing 0.1 μM Acetosyringone for two days. For selection of transformants, the calli were transferred to NB medium containing 80 mg/L G-418 and 500 mg/L cefotaxime, maintained for 3 weeks, and then transferred to NB medium containing 150 mg/L G-418 and 500 mg/L cefotaxime and maintained for another three weeks. After selection, the calli were transferred to SR medium for shoot regeneration and RM medium (1/2 MS salts containing 0.5 mg/L NAA) for root development. Regenerated plants were eventually transferred to soil and grown in the greenhouse at 30°C during days and 24°C at night. Plant height was measured at maturity and *glu* dwarf mutant was identified from the T1 segregation of these T-DNA insertional rice lines.

Measurement of shoot and root elongation

Surface-sterilized seeds of wild type and mutant plants were soaked in water for 3 days at 37°C and then were grown in the media containing 0.5% Murashige and Skoog basal salt at 27°C under continuous light. Elongation of shoot and root was measured at 2-day interval.

Hormone treatments

The plant hormone treatments were performed as described (Chen *et al.*, 2003). Briefly, Seeds of rice (*Oryza sativa* L.) were embedded in water at 37°C overnight and grown hydroponically at 25°C with a photoperiod of 12 h for approximately 3 weeks; the plants were transferred to the solution containing 30 μM GA_3, 1 μM BR, 100 μM IAA, or 100 μM ABA, respectively. For ethylene treatment, plants were sprayed with 20% ethrel and then grown in container. In each case, the plants were subjected to the treatments for various periods, frozen in liquid nitrogen and stored at –80°C for RNA extraction.

Light and scanning microscopy

For light microscopy, developed culms were fixed and embedded, and 10-μm sections were prepared on a microtome based on a previous description (Xie *et al.*, 1999). The sections were stained with safranin-fast green contrast-stain method. For scanning electron microscopy, samples were prepared as described previously (Li *et al.*, 2003).

Carbohydrate measurement

Cell walls were prepared according to the method described by Zablackis *et al.* (1995). The leaves of rice were ground into fine powder in liquid nitrogen, washed in phosphate buffer (50 mM, pH 7.2) for three times. After centrifugation of the homogenate, the pellet was extracted with phenol–methanol (1:1, v/v) to remove lipid and protein and then extracted twice with 70% ethanol at

70 °C for 1 h, and dried under vacuum. The dried cell wall materials were weighed for cellulose content. The cellulose content was assayed according to the methods described by Selvendran and O'Neill (1987) and Whatman 3MM paper was used as the standard. The measurement of pectin content was performed in the Chinese Academy of Forestry Sciences (Beijing), following the National Standard For Determination of Pectin Content in People's Republic of China. Briefly, 5 g samples were first incubated for 3 h in a mixture of ethanol:benzene (1:3, v/v) in a boiling-water bath, then the samples were taken out, dried and extracted in 1% (g/v) ammonium oxalate for 3 h in a boiling water bath. The extractant was collected. The residues were further extracted in 0.5% (g/v) ammonium oxalate for 3 h in a boiling-water bath. The extractant was collected and combined. The residues were also washed with hot water for three times and filtered. The filtrate was combined with the previous extractant, and further concentrated. The concentrate was adjusted to 100 ml with water. Twenty-five milliliter of this solution was slowly added, under shaking, with 90 ml of ethanol containing HCl [ethanol:HCl (ρ_{20} = 1.19 g/ml) = 1000 ml:11 ml]. The mixture was stayed over night and the pelleted pectin was filtered. The pelleted pectin was further washed with ethanol containing HCl [ethanol:HCl (ρ_{20} = 1.19 g/ml):water = 1000:11:250, ml/ml/ml] until the washings did not contain oxalate. The pelleted pectin was dissolved in 50 ml of hot ammonium hydroxide solution [50 ml hot water plus 1 ml ammonium hydroxide (ρ_{20} = 0.90 g/ml)], and 25 ml of diluted ammonium hydroxide solution [100 ml water plus several drops of ammonium hydroxide (ρ_{20} = 0.90 g/ml)] was further added. After boiled for several min, the solution was filtered and the filtrate was collected. The original filter holding the pelleted pectin was boiled with hot water for three times, and each time the solution was filtered. All the filtrate was collected, combined and added with 100 ml of 0.1 M NaOH. The solution was mixed well and stayed for 12 h. Acetic acid solution (50 ml, 1 M) was added, mixed well and stayed for 5 min. Finally, 50 ml of 1 M $CaCl_2$ solution was added, mixed and stayed for 1 h. After boiled for 5 min, the mixture was filtered, and the pellet was washed. The pelleted pectin (Ca salt) on the filter was dried at 105°C, weighed and calculated. Each result was repeated by three reactions.

Tail-PCR, 5′ and 3′RACE

Tail-PCR was carried out as described (Liu *et al*., 1995). One microgram of total DNA was added to reaction tubes containing 1X PCR buffer, 0.2 mM each dNTPs, 0.15 μM specific primer (LL5: 5′-AATGCAGCCTCGTGCGGA-3′; LL3: 5′-GCTCGAATTCAATTCGGCGT-3′; LL2: 5′-TCAGTACATTAAAAACGTCCGCAA-3′) and 2.5 μM arbitrary primer (AD1: 5′-AGTGNAGAANCAAAGG-3′; AD2: 5′-TCGTNCGNACNTAGGA-3′; AD3′: 5-NTCGASTWTSGWGTT-3′; AD4: 5′-NGTCGASWGANAWGAA-3′; AD5: 5′-TGWGNAGWANCASAGA-3′; AD6: 5′-AGWGNAGWANCAWAGG-3′; AD7: 5′-CAWCGICNGAIASGAA-3′; AD8: 5′-WGTGNAGWANCANAGA-3′), and 0.5 units of Taq polymerase. The thermal cycling conditions and procedures of first reaction PCR, secondary reaction PCR, and the tertiary PCR are the same as one described by Liu *et al*., (1995). The products of the tertiary PCR were directly used for sequencing using the primer LL2: 5′-TCAGTACATTAAAAACGTCCGCAA-3′. The 5′ and 3′ ends of the *OsGLU1* cDNA were amplified as described previously (Zhang *et al*., 2003). Nested primers for 5′-RACE were OsGLU1-5-1 and OsGLU1-5-2 (5′-GTGGTAGCACCATGTACCAGCTTCTG-3′ and 5′-GTCAATGTCCTCTGGTCTCATCCAAC-3′); Nested primers for 3′-RACE were OsGLU1-3-1 and OsGLU1-3-2 (5′-GGATGTATCTCGCAACCGGCAATTCA-3′ and 5′-GGTCCAGACTACGGTGTATTCAGCTG-3′). The RACE-PCR products were cloned into the pGEM-T vector (Promega, Madison, WI) and sequenced.

DNA Isolation, RNA isolation, Reverse Transcription, and RT-PCR

DNA extraction was performed as described previously (Chen *et al*., 1991). Total RNA was extracted from all kinds of materials according to the method described by Zhang *et al*. (1996). For reverse transcription (RT)-PCR, first-strand cDNA was reverse transcribed from total RNA with oligo(dT) as the primer and used as the template to amplify the transcripts with a profile

(30 cycles) of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. Each RT-PCR result was repeated with three reactions and using different RNA preparations. Primers for *OsGLU1* were *OsGLU1*F 5′-CTCGGATCCGGCAGTCGGGCG3′ and *OsGLU1*R 5′-ATGAGCCCAAATGCAG GTTGCATGCTG3′. Primers for actin were ActF 5′-GGAACTGGTATGGTCAAGGC-3′ and ActR 5′-AGTCTCATGGATAACCGCAG-3′.

Results

Construction of a multifunctional T-DNA tagging vector

A novel T-DNA tagging system was developed for both gene trapping and activation tagging. The binary vector pCAS04 adopted for mutagenesis carries the promoterless β-glucuronidase (GUS) reporter gene immediately next to the right border of T-DNA, and also harbors a rice *actin1* promoter with 5′-leader intron next to the left border. The selectable marker gene, *nptII*, under control of maize ubiquitin promoter was placed between the *GUS* gene and the rice *actin1* promoter. This vector can be used not only for T-DNA inactivation, but also for gene trapping and activation tagging.

Phenotype and genetic analysis of the glu *mutant*

The pCAS04 plasmid was transformed into rice and about 20,000 T-DNA insertional lines for rice varieties Nipponbare or Lanshen were obtained. A rice dwarf mutant *glu* in the Lanshen background was identified from screening of the segregated individuals of these T-DNA insertional lines (Figure 1A). *glu* showed a typical dwarf phenotype of short internode (Figure 1C). The leaves of *glu* were also shorter compared with wild-type plants (Figure 1D). When germinated in light for 7 and 14 day respectively, the shoots and roots of *glu* were shorter than those of wild-type plants (Figure 1B). The difference between wild-type plants and the *glu* mutants was more obvious for shoots than that for roots after 15 day germination (Figure 1E, F). The segregation of the self-pollinated progeny of *glu* T1 heterozygous plants showed a ratio of 3:1, with 136 plants of wild-type appearance and 40 plants of *glu* phenotype ($\lambda^2 = 0.3305$; $p < 0.05$), suggesting that the dwarf phenotype was controlled by a single recessive nuclear locus.

The light microscopy analysis was conducted to observe the difference of cell structure between *glu* and wild-type rice. Transverse sections along the elongation zone of first internode showed the reduction of cell expansion in the *glu* mutant compared with wild-type rice plants (Figure 2A, B). Longitudinal sections displayed that cells in the internodes were narrower and slightly shorter in the *glu* than that in the wild-type rice (Figure 2B, D). Scanning electron microscopy of the leaf from 14-day-old seedlings revealed small vessel elements in the vascular bundle of the *glu* compared with wild-type rice (Figures 2E, F).

The glu mutant has an altered cell wall composition

To analyze whether the cellular phenotype and the reduced cell elongation in *glu* mutants resulted from altered cell wall composition, cell wall materials from the leaves of *glu* and wild-type rice were isolated, and then the cellulose and pectin contents were determined. The result in Figure 3A clearly demonstrated a 9% reduction in the cellulose content in the *glu* compared with that in the wild-type rice, implying that the tagged gene may have a role in cellulose biosynthesis. In contrast, the pectin content of cell wall in the *glu* plants was increased by nearly one-fold when compared with that of the wild type (Figure 3B). These results indicate that the cell wall composition was affected in the *glu* mutant.

The dwarf phenotype is resulted from T-DNA insertion mutation of the OsGLU1 *gene*

By Southern analysis using *NPTII* gene as a probe, four independent T-DNA insertions were identified in *glu* mutant (data not shown). Three T-DNA flanking sequences were obtained via Tail-PCR method, with two representing non-coding or non-regulatory sequences and one showing homology to the endo- β-1,4-D-glucanase gene from Arabidopsis. This T-DNA tagged gene was designated as *OsGLU1*.

The full length of *OsGLU1* cDNA was obtained by both 5′- and 3′-RACE methods, and

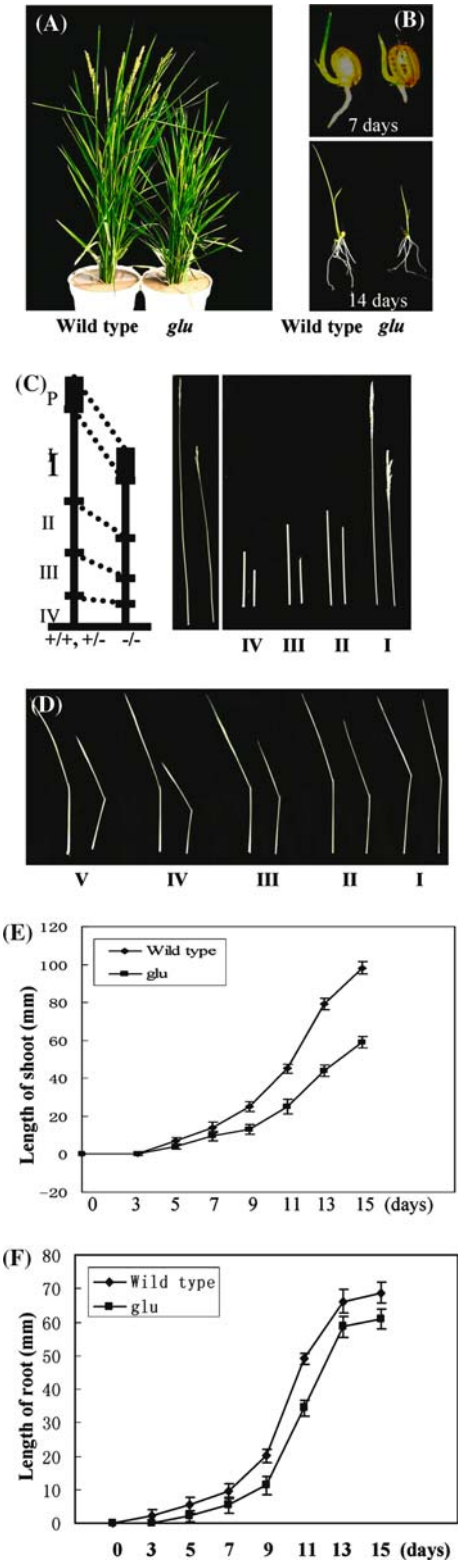

Figure 1. Phenotype of the *glu* mutants. (A) Wild-type plant (left) and *glu* mutant (right). (B) Shoot and root morphology of 1- and 2-week-old plants, wild type (left) and *glu* (right). (C) Morphological and schematic representation of the various elongation patterns of internodes in the wild type (left) and the *glu* mutant (right). The number of each internode is indicated. P: panicle; +/+: wild-type plant; +/−: heterozygous plant; −/−: *glu* mutant. (D) Leaf morphology of wild type (left) and *glu* (right). The number of each internode is indicated. (E) Reduced shoot growth in the *glu* mutant. Shoot lengths were measured every two days from day 3 to day 15 [$n = 50$]. (F) Reduced root growth in the *glu* mutant. Root lengths were measured every two days from day 3 to day 15 ($n = 50$).

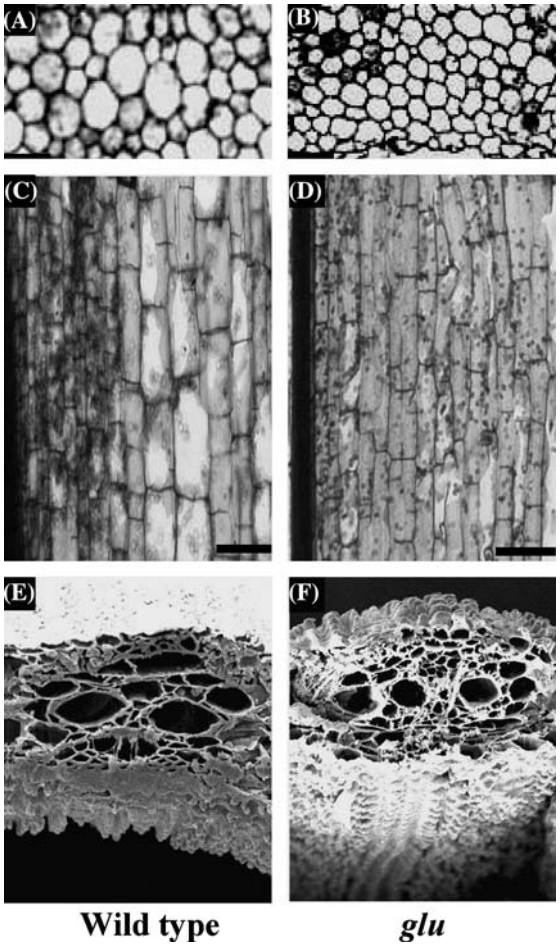

Figure 2. Light and scanning electron microscopic analysis of the *glu* mutant and wild-type plant. Transverse sections (A, B) and longitudinal sections (C, D) along elongation zone of the first internode after Calcofluor staining were compared in wild type (A, C) and *glu* (B, D). All cells of the *glu* mutants showed reduced expansion or elongation compared with the wild type. The scanning electron micrographs (SEM) of the transverse sections of 10-day-old leaf were presented for wild type (E) and glu (F). Bars indicate 50 μm for a b, c, d, and indicate 10 μm for E and F.

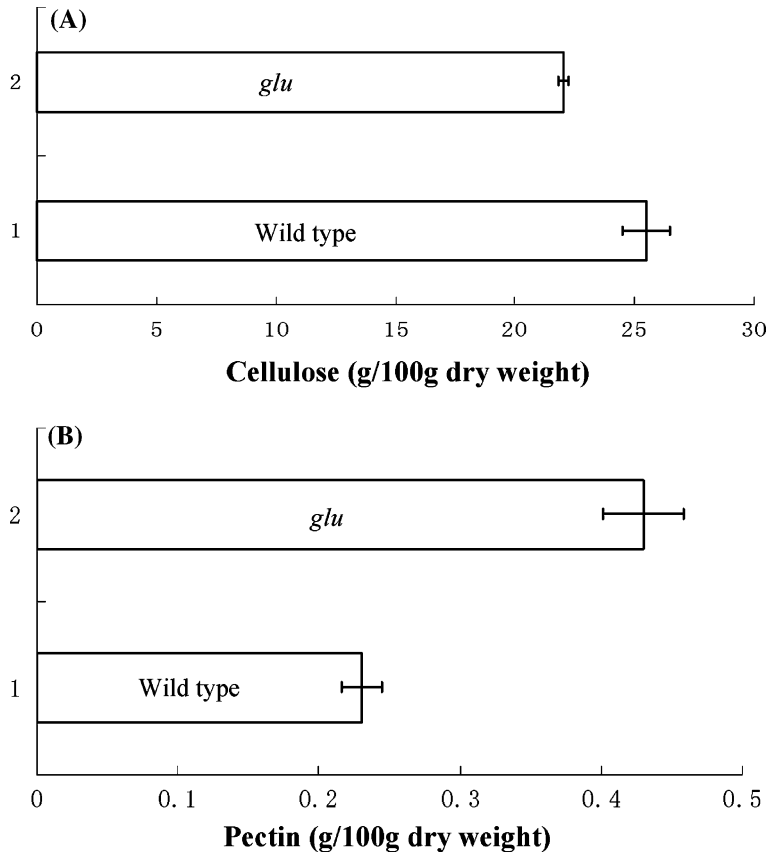

Figure 3. Cell wall composition of wild-type and *glu* mutant plants. (A) The content of cellulose fraction in cell walls prepared from leaves of mature wild type and *glu* mutant plants. (B) The content of pectin in cell walls prepared from leaves of mature wild type and *glu* mutant plants. Each result was repeated by three reactions ($n = 3$).

the 2230 bp full-length *OsGLU1* gene encodes a protein of 619 amino acids (Figure 4). Comparison of the genomic sequence of the *OsGLU1* from database with the *OsGLU1* cDNA revealed that there were 5 introns and 6 exons and the T-DNA was inserted in the second intron of the *OsGLU1* (Figure 5A).

To analyze whether the dwarf phenotype co-segregated with the T-DNA insertion in the *OsGLU1* gene, genetic analysis of T1 plants of *glu* was carried out. Three primers P1, P2, and P3 were designed (Figure 5B) and two PCR reactions with primer pairs P1 and P2, P1 and P3 were carried out, respectively. The T1 plants No. 6–20 showed wild-type phenotype, whereas the plants No.1–5 displayed the dwarf phenotype. The plants 1–5 were homozygous for T-DNA insertion because a 600-bp band was amplified using the P1 and P2 primers and no 700 bp DNA band was amplified with P1 and P3 primers. In plants No. 7, 8, 10, 11, 17 and 19, a 700-bp band was amplified using P1 and P3 primers, whereas no 600 bp band was amplified, indicating that these plants were homozygous as wild types. Both the 600- and 700-bp bands were amplified from plants No. 9, 12, 13, 14, 15, 16 and 18, indicating that they were heterozygous (Figure 5C). These results indicate that the homozygous T-DNA insertion at the *OsGLU1* co-segregated with the dwarf phenotype of *glu* mutant, and the wild-type phenotype and dwarf phenotype segregated at a ratio of 3:1. In addition, there was no segregation in T2 generation of the dwarf plants No. 1 – 5.

Expression of *OsGLU1* was also examined by RT-PCR using the P1 and P3 primers and there were no transcripts of *OsGLU1* in the homozygous *glu* plants. However, the *OsGLU1* transcripts were displayed in the heterozygous and wild type plants

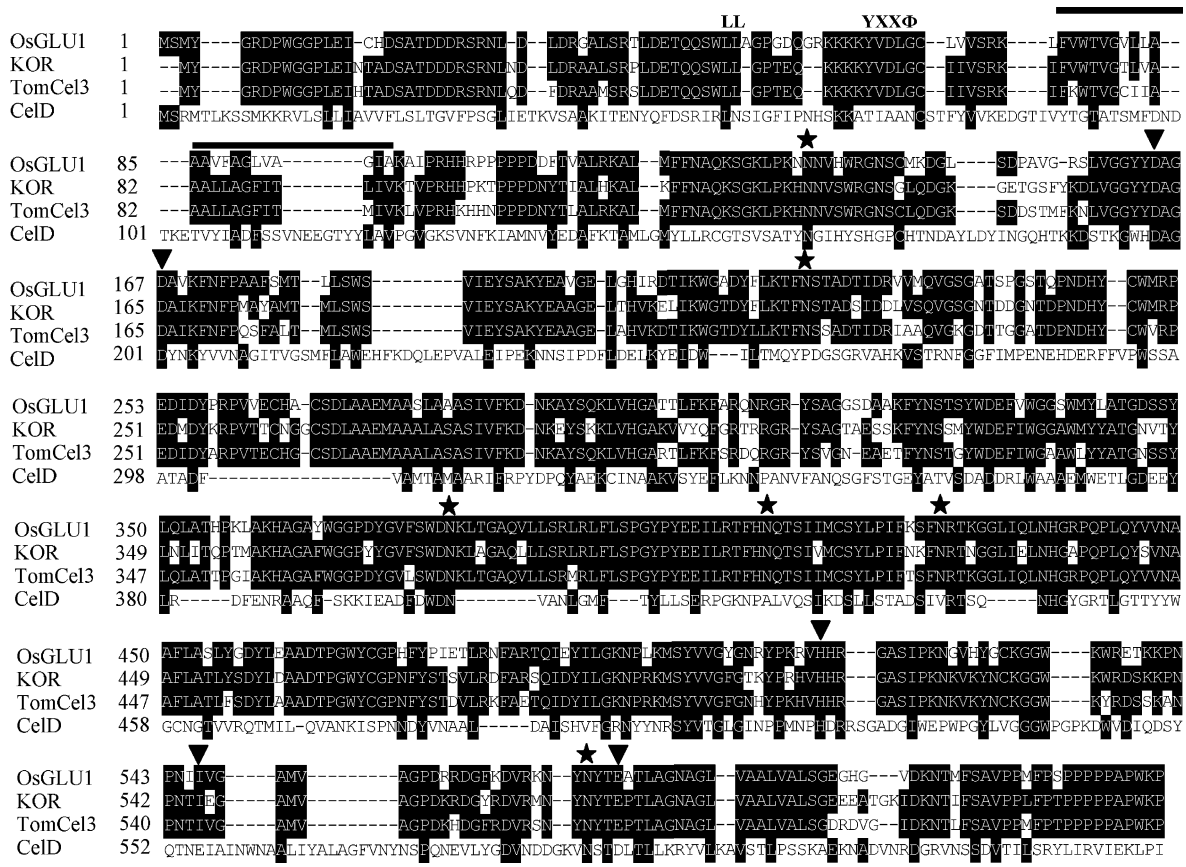

Figure 4. Alignment of OsGLU1 with plant and bacterial EGases. KOR (AAC83240) is from Arabidopsis, TomCel3 (T07612) is from tomato and CelD (P04954) is from *C. thermocellum*. ▼ Represents the residues essential for catalytic activity identified in CelD, which are also conserved in OsGLU1 (D198/201, H516 and E555); * indicates the six predicted glycosylation sites. The thick black line overlines the predicted transmembrane domain in OsGLU1. The conserved polarized targeting signals are highlighted (LL and YXXϕ).

(Figure 5D). These results further demonstrate that T-DNA insertion in the *OsGLU* gene resulted in the dwarf phenotype of *glu* plants.

The 619-aa OsGLU1 contains a transmembrane domain and a glucanase (EGase) domain. In comparison with other endo-β-1,4-D-glucanases, it showed high similarity with endoglucanase TomCel3 (76%) from tomato and KOR (75%) from Arabidopsis. Similar to TomCEL3 and KOR, OsGLU1 did not contain a predicted cleavable signal peptide, which was present in *Clostridium thermocellum* endoglucanase (CelD) (Figure 4). Alternatively, the nearby sequence may fulfill the signal peptide function. In the CelD, site-directed mutagenesis and chemical modification techniques have identified four amino acids (D198/201, H516 and E555) as potentially important residues in catalysis activity (Chauvaux *et al.*, 1992). Three (D198/201, H516) of these four residues were also conserved in the OsGLU1 and other plant EGases compared (Figure 4). The fourth residue E555 was replaced by I555 in plant. However, conserved E residue in the nearby position in plant endo-β-1,4-D-glucanases (E571 in OsGLU1, E570 in KOR, and E568 in TomCel3) were identified. The OsGLU1 protein also contained six potential N-glycosylation sites and a conserved polarized targeting signal.

Complementation of the glu mutant and phenotypic analysis of OsGLU1 *RNAi plants*

To further confirm that *OsGLU1* was located on the *glu* locus, complementation assay of the *glu* mutant by introduction of the *OsGLU1* gene to the mutant, and also the suppression of the wild-type

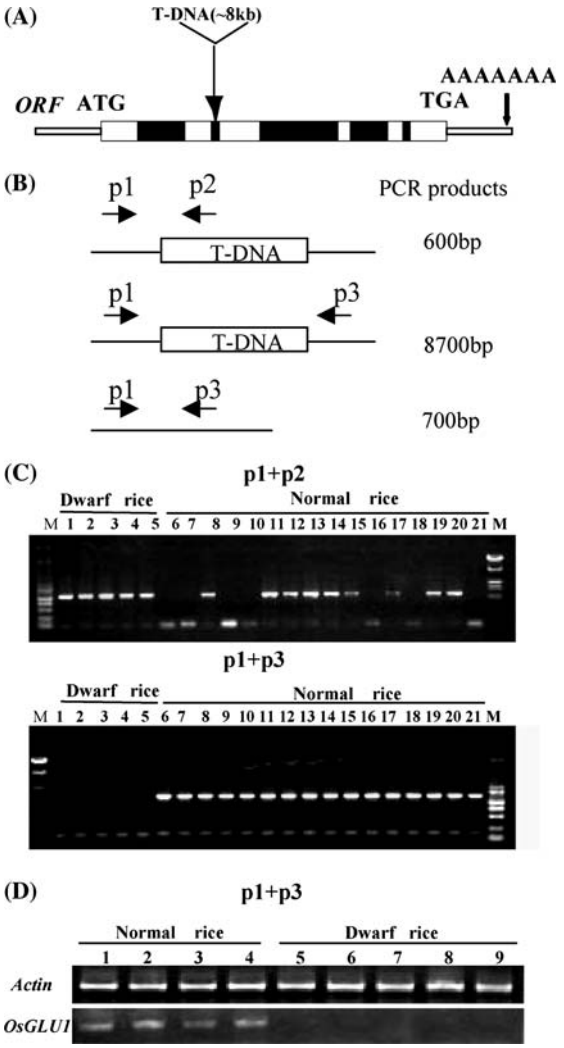

Figure 5. Segregation of the T-DNA insertion in *OsGLU1* with the dwarf phenotype. (A) Schematic representation of the intro-exon organization of the *OsGLU1* and the T-DNA insertion position in *glu* mutant. Exons are indicated with empty boxes, and introns with filled boxes. (B) Schematic diagrams of the genotyping. P1, forward primer in *OsGLU1*; P2, reverse primer in T-DNA; P3, reverse primer in *OsGLU1*. The P1 and P2 primers produced the 600-bp PCR fragment from the T-DNA inserted DNA template; P1 and P3 primers produced 700-bp PCR bands from the wild-type DNA, while no PCR band was obtained from the T-DNA inserted template because the expected fragment (6.0 kb) was too large to amplify. (C) PCR analysis of the twenty T1 plants. Samples 1–5 amplified the 600-bp fragment only and were *glu* homozygous; Samples 7, 8, 10, 11, 17, 19 amplified the 700-bp band only and were wild type; Samples 9, 12, 13, 14, 15, 16, 18 amplified both bands and were *glu* heterozygous. M (left), the lambda DNA size marker cut with *Eco*RI and *Hind*III; M (right), 100 bp DNA size marker. (D) RT-PCR analysis of the *OsGLU1* transcript. Samples 1–4 were *glu* homozygous; samples 5–9 were *glu* heterozygous or wild type.

phenotype by *OsGLU1* RNAi approach were performed.

The binary vector pZFGLU1, including the entire *OsGLU1* coding region and also 127bp 5′- and 153 bp 3′-flanking sequences under the control of CaMV 35S promoter was constructed (Figure 6A) and the *glu* plants was transformed. As a control, the empty vector pZH01 without *OsGLU1* was also used for transformation. Twenty-four independent transgenic lines harboring pZFGLU1 were obtained, among which six transgenic plants complemented the mutation and displayed the wild-type phenotype (Figure 6B, C). However, 20 independent transgenic lines harboring the control vector pZH01 without *OsGLU1* cannot complement the *glu* phenotype. The expression analysis revealed that the *OsGLU1* was expressed in the *glu* mutant harboring the vector pZFGLU1. In contrast, no expression of the *OsGLU1* was detected in the *glu* mutant harboring a control vector pZH01 (Figure 6F).

The RNAi vector pZAGLU1 with 500-bp of the *OsGLU1* fragment in sense and antisense orientation separated by an intron were assembled and inserted into cassette with CaMV 35S promoter and Nos terminator derived from a pCambia binary vector. Fourteen transgenic plants with pZAGLU1 were obtained. Figure 6D and e indicated that three transgenic plants ad2, ad3, and ad4 displayed dwarf phenotype varying in severity, and the transgenic plant ad1 showed wild type phenotype. The expression of the *OsGLU1* in these four transgenic rice plants was analyzed (Figure 6G). In ad1 transgenic line, the *OsGLU1* expression is similar to that in wild type, whereas no expression was observed in the transgenic line ad4. Expression of the *OsGLU1*1 was reduced in the ad2 and ad3 transgenic rice. These results confirmed that the *glu* mutant phenotype was caused by the loss-of-function mutation in the *OsGLU1* gene.

Regulation of OsGLU1 *mRNA level*

The *OsGLU1* expression was also examined in rice seedlings treated with various plant hormones, including indole-3-acetic acid, GA, ethylene, BR, and abscisic acid. It was shown that the mRNA level of *OsGLU1* was increased in shoot treated with GA or BR (Figure 7A, B), whereas no significant changes were observed when treated

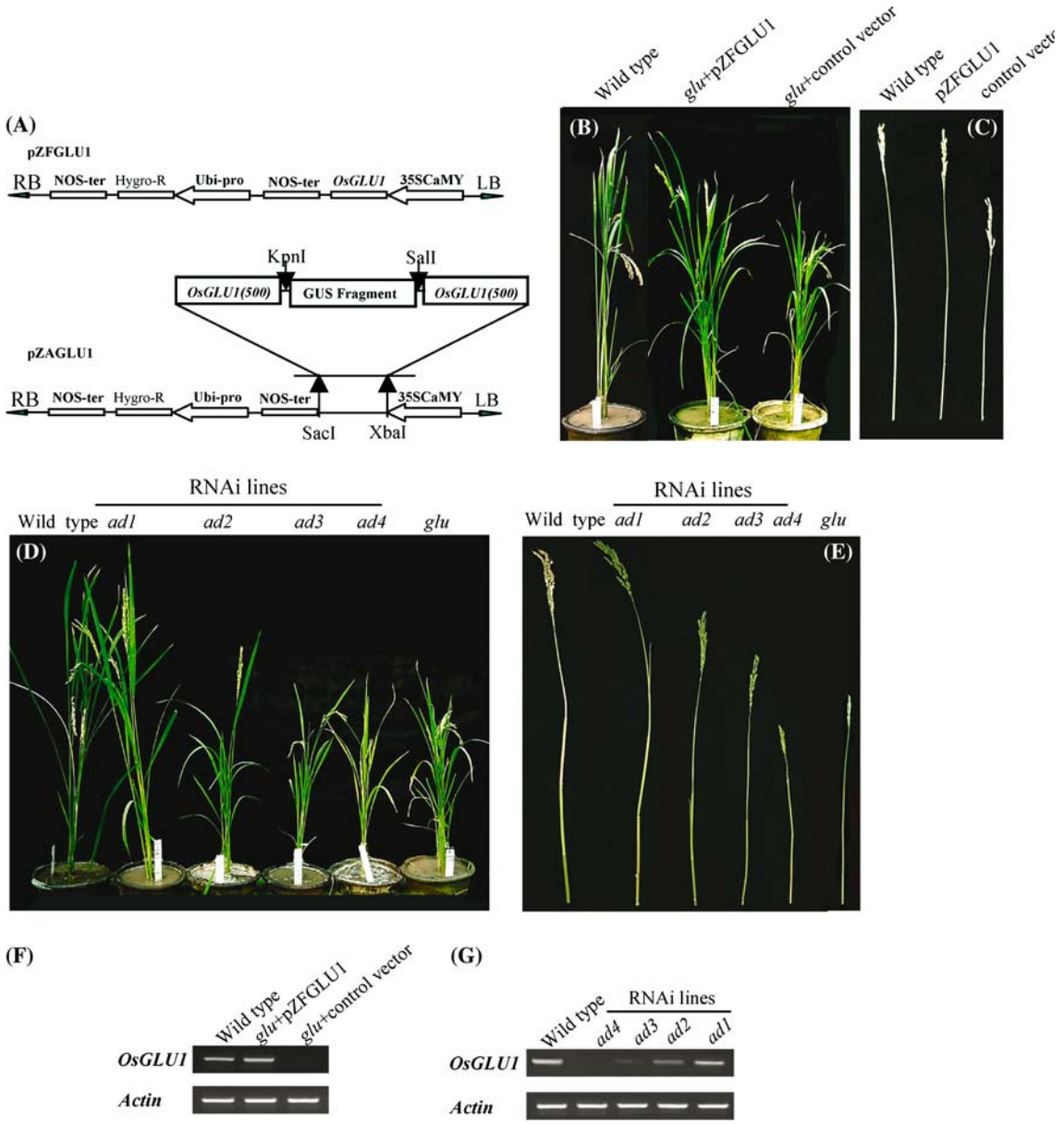

Figure 6. *OsGLU1* complementation and RNAi phenotype of the rice plants. (A) Schematic diagrams of the two constructs. Top vector pZFGLU1 is used for complementation and bottom vector pZAGLU1 is used for RNAi analysis. (B) *OsGLU1* complementation of the *glu* dwarf phenotype. Phenotype was compared among the *glu* plant overexpressing the wild-type *OsGLU1* gene (middle), *glu* plant introduced with a vector only (right), and wild type (left). (C) Internode morphology of wild-type plant (left), *glu* plant overexpressing the wild-type *OsGLU1* gene (middle), and *glu* plant introduced with a vector only (right). (D) *OsGLU1* RNAi plants with wild type-like phenotype (*ad1*), intermediate dwarf phenotype (*ad2, ad3*), and severe dwarf phenotype (*ad4*) in comparison with a wild-type plant and a *glu* mutant plant. (E) Internode morphology of wild-type plant, *glu* mutant and *OsGLU1* RNAi plants with wildtype-like (*ad1*), intermediate (*ad2* and *ad3*), and severe dwarf phenotype (*ad4*). (F) Expression of *OsGLU1* in the complemented plants. (G) Expression of *OsGLU1* in the RNAi plants.

with indole-3-acetic acid, ethylene or abscisic acid (Figure 7D–F). The expression level of *OsGLU1* in root was only increased upon treatment with brassinosteroids (Figure 7C).

OsGLU1 *belongs to a multigene family in rice*

A genome-wide search of DNA and protein databases revealed that at least 10 *OsGLU1*-like

genes (*OsGLU1* to *OsGLU10*) existed in rice genome localized on different chromosomes (Table 1). These genes encoded proteins of various lengths, ranging from 499 to 628 amino acids. Recently, Libertini *et al*. (2004) reported that 15 endoglucanase genes were present in rice genome. We thus added the additional five genes named as *OsGLU11* to *OsGLU15* in the present list (Table 1). To determine the evolutionary distances among the 15 rice OsGLU1-like proteins and KOR in Arabidopsis, CEL3 in tomato, and CelD in *C. thermocellum*, phylogenetic analysis was performed with MegAlign program. Figure 8A showed that these proteins could be classified into four main clusters. One cluster contained OsGLU4, OsGLU8, OsGLU12, OsGLU13, OsGLU14 and OsGLU15. Another cluster contained OsGLU1, OsGLU2, OsGLU3, KOR and CEL3. The third cluster contained OsGLU5, OsGLU6, OsGLU7, OsGLU9, OsGLU10 and OsGLU11.The CelD from *C. thermocellum* did not cluster with any others and thus represented a distinct group. The genomic structures of the *OsGLU1* to *OsGLU10* were also illustrated in Figure 8B, and each gene had different numbers of introns and exons. All proteins of the OsGLU family contained the EGase domain. The OsGLU1, OsGLU2 and OsGLU3 contained a predicted highly hydrophobic transmembrane motif in the N-terminal and belonged to the type II integral membrane protein anchored in the membrane. Other 12 proteins OsGLU4 to OsGLU15 belonged to the secretive protein anchored in the cell wall because they did not contain a predicted highly hydrophobic transmembrane motif but contained the predicted cleavable signal peptide (data not shown).

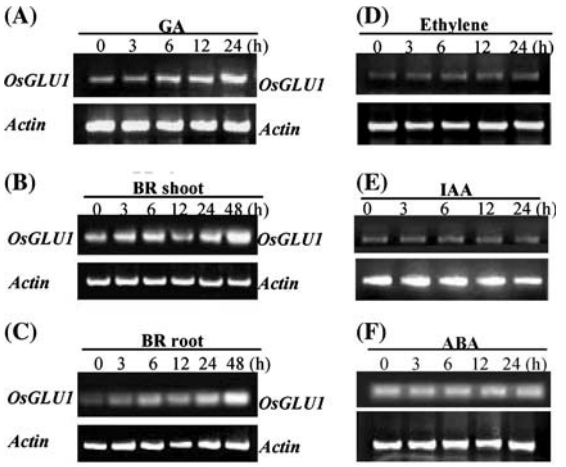

Figure 7. Expression of *OsGLU1* in response to different hormone treatments. (A), (B), (E), and (F) *OsGLU1* expression was examined in shoots of two-week-old rice seedlings incubated for the indicated times in solutions of 30 μM GA_3 (A), 1 μM BR (B), 100 μM IAA (E), or 100 μM ABA (F), respectively. (C) *OsGLU1*expression in roots of two-week-old rice seedlings incubated for 0, 3, 6, 12, 24 h in solutions of 1 μM BR. (D) *OsGLU1* expression in shoots of 2-week-old rice seedlings sprayed with 20% ethrel (ethylene treatment) for 0, 3, 6, 12, 24 h. The total RNAs isolated from these samples were subjected to RT-PCR analysis. Amplification of actin transcript was used as an internal control.

Table 1. Rice *OsGLU* gene family.

Locus	Accession no.	Gene position	EST accession no.	Chr.	Amino acids
OsGLU1	AC137547	98011 – 101961	CA759902; C97417	3	619
OsGLU2	AC118133	55318 – 58602	AU093798; D46633	3	620
OsGLU3	AL606627	101221 – 104546	BE040704; AU173081	4	623
OsGLU4	BX548155	33942 – 36295		4	500
OsGLU5	AP002745	131177– 134622	C72268; NM188491	1	618
OsGLU6	AL606457	103490 – 106931		4	625
OsGLU7	AP002745	108451 – 110905	E1328	1	640
OsGLU8	AP003722	78996 – 81612		1	499
OsGLU9	AP004463	52849 – 54438		8	529
OsGLU10	AP005112	41268 – 44659	BI812972; CD670721	2	531
OsGLU11	AP007149	25355 – 33915		9	532
OsGLU12	AP005682	90948 – 94960		9	515
OsGLU13	AP005619	82171 – 87728		6	497
OsGLU14	AP004885	68190 – 71251		2	503
OsGLU15	AP004689	120434 – 124434		8	516

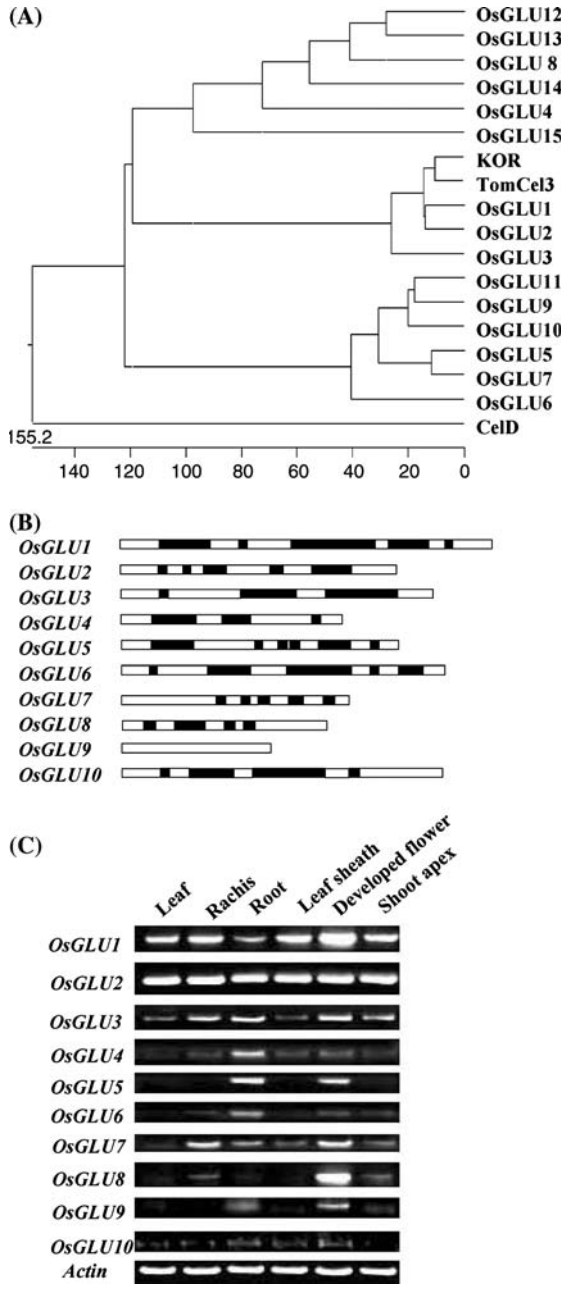

Figure 8. The *OsGLU* gene family in rice and their expression pattern. (A) Phylogenetic tree of OsGLU family proteins, KOR (AAC83240) in Arabidopsis, TomCel3 (T07612) in tomato, and CelD (P04954) in *C. thermocellum*. The scale bar is an indicator of genetic distance based on branch length. (B) Intron/exon structure of rice *OsGLU* family members. Exons are indicated with open boxes, and introns are indicated with filled boxes. (C) Organ-specific expression of rice *OsGLU* family genes revealed by RT-PCR. Amplification of actin transcript was used as an internal control.

Organ-specific expression of the OsGLU *genes*

To examine the expression patterns of the *OsGLU* gene family in different organs, total RNAs were extracted from leaf, rachis, root, shoot apex, leaf sheath, and developing flower of rice plants and RT-PCR analysis was carried out by using specific primers for each *OsGLU* gene. The results in Figure 8C demonstrated that *OsGLU1*, *OsGLU2*, *OsGLU3* and *OsGLU10* showed constitutive expression patterns in all the organs tested, and the *OsGLU4, OsGLU5, OsGLU6, OsGLU9* were abundant in roots and developing flowers of plants. The other two genes *OsGLU7* and *OsGLU8* showed relatively higher expression in rachis and developing flowers. These different expression patterns indicated multiple functions of these genes in different processes of plant growth and development. Specific and combinational expression of these genes may be essential for the formation or function of a given organ.

Discussion

In the present study, an insertional mutant *glu* with the dwarf phenotype was screened out from rice T-DNA tagging pool and the tagged gene was identified to be *OsGLU1* encoding an endo-β-1,4-D-glucanase. The endo-β-1,4-D-glucanase can hydrolyze β-1,4-linkages behind unsubstituted glucose residues and is involved in the formation of cell wall structure (Molhoj *et al*., 2001b). Mutation of this gene disrupted cell wall and affected the plant growth. Consistent with the *OsGLU1* mutation, transverse and longitudinal sections along the elongation zone of internode from *glu* plants showed short and narrow cells in comparison with the wild type plants. *glu* plants also showed the reduction of the cellulose content and increase of pectin content, suggesting that the mutation in the *glu* was involved in the structural change of cell walls.

Genetic analysis of T1-population of *glu* showed that the T-DNA insertion in *OsGLU1* co-segregated with the dwarf phenotype of the *glu* mutant. Expression analysis revealed that there was no expression of *OsGLU1* in the mutant *glu*, consistent with the recessive nature of the mutant phenotype. The *glu* mutant overexpressing the *OsGLU1* gene exhibited wild-type like phenotype,

indicating that the *OsGLU1* was able to completely rescue the mutant phenotype. Transgenic rice expressing the inverted repeats of 500 bp *OsGLU1* portions showed RNA silencing and displayed the dwarf phenotype. These results demonstrated that mutation of *OsGLU1* was the primary cause of the dwarf phenotype in the *glu*.

In Arabidopsis, it has been reported that mutation of *KOR*, a homolog of *OsGLU1*, caused dwarfism (Nicol *et al*., 1998; Zuo *et al*., 2000). The dwarf mutant *kor1-1* and *kor1-2* were obtained from a T-DNA-tagged population of Arabidopsis. KOR was located primarily in the plasma membrane by immunoblot analysis of membrane fraction with anti-KOR antiserum and has been linked to cell expansion in the weak *kor-1* (Nicol e*t al*., 1998). However KOR-GFP fusion protein was localized to growing cell plates of tobacco BY2 cells. Results from another mutant, *kor-2* suggest that KOR played a critical role during cytokinesis (Zuo *et al*., 2000). Function of KOR has also been studied in two temperature-sensitive mutants *rsw2-1* and *acw1* (Lane *et al*., 2001; Sato *et al*., 2001). Sequencing *KOR* from *rsw2-1* and *acw1* showed that both *KOR* genes from the two mutants had the same mutation in which a larger Arg replaced a small Gly at the 429th position. The shoots of *rsw2-1* seedling had a reduction in the content of cellulose and accumulated a short chain, readily extractable glucan, suggesting that the endo-β-1,4-D-glucanase is involved in cellulose synthesis in primary cell walls, and also cell elongation and cytokinesis of Arabidopsis (Lane *et al*., 2001). Analysis of the cell walls of *acw1* grown at 31°C showed that the cellulose content was reduced by 40% and the pectin content was increased by 162% compared with that of wild-type. Field emission scanning electron microscopy revealed that the structure of cellulose bundles was affected in *acw1* (Sato *et al*., 2001). These studies revealed that the endo-β-1,4-D-glucanase was important for the growth of Arabidopsis. Consistent with these observations, the present study also demonstrated that mutation of the *OsGLU1* affected the cell wall components and then altered the plant height. The amino acid sequence of OsGLU1 is 75% identical to the Arabidopsis KOR and 76% identical to the tomato Cel3. Conservation of the sequence between dicots and monocots suggests a functional specialization preceding the divergence between monocots and dicots in evolution.

In the dwarf mutant *glu*, the T-DNA was inserted in the second intron of the *OsGLU1*. The actin promoter located at the left border of the T-DNA can drive the transcription of about 1.4 kb 3′-*OsGLU1* gene. Translation starting from an initiation codon resulted in a wrong reading frame of 1.4 kb in 3′-*OsGLU1* gene. Therefore insertion of T-DNA resulted in loss-function of *OsGLU1*. OsGLU1 is involved in the growth and development of rice plant, resembling KOR in Arabidopsis.

It has been shown that the endo-β-1,4-D-glucanase can be classified into two subfamily (Nicol *et al*., 1998). One subfamily of EGase in plants and bacteria are soluble secreted enzymes, containing N-terminal signal peptide and EGase domain, and are localized to the periplasm where they have function as cell wall-loosening enzymes to modify the cell wall. In Arabidopsis, there are at lease 17 genes encoding that subfamily of EGase. Another subfamily of EGase is integral membrane proteins, which have a predicted N-terminal transmembrane anchor motif (Brummell *et al*., 1997). OsGLU1, like KOR and Cel3, belongs to the latter, and it contains six potential N-glycosylation sites and the conserved polarized targeting signals.

In Arabidopsis, membrane-anchored EGases belong to a small gene family of three genes: *KOR1*, *KOR2* and *KOR3* (Nicol *et al*., 1998; Zuo *et al*., 2000; Molhoj *et al*., 2001a). *KOR1* is expressed throughout the plant, whereas *KOR2* is expressed in root hairs within root differentiation zone, proximal parts of leaves and floral organs, and trichomes. Interestingly, *KOR3* is expressed in the trichome support cells (Molhoj *et al*., 2001a). In rice, in addition to the *OsGLU1*, 14 additional *OsGLU1* homologous genes are also identified according to the present study and previous report (Libertini *et al*., 2004). Based on phylogenetic analysis, the *OsGLU* family can be divided into three subfamilies. OsGLU1, OsGLU2, OsGLU3, KOR1, and TomCel3 belong to the membrane-anchored EGase. *OsGLU1*, *OsGLU2* and *OsGLU3* are constitutively expressed in all the organs examined. In contrast, the other seven *OsGLU* genes identified presently encode the soluble secreted EGase. They exhibited different organ-specific expressions. Except that *OsGLU8* had no

expression in roots, the other nine *OsGLU* genes were expressed in developing flowers and roots, suggesting that *OsGLUs* play important roles in flower and root development. OsGLU1 showed highest similarity to KOR1 and TomCel3 among all *OsGLUs* examined. Three membrane-anchored EGase genes *OsGLU1*, *OsGLU2* and *OsGLU3* in rice had similar expression pattern, suggesting that they possibly had functional redundancy. However, different expression patterns were observed for the three membrane-anchored EGase genes *KOR1*, *KOR2* and *KOR3* in Arabidopsis. The divergence in expression patterns between membrane-anchored EGases in rice and Arabidopsis may indicate specific functions for these genes in different plant species referring to dwarfism.

The expression of *OsGLU1* was induced by GA and BR. However, indole-3-acetic acid, ethylene, abscisic acid did not affect the expression of *OsGLU1*, suggesting that *OsGLU1* gene may act in altering the structure of the cell wall in response to GA and BR. Many mutants with GA and BR biosynthesis affected internode elongation and had dwarf phenotype. It has been reported that GA and BR regulated some enzymes that are involved in the construction of the cell wall. Transcriptions of *KOR* and *TomCel3* were not induced by auxin, ethylene, and GA, whereas reduction of *KOR* mRNA level was observed in BRs-deficient mutant. *TCH4* and *BRU1*, encoding a xyloglucan-endotransglycosylase (XET)-homolog, were regulated by BR (Kauschmann *et al*., 1996). Expression of two rice *XET*-related genes, *OsXTR1* and *OsXTR3*, can be increased upon treatment with either GA or BR (Uozu *et al*., 2000). XETs are thought to be the cell wall-loosening enzyme for cell elongation. Transgenic rice expressing RNAi construct *OsXTR8* exhibited repressed growth (Jan *et al*., 2004). These studies suggest that some enzymes such as OsGLU1 may be involved in the construction of cell wall through the regulation of GA and BR.

Dwarfism is one of the most important agronomic traits that result in lodging resistance and high yield. OsGLU1 may play a significant role in the control and regulation of plant height. Manipulation of this gene in crops should be beneficial for the improvement of the relevant agronomic traits.

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