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A new genetic factor for root gravitropism in rice (*Oryza sativa* L.)*

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Abstract: Root gravitropism is one of the important factors to determine root architecture. To understand the mechanism underlying root gravitropism, we isolated a rice (Xiushui63) mutant defective in root gravitropism, designated as *gls1*. Vertical sections of root caps revealed that *gls1* mutant displayed normal distribution of amyloplast in the columella cells compared with the wild type. The *gls1* mutant was less sensitive to 2,4-dichlorophenoxyacetic acid (2,4-D) and α-naphthaleneacetic acid (NAA) than the wild type. Genetic analysis indicated that the phenotype of *gls1* mutant was caused by a single recessive mutation, which is mapped in a 255-kb region between RM16253 and CAPS1 on the short arm of chromosome 4.

Key words: *Oryza sativa* L., Root gravitropism, Genetic factor
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INTRODUCTION

Rice is an important food crop in the world. Rice root system is mainly composed of primary, adventitious, and lateral roots, which play vital roles in acquiring water and nutrients and in anchoring plants in the soil. Root gravitropism is one of the important factors to determine root architecture, and root gravitropic sensitivity affects spatial distribution of root system in the soil and consequently the nutrient uptake efficiency (Lynch, 1995; Rubio *et al*., 2001).

The Cholodny-Went theory proposes that lateral auxin transport and its asymmetrical redistribution across organs upon gravistimulation are essential for normal gravitropic curvature responses (Chen *et al*., 2002; Blancaflor and Masson, 2003; Perrin *et al*., 2005; Harrison and Masson, 2008). Yet the mechanisms that mediate gravity perception in plants remain poorly understood. The starch-statolith hypothesis postulates that gravity perception in plants is mediated by the sedimentation or pressure/tension exerted by starch-filled statoliths within the gravity-perceiving columella cells in the root caps and the endodermal starch sheath cells in shoots (Morita and Tasaka, 2004; Stanga *et al*., 2009). Numerous studies have shown that amyloplasts are important in gravity perception. Starch deficient *pgm1* mutant lacks a normal response to gravistimulation compared with the wild type (WT) (Caspar and Pickard, 1989; Kiss *et al*., 1989), and mutants with intermediate levels of starch are more gravisensitive than starchless mutants but are less sensitive than the WT (Kiss *et al*., 1997), while the starch excess mutant *sex1* displays an increased sensitivity to gravistimulation (Vitha *et al*., 2007).

Until now, there has been no definitive description of the mechanism that senses the position or movement of amyloplasts within the statocytes (Perrin *et al*., 2005). The cytoskeleton is proposed to interact with the sedimenting amyloplasts in the processes of gravity perception and signal transduction (Baluška and Hasenstein, 1997). It has been proposed that sedimenting amyloplasts may activate mechano-sensitive ion channels at the plasma membrane or the endoplasmic reticulum by sedimentation onto them, by exerting pressure on the actin cytoskeleton, or by disrupting dense actin network

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(Harrison and Masson, 2008).

Auxin regulates a variety of growth and development processes, including cell elongation, cell division, lateral root formation, and tropic responses (Hobbie, 1998). In *Arabidopsis* many auxin signal related mutants, such as *axr1* (Lincoln *et al*., 1990), *axr2* (Wilson *et al*., 1990), *axr3* (Leyser *et al*., 1996), *axr4* (Hobbie and Estelle, 1995), *axr5* (Yang *et al*., 2004), and *axr6* (Hobbie *et al*., 2000), display pleiotropic phenotypic defects, including decreased lateral root number, agravitropic response, and suppressed root inhibition, providing the evidences that auxin plays a pivotal role in these processes. In rice, auxin resistant mutants *lrt1* (Hao and Ichii, 1999) and *lrt2* (Wang *et al*., 2006) were isolated, and both mutants showed defects in lateral root formation and altered root gravitropic response, suggesting that auxin is required for normal root growth.

In this study, we isolated and characterized a rice mutant defective in root gravitropism, designated *gls1* (*gravitropism loss 1*). The *gls1* mutant displayed decreased sensitivity to auxin. We mapped *GLS1* using both simple sequence repeats (SSRs) and cleaved amplified polymorphic sequence (CAPS) markers.

MATERIALS AND METHODS

Plant growth conditions and mutant isolation

Hydroponic culture was carried out using rice (*Oryza sativa* L.) culture solution (Yoshida *et al*., 1976). Paper pouch culture was conducted using blue no-phosphorus paper enclosed by plastic bag filled with 100 ml rice culture solution, the germinated seeds were transferred onto the paper after sterilization, and the bags were hanged vertically in the growth chamber. Phenotypic characterization of the WT and mutant was performed in a growth chamber at 30/22 °C (day/night) and 60%~70% humidity under a photoperiod of 12 h.

The *gls1* mutant was isolated from an ethyl methane sulfonate (EMS)-generated rice mutant library (*Oryza sativa* L. *japonica* cv. Xiushui63) under rice culture solution. On Day 4, plants defective in root gravitropism were transferred to the soil. Progeny was then re-tested under the same conditions.

Microscopic analysis

For microscopic analysis of amyloplast sedimentation, the *gls1* and WT seedlings were cultivated for 6 d in nutrient solution, then kept vertical in solution (for control) or horizontally placed (for gravistimulation) in plastic net floating in culture solution for 1 h. Root tips from vertically and horizontally grown plants were fixed overnight at 4 °C in 2.5% (v/v) glutaraldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.2) and then washed three times for 30 min in the same buffer. Root samples were then postfixed in osmium tetroxide (OsO_4) for 4 h at room temperature and washed for 30 min in the same buffer. Samples were dehydrated through a gradient ethanol (30%, 50%, 70%, 80%, 90%, 95% (v/v) and three changes of 100% ethanol) for 30 min each, and then infiltrated through ethanol. The samples were embedded in pure Spurr resin, and polymerized overnight at 70 °C. Semithin sections (2 μm thick) were made using glass knives on a power Tome XL microtome (RMC-Boeckeler Instruments, Tucson, AZ, USA) and stained with 0.1% (w/v) methylene blue for 3~5 min at 70 °C. The samples were rinsed with distilled water and visualized with a Zeiss Axiovert 200 microscope with a color charge-coupled device camera (Zeiss, Jena, Germany) (Jia *et al*., 2008).

Exogenous auxin and 1-*N*-naphthylphthalamic acid treatments

The *gls1* and WT seeds were germinated in distilled water, and placed in plastic net floating in rice culture solution (α-naphthaleneacetic acid (NAA) at 0.01 μmol/L or 0.1 μmol/L, 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.01 μmol/L or 0.1 μmol/L, 1-*N*-naphthylphthalamic (NPA) at 0.5 μmol/L). The seedlings were sampled on Day 10 after germination for NAA and 2,4-D treatments. On Day 7 after germination for the NPA treatment, primary root length and shoot height were measured by counter ruler, and lateral root length and number were analyzed using a scanner connected to an image-analysis system WinRHIZO (Regent, Canada).

Phenotypic analysis of *gls1* mutant and mapping of *GLS1* gene

The root tips and surfaces were examined and photographed using a Leica MZ95 stereomicroscope

with a color charge coupled device (CCD) camera. Root lengths and numbers were analyzed as described as above.

For genetic analysis and mapping, *gls1* mutant was crossed with Kasalath (*Oryza sativa* L. *indica* cv. Kasalath; WT seeds provided by International Rice Research Institute (IRRI)), and F2 progenies showing *gls1* mutant phenotype were selected. *GLS1* gene was mapped with SSRs and CAPS markers using 265 F_2 mutant plants.

RESULTS

Isolation and characterization of *gls1* mutant

Seeds from the M2 generation of an ethyl methane sulfonate-mutagenized population of rice (Xiushui63) were germinated and grown in rice nutrient solution (Yoshida *et al*., 1976) to screen mutants with abnormal root development. A rice mutant defective in root gravitropism on Day 4 after germination was isolated and designated as *gls1*. Under paper pouch culture, *gls1* mutant led to a loss of root gravitropism on Day 10 after germination (Fig.1a). Under nutrient solution culture, the *gls1* mutant exhibited impaired primary root gravitropism on Day 4 (Fig.1b) and loss of gravitropism of the whole root system on Day 40 (Fig.1c).

Under paper pouch culture for 10 d, the primary root length and the shoot height of the mutant were about 61% and 81.5% of those of the WT, respectively. Average lateral root length of the mutant was 0.6 times longer than that of the WT (Table 1). No significant (P>0.05) differences between the mutant and the WT plants were observed in adventitious root number, average adventitious root length, or lateral root number evaluated (Table 1).

Sedimentation of amyloplasts was analyzed at a cellular level for the WT and the *gls1* mutant. Vertical sections from root tips of 6-d-old seedlings grown in nutrient solution indicated that the sedimentation of amyloplasts in response to gravity in the mutant root cap was similar to that in the WT without gravistimulation (Figs.2e~2h), and with the treatment of 1-h gravistimulation, the *gls1* mutant showed normal amyloplast sedimentation in the columella cells compared with the WT (Figs.2a~2d). We therefore concluded that amyloplasts in root cap of the *gls1* mutant could normally sediment toward the direction of gravity vector compared with those in the WT, although we cannot yet exclude the possibility that the mutation affects the rate of amyloplast sedimentation relative to the WT.

Auxin responses of *gls1* mutant

To examine the effect of exogenous auxin on the *gls1* mutant root growth, seeds of the WT and the *gls1* mutant were germinated and grown in nutrient solution supplied with different concentrations of synthetic auxin analogue, NAA and 2,4-D.

Treatments with 0.01 and 0.1 μmol/L NAA significantly decreased the length of primary root in both WT and *gls1* mutant, but the *gls1* mutant was less sensitive than the WT. At 0.01 μmol/L NAA, the primary root lengths of the WT and the *gls1* mutant were decreased by 22% and 7.9%, respectively. At 0.1 μmol/L NAA, the primary root lengths of the WT and the *gls1* mutant were decreased by 73.2% and 51.1%, respectively (Figs.3a~3d). Treatments with 0.01 and 0.1 μmol/L NAA also significantly decreased lateral root numbers in both WT and *gls1* mutant. At 0.01 μmol/L NAA, the lateral root numbers of the WT and the *gls1* mutant were decreased by 31.5% and 15.9%, respectively, whereas at 0.1 μmol/L NAA, the lateral root numbers of WT and *gls1* were decreased by 61.1% and 30.8%, respectively (Figs.3g and 3h).

Table 1 Characterization of 10-d-old seedlings of the WT (Xiushui63) and *gls1* mutant under paper pouch

Plant	Primary root length (cm)	Shoot height (cm)	Adventitious root No.	Average AR length (cm)	Lateral root No.	Average LR length (cm)
WT	20.56±1.40	9.28±0.16	4.20±0.86	6.74±0.34	225.60±18.80	0.14±0.02
gls1	12.54±2.46*	7.56±0.35*	4.40±0.25	7.22±0.45	213.40±25.09	0.22±0.03*

Data represent the mean±SE of 5 plants in each line. AR and LR stand for adventitious root and lateral root, respectively. Asterisks indicate significant differences between *gls1* mutant and the WT (*P<0.05, Student's t-test)

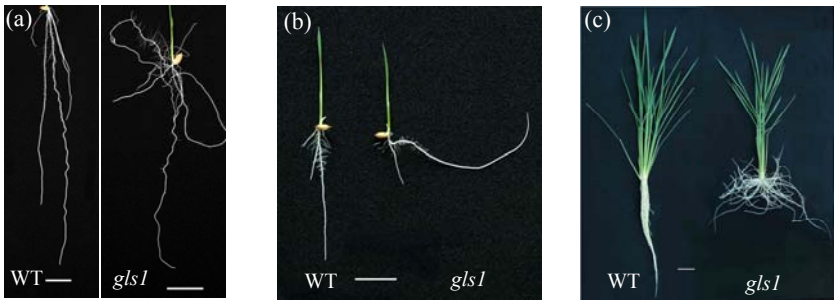

Fig.1 Phenotypes of *gls1* mutant. (a) Root phenotypes of WT and *gls1* in 10-d-old plants grown in paper pouch; (b) 4-d-old WT and *gls1* seedlings grown in rice nutrient solution; (c) 40-d-old WT and *gls1* plants grown under rice nutrient solution culture

Bar=2 cm

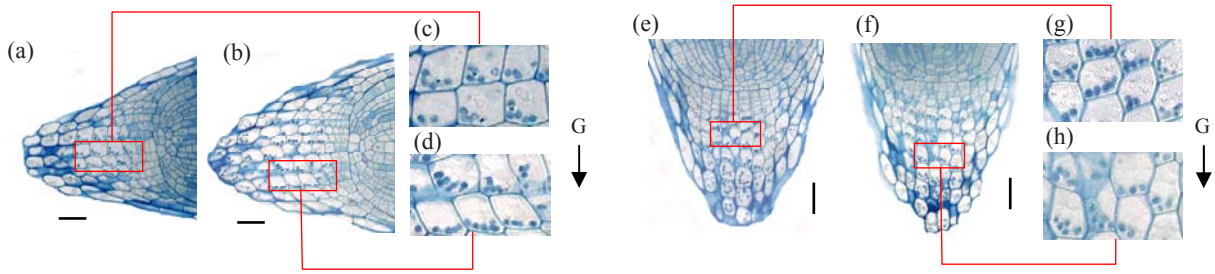

Fig.2 Amyloplast sedimentation in response to gravity. (a), (b) Amyloplast sedimentation in root cap of 6-d-old seedlings from (a) the WT and (b) the *gls1* mutant treated with 1-h gravistimulation; (c), (d) Magnified sections (3×) of (a) the WT and (b) the *gls1* mutant, respectively, showing amyloplast sedimentation in reponse to gravistimulation; (e), (f) Amyloplast sedimentation in root cap of 6-d-old seedlings from vertically grown (e) WT and (f) *gls1* mutant; (g), (h) Magnified sections of (e) the WT and (f) the *gls1* mutant, respectively, showing amyloplast sedimentation in reponse to gravity

Bar=50 μm; The arrows show the direction of gravity vector (G)

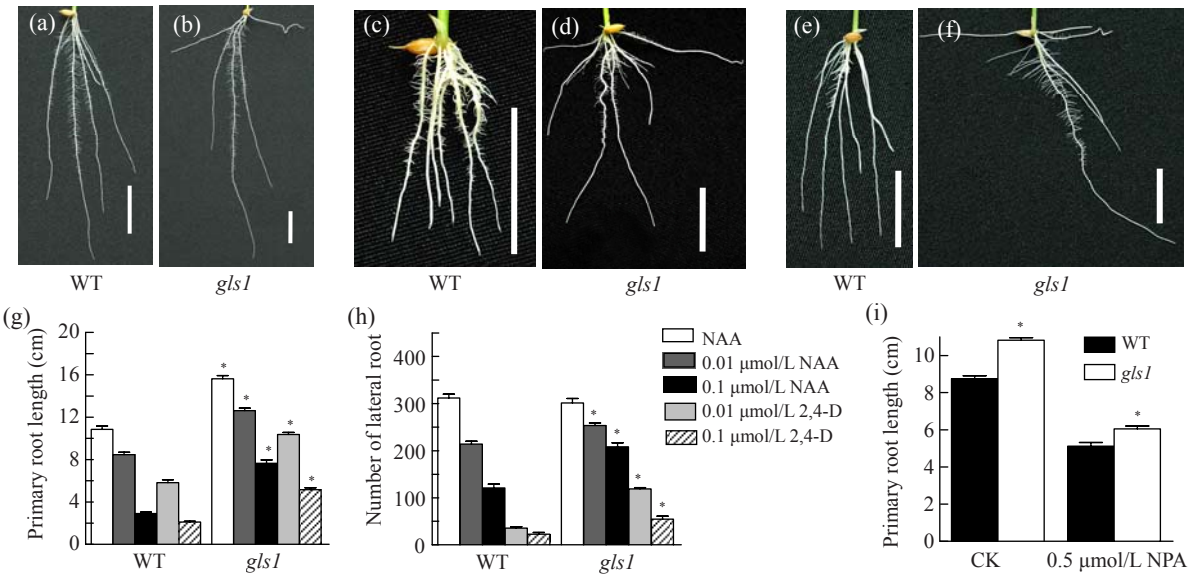

Fig.3 Effect of auxin treatment on *gls1* mutant. (a), (c), (e) Root phenotypes of WT under (a) control, (c) 0.01 μmol/L 2,4-D and (e) 0.1 μmol/L NAA; (b), (d), (f) Root phenotypes of *gls1* mutant under (b) control, (d) 0.01 μmol/L 2,4-D and (f) 0.1 μmol/L NAA; (g), (h) Auxin repression of root development was quantified by measuring (g) the primary root length and (h) lateral roots number; (i) Effect of NPA on the primary root elongation in the WT and the *gls1* mutant

The values of 5 seedlings were averaged; Asterisks indicate significant differences between the *gls1* mutant and the WT at various treatments ($^*P<0.05$, Student's *t*-test); Bar=2 cm

Treatments with 0.01 and 0.1 μmol/L 2,4-D significantly decreased the primary root length in both the WT and *gls1* mutant. At 0.01 μmol/L 2,4-D, the primary root lengths of the WT and the *gls1* mutant were decreased by 44.3% and 34.7%, respectively, and at 0.1 μmol/L 2,4-D, the primary root lengths of the WT and the *gls1* mutant were decreased by 79.9% and 67.5%, respectively (Figs.3a, 3b, 3e, and 3f). Treatments with 0.01 and 0.1 μmol/L 2,4-D also significantly decreased the lateral root number in both the WT and *gls1* mutant. At 0.01 μmol/L 2,4-D, the lateral root numbers of the WT and the *gls1* mutant were decreased by 89.9% and 57.4%, respectively. At 0.1 μmol/L 2,4-D, the lateral root numbers of the WT and the *gls1* mutant were decreased by 93.6% and 80.3%, respectively (Figs.3g and 3h). In addition, the response of *gls1* roots to auxin transport inhibitor NPA was similar to that of the WT (Fig.3i). Taken together, we concluded that the *gls1* mutant is insensitive to auxin compared with the WT.

Genetic analysis and *GLS1* gene mapping

For genetic analysis, seeds of F_1 progeny were derived from a cross between the *gls1* mutant and the *indica* cv. Kasalath. The F_1 seedlings showed the WT phenotype. F_2 progenies displayed a ratio of 3:1 $[151:32, \chi^2 = 0.021 < \chi^2_{0.05} = 3.841]$ segregation of the WT:mutant phenotype, suggesting that the mutant phenotype was controlled by a single recessive gene.

For mapping *GLS1*, we used 120 SSR markers distributed with 10~30 cM intervals on 12 chromosomes. By using 265 F_2 mutant plants, the *GLS1* was mapped on chromosome 4 linked to RM16260. To further mapping, markers RM16285, RM16253, and the new generated CAPS1 (forward primer: CTAAA CTGGCCTAGATCCTTTTC; reverse primer: GTAA TGCATCATACAACATGA; restriction enzyme, ClaI) on the flanked region were used. The mapping result shows that the *GLS1* gene was located in a 255-kb region between RM16253 and CAPS1 on the short arm of chromosome 4 (Fig.4). There were 24 putative genes (including 2 miRNAs) and many other small RNAs (http://sundarlab.ucdavis.edu/cgi-bin/smrnabrowse/rice2/) in this 255-kb region based on the database (http://www.tigr.org) (Table 2).

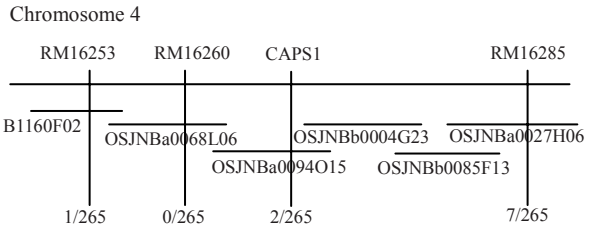

Fig.4 The result of *GLS1* mapping

Chr: chromosome; CAPS: cleaved amplified polymorphic sequence

Table 2 Annotation of candidates *GLS1* gene*

Locus name	Annotation
RM16253 (63 061 bp)	
LOC_Os04g01110.1	BED zinc finger family protein
LOC_Os04g01130.1	MRG family protein
LOC_Os04g01140.1	Cytochrome P450 family protein
Osa-mir806f	
Osa-mir806h	
LOC_Os04g01150.1	Phagocytosis and cell motility protein ELMO1
LOC_Os04g01160.1	Zinc ion binding, ubiquitin-protein ligase
LOC_Os04g01170.1	Unknown
RM16260 (167 710 bp)	
LOC_Os04g01230.1	Phosphoglycerate mutase
LOC_Os04g01240.1	Unknown
LOC_Os04g01250.1	Amidase family protein
LOC_Os04g01270.1	Unknown
LOC_Os04g01280.1	Glycosyltransferase family
LOC_Os04g01290.1	PCI domain containing, proteasome family protein
LOC_Os04g01300.1	Rhomboid protein-related contains 6 transmembrane domains
LOC_Os04g01310.1	D-mannose binding lectin family, tyrosine kinase
LOC_Os04g01320.1	D-mannose binding lectin family, tyrosine kinase
LOC_Os04g01330.1	Unknown
LOC_Os04g01354.1	Chalcone and stilbene synthases
LOC_Os04g01380.1	Unknown
LOC_Os04g01390.1	Unknown
LOC_Os04g01460.1	Unknown
LOC_Os04g01470.1	*O*-methyltransferase family
LOC_Os04g01480.1	Zinc finger family protein
CAPS1 (318 241 bp)	

* http://www.tigr.org; BED: Drosophila BEAF and DREF related domain; MRG: human MORF4 related gene; ELMO1: the engulfment and cell motility 1 gene; PCI: proteasome-COP9-initiation factor

DISCUSSION

In this study, we isolated and characterized a root *gls1* mutant. The *gls1* mutant displays a defect in

root gravity response and decreased sensitivity to auxin. The phenotype is controlled by a single recessive mutation. There is a great deal of cell biological and physiological evidence demonstrating that starch-containing amyloplasts in columella cells of the root cap are significant for gravity sensing (Chen *et al*., 2002; Boonsirichai *et al*., 2002). Mutants defective in starch synthesis are less sensitive to the gravity and lead to root gravitropism loss to some extent (Vitha *et al.*, 2000). The sedimentation of amyloplasts within the columella cells is proposed to constitute one of the initial acts of gravity sensing in roots (Chen *et al*., 2002; MacCleery and Kiss, 1999). Our results show that the differentiation of amyloplasts in columella cells of the *gls1* mutant is normal compared with the WT, and that the amyloplasts can normally sediment toward the direction of gravity vector in the *gls1* mutant (Figs.2a and 2h). Therefore the sedimentation of amyloplasts in *gls1* mutant cannot explain the mechanisms underlying *gls1* mutant phenotype.

The plant hormone auxin regulates a variety of physiological processes, including tropic responses, lateral root formation, and primary root growth (Woodward and Bartel, 2005). NAA at 0.1 μmol/L and 2,4-D at 0.01 μmol/L significantly decreased the lateral root number and the primary root length in the WT, while the *gls1* mutant is less sensitive to the two types of auxin stimuli (Figs.3a~3f). In *Arabidopsis*, auxin influx carrier mutant *aux1* is an auxin-resistant mutant and shows aberrant root gravitropism response phenotype. The exogenously added auxin (NAA) can rescue defects in *aux1* (Marchant *et al*., 1999; Marchant *et al*., 2002), NAA corrected the aberrant gravitropic response to the normal level, and *aux1* was not resistant to NAA at all (Yamamoto and Yamamoto, 1998). It has been reported that (NAA) treatment is able to rescue the mutated phenotypes occurring in the *OsPIN1* RNAi rice plants (Xu *et al*., 2005). Auxin efflux carrier mutant *agr1* is involved in root gravitropism, and confers increased root growth sensitivity to auxin (Chen *et al*., 1998). In *Arabidopsis* several auxin-resistant mutants, such as *axr4* and *axr6*, have been reported to display defects in root gravitropic response and lateral root development (Hobbie and Estelle, 1995; Hobbie *et al*., 2000), confirming that auxin functions in regulating root gravitropism. In our study, the *gls1* mutant was insensitive to auxin compared with the WT (Figs.3a~3h), and *gls1* showed the normal sensitivity to the auxin transport inhibitor NPA compared with the WT (Fig.3i). These results suggest that *gls1* mutant is involved in auxin response process.

In rice, little information has been so far available on the molecular mechanisms underlying root gravitropism. The isolation and characterization of the *gls1* mutant will enable us to investigate the function of the *GLS1* gene and to better understand the mechanisms for auxin-mediated regulation of root gravitropism. By searching the Institute for Genomic Research (TIGR) Rice Genome Annotation Database (http://www.tigr.org), we have found several candidate genes in the *GLS1* locus, which are related to auxin signaling pathway (Table 2). Currently, we are conducting a high-resolution mapping of *GLS1* toward its cloning and functional analysis.

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