Cell Research (2009) 19:1110-1119.

www.nature.com/cr

ORIGINAL ARTICLE

Adventitious root formation in rice requires OsGNOM1 and is mediated by the OsPINs family

Shiping Liu[1, 2], Jirong Wang[1], Lu Wang[1], Xiaofei Wang[1], Yanhong Xue[1], Ping Wu[1], Huixia Shou[1]

[1]*State Key Laboratory of Plant Physiology and Biochemistry, College of Life Science, Zhejiang University, Hangzhou 310058, China;* [2]*College of Chemistry and Life Science, China Three Gorges University, Yichang 443002, China*

The fibrous root system in cereals comprises primarily adventitious roots (ARs), which play important roles in nutrient and water uptake. Current knowledge regarding the molecular mechanism underlying AR development is still limited. We report here the isolation of four rice (*Oryza sativa* L.) mutants, from different genetic backgrounds, all of which were defective in AR formation. These mutants exhibited reduced numbers of lateral roots (LRs) and partial loss of gravitropism. The mutants also displayed enhanced sensitivity to *N*-1-naphthylphthalamic acid, an inhibitor of polar auxin transport (PAT), indicating that the mutations affected auxin transport. Positional cloning using one of the four mutants revealed that it was caused by loss-of-function of a guanine nucleotide exchange factor for ADP-ribosylation factor (OsGNOM1). RT-PCR and analysis of promoter::GUS transgenic plants showed that *OsGNOM1* is expressed in AR primordia, vascular tissues, LRs, root tips, leaves, anthers and lemma veins, with a distribution pattern similar to that of auxin. In addition, the expressions of *OsPIN2*, *OsPIN5b* and *OsPIN9* were altered in the mutants. Taken together, these findings indicate that OsGNOM1 affects the formation of ARs through regulating PAT.

***Keywords*:** *Oryza sativa* L., adventitious root, OsGNOM1, polar auxin transport

Cell Research (2009) **19**:1110-1119. doi: 10.1038/cr.2009.70; published online 23 June 2009

Introduction

In contrast to the tap root system of *Arabidopsis thaliana*, monocot cereals consist almost entirely of a complex fibrous root system, comprising a mass of adventitious roots (ARs) that develop from the stem base postembryonically. Rice (*Oryza sativa* L.) is a model cereal crop with seminal roots that die during the growing period [1]. Thus, lateral roots (LRs) and AR are the key determinants of nutrient and water use efficiency in rice.

A number of *Arabidopsis* mutants with defects in root development have been characterized [2, 3]. These research efforts have comprehensively documented the molecular control and hormonal regulation of root initiation and development in dicotyledonous plants [3-5]. In recent years, an increasing number of cereal mutants with impaired root formation have been discovered [6-9]. However, due to the complex structure and intricate regulation of their root systems, the mechanism of AR development is still far from clear.

Phytohormone is required for the establishment of appropriate root architecture. It affects the number and length of LR and AR [2]. Many genes required in root development have connections with the auxin-signaling pathway [4, 10], including the Aux/IAA family [11], the auxin response factor family [12] and certain hormone-related transporters [13]. An LOB domain-containing transcription factor controlled by auxin responsive factor was known to play a critical role in the formation of AR primordia in rice and maize (*Zea mays* L.) [8-9, 14].

GNOM is a large guanine nucleotide exchange factor (GEF) for ADP-ribosylation factor (ARF), a small GTPase. GNOM affects polar auxin transport (PAT) and development in *Arabidopsis* [15-18]. Over 20 allelic mutants of *gnom* have been identified [19-21]. Most of these mutants exhibited severe defects in both early embryonic structure and subsequent post-embryonic development such as LR formation and gravitropism, resulting in lethality [19, 22, 23]**.** GNOM is found in

Correspondence: Ping Wu[a], Huixia Shou[b]
[a]Fax: +86 571 88206146;
E-mail: clspwu@zju.edu.cn
[b]E-mail: huixia@zju.edu.cn
Received 15 December 2008; revised 9 March 2009; accepted 24 March 2009; published online 23 June 2009

endosomal compartments and catalyzes guanine nucleotide exchange through its SEC7 domain, which is highly conserved among animals, plants and fungi [21, 24-26]. Except for GL1 in *Arabidopsis*, most large ARF-GEFs in plants (including GNOM) are predicted to be sensitive to the fungal toxin brefeldin A (BFA), a specific inhibitor of membrane protein trafficking [27, 28]. BFA treatment can change the cellular localization of GNOM, yielding a mutant phenotype similar to that of *gnom* mutants [18]. BFA also regulates vesicle formation by activating ARF GTPases on specific membranes [18, 27, 29]. In *gnom* mutants, the auxin efflux carrier component PIN1 (PIN-FORMED 1) is mislocated, suggesting that GNOM regulates the cellular distribution and trafficking of PIN1 in *Arabidopsis* [30-32].

In this study, we isolated four lethal rice mutants from mutant libraries generated from japonica variety Zhonghua11 and from indica variety Kasalath. These mutants have reduced numbers of LR and lack AR. The phenotypes are similar to those of wild-type (WT) plants treated with *N*-1-naphthylphthalamic acid (NPA), an inhibitor of PAT. Using a positional cloning strategy, we identified that the *OsGNOM1* gene is responsible for the mutations. We also found that the transcript levels of *OsPIN2*, *OsPIN5b* and *OsPIN9* were altered in mutants in the basal portion of the shoots where the AR primordia are initiated, suggesting that OsGNOM1 is required to maintain the normal distribution of auxin during the initiation of AR.

Results

Isolation and characterization of gnom1 rice mutants

Four rice mutants, here designated as gnom1-1 to gnom1-4 (see later for the cloning of *OsGNOM1*), were isolated from mutant libraries generated from japonica variety Zhonghua11 (*gnom1-1*) and indica variety Kasalath (*gnom1-2*, *gnom1*-3 and *gnom1*-4), as described in Table 1. These mutants lacked AR during the early stages of growth and exhibited a reduced number of LR and partial agravitropism (Figure 1A to 1D, and Supplementary information, Table S1). A small number of AR emerged at 6 weeks after germination (Figure 1E), whereas no mutants survived longer than 2 months. When all roots from the base of the stem were surgically removed, the WT seedlings initiated new roots in 7 days but the mutants did not (Figure 1F), indicating that these mutants had lost the ability to regenerate new roots.

Longitudinal and cross-sections obtained from the basal portion of the stem of 5-day-old seedlings showed that AR primordia formation was impaired in the *gnom1* mutants (Figure 1G to 1J). Methylene blue staining [33] of seminal roots showed that the number of LR primordia was also significantly reduced (Figure 1K and 1L).

Genetic analysis

The *OsGNOM1* locus was cloned using a map-based cloning strategy. The F_2 segregation population derived from a cross between a heterozygous *gnom1-1* and the indica cultivar Kasalath was used for mapping. AR development was normal in the F_1 generation, indicating that a recessive mutation controlled the phenotype. *OsGNOM1* was mapped to the long arm of chromosome 3 between the single sequence repeat (SSR) marker RM15621 and a sequence-tagged site (STS) marker STS18 using 120 mutants (Figure 2A). To finely map this locus, 962 mutant seedlings were further analyzed using three newly developed STS markers: STS2, STS14 and STS19. Sequencing the genomic region between STS2 and STS14 in WT and mutant plants revealed that there was a guanine deletion in the third exon of the *OsGNOM1* gene (accession number: EAY91294.1, Figure 2B); the deletion results in a frame shift. Subsequent sequencing of *OsGNOM1* in three other mutants (*gnom1-2*, *gnom1-3* and *gnom1-4*) confirmed mutations in the *OsGNOM1* gene. *gnom1-2* has a C/A substitution at position 4 447, resulting in premature termination of protein synthesis (Figure 2B and Table 1). *gnom1-3* and *gnom1-4* have GA and TG deletions at positions 5 799 and 3 107, respectively (Figure 2B and Table 1). These deletions result in frameshift and the formation of non-functional *OsGNOM1* proteins.

Alignment of OsGNOM1 with GNOM in Arabidopsis revealed the presence of three exons encoding six conserved domains (Figure 2B). Both *gnom1-1* and *gnom1-2* involves mutations in the HDS1 (homology downstream of SEC7) domain, while *gnom1-3* and *gnom1-4* have a

Table 1 Features of *gnom1* alleles in rice

Mutant name	*gnom1-1*	*gnom1-2*	*gnom1-3*	*gnom1-4*
Genetic background	Zhonghua 11, Japonica	Kasalath, Indica	Kasalath, Indica	Kasalath, Indica
Mutagen	Tissue culture	EMS	γ radiation	γ radiation
Mutation	G deletion	C/A substitution	GA deletion	TG deletion
Position	4 328	4 447	5 799	3 107
Domain	HDS1	HDS1	HDS3	HUS

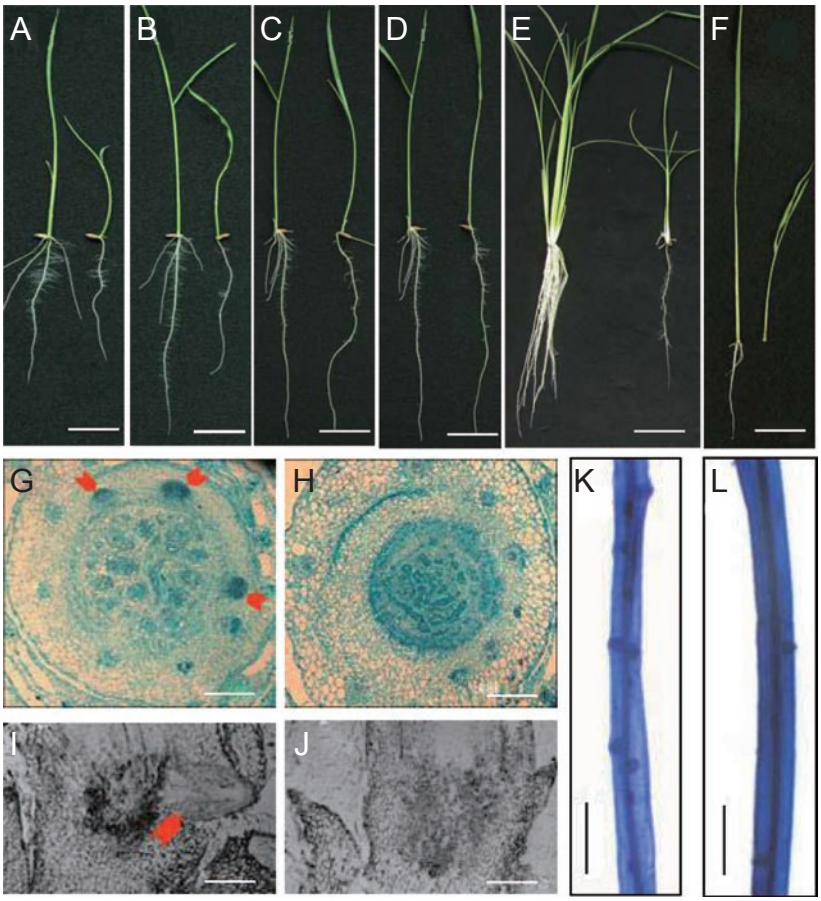

Figure 1 Phenotypes of rice *gnom1* mutants. **(A-D)** Seven-day-old seedlings representing *gnom1-1* to *gnom1-4*. The mutant is on the right and wild type is on the left. Bars = 2 cm. **(E)** Six-week-old seedlings (left: Zhonghua 11; right: *gnom1-1*). Bar = 5 cm. **(F)** WT (left) and *gnom1-1* (right) at 1 week after surgically removing roots, Bar = 2 cm. **(G-H)** Stem-base cross-sections of the WT **(G)** and *gnom1-1* **(H)**; the arrows show the AR primordia. Bars = 100 μm. **(I-J)** Stem-base vertical sections of the WT **(I)** and *gnom1-1* **(J)**; the arrows show the AR primordia. Bars = 200 μm. **(K** and **L)** Methylene blue staining of seminal roots of WT **(K)** and *gnom1-1* **(L)**. Bars =200 μm.

mutation in the HDS3 and HUS (homology upstream of SEC7) domains (Figure 2B and Table 1), respectively.

To confirm the positional cloning of these mutants, we developed two dCAPS (derived cleavage amplify polymorphism) markers, C1 for *gnom1-1* and C2 for *gnom1-2* (Supplementary information, Figure S1). The heterozygote mutants, homozygote mutants and WT genotypes can be easily distinguished in the segregation population using these dCAPS markers (Figure 2C). Moreover, the mutation of *OsGNOM1* in *gnom1-3* was also confirmed by *Cel* I digestion, a restriction enzyme that digests DNA specifically at mismatch sites (Figure 2D).

Genetic complementation of the *gnom1-1* mutant was carried out by introducing a 4.8-kb WT cDNA fragment of *OsGNOM1* into modified pCAMBIA 1300 under control of the 35S promoter. After Agrobacterium tumfaciens-mediated transformation, fifteen independent transgenic lines were obtained and confirmed by RT-PCR (Figure 2E). These transgenic plants showed normal development of AR, indicating that the mutant phenotype was completely complemented.

Effect of gnom1 mutation on callus initiation

Although the *gnom1-1* mutant in the japonica background did not affect callus induction, the *gnom1-2* mutation in the indica background significantly reduced the rate of callus induction, as compared to that of the WT counterpart (Figure 3A and 3C). Using the C1 and C2 dCAPS markers, we were able to distinguish the homozygous mutant calli from the heterozygotes and calculate the frequency of the homozygous mutant genotype among the calli generated from the mixed seeds produced by heterozygotic plants. Results showed that the frequency of homozygous mutant callus in the in-

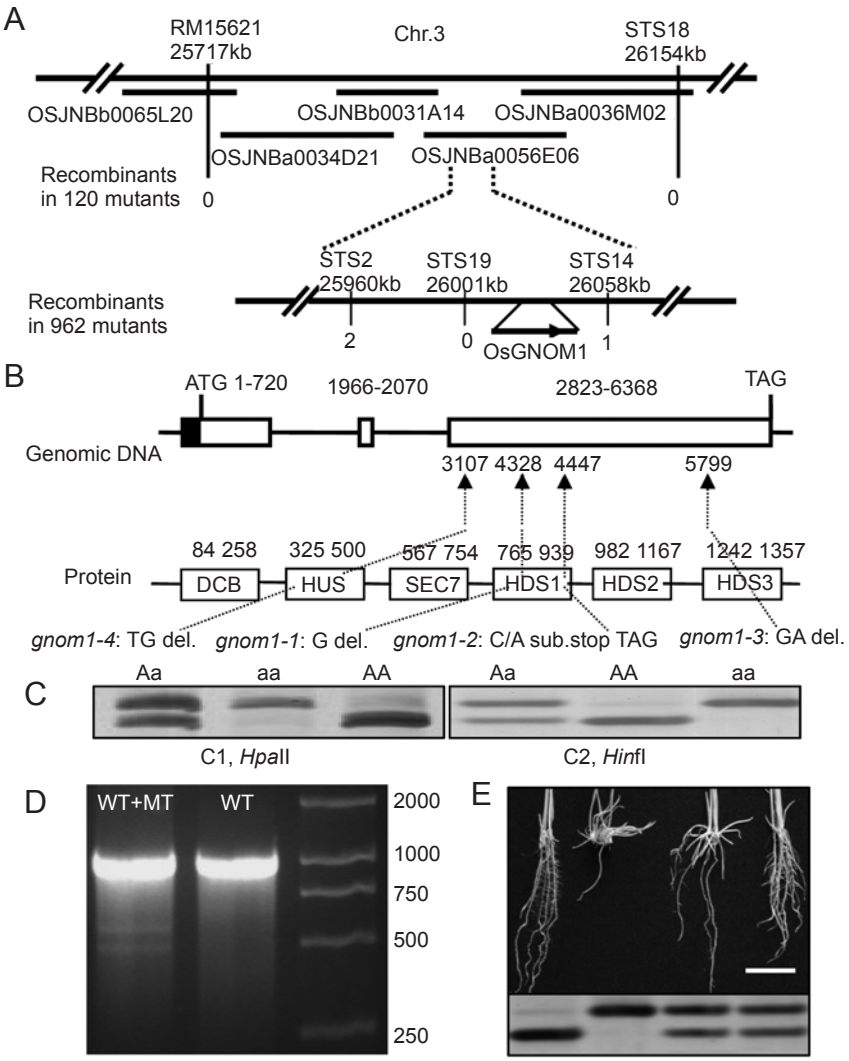

Figure 2 Map-based cloning of the *OsGNOM1* gene. **(A)** Fine mapping of the *OsGNOM1* gene between the markers STS19 and STS14 within the BAC clone OSJNBa0056E06. BAC, bacterial artificial chromosome. The number of recombinants is marked corresponding to the molecular markers. **(B)** Gene and protein structure of the *OsGNOM1*. The mutation sites of four alleles (*gnom1-1* to *gnom1-4*) are marked by the arrow. **(C)** Confirmation of *gnom1-1* and *gnom1-2* by the dCAPS markers C1 and C2. **(D)** The *OsGNOM1* DNA fragments of *gnom1-3* and Kasalath digested with *Cel* I. **(E)** Complementation analysis of the *gnom1-1* mutant. From left to right in the upper panel: WT plant, *gnom1-1* mutant, two lines of *gnom1-1* mutant harboring exogenous OsGNOM1. Bar = 2cm. The bottom panel: C1 PCR products after *Hpa*II digestion using cDNA for *OsGNOM1* in PAGE gel.

dica background was significantly lower than the ratio predicted by Mendelian segregation (Figure 3D). Furthermore, in contrast to the normal morphology of indica calli, those homozygous for *gnom1-2* were dispersed and fragile (Figure 3B). However, no difference was observed between WT and *gnom1-1* calli in the japonica genetic background, suggesting that OsGNOM1 may play a different role in the indica and japonica subspecies.

Effect of gnom1 mutation on PAT

When treated with NPA, the inhibitor of PAT, the WT seedlings exhibited a phenotype similar to that of *gnom1*. As shown in Figure 4A, neither the NPA-treated WT seedlings nor the *gnom1* seedlings had AR. The aerial parts of both groups of seedlings grew in a curly manner, while the roots in both groups displayed a loss of gravitropism (Figure 4A and 4B). In addition, the mutants were more sensitive to NPA than the WT plants (Figure 4A), indicating that PAT was impaired in the mutants. Howev-

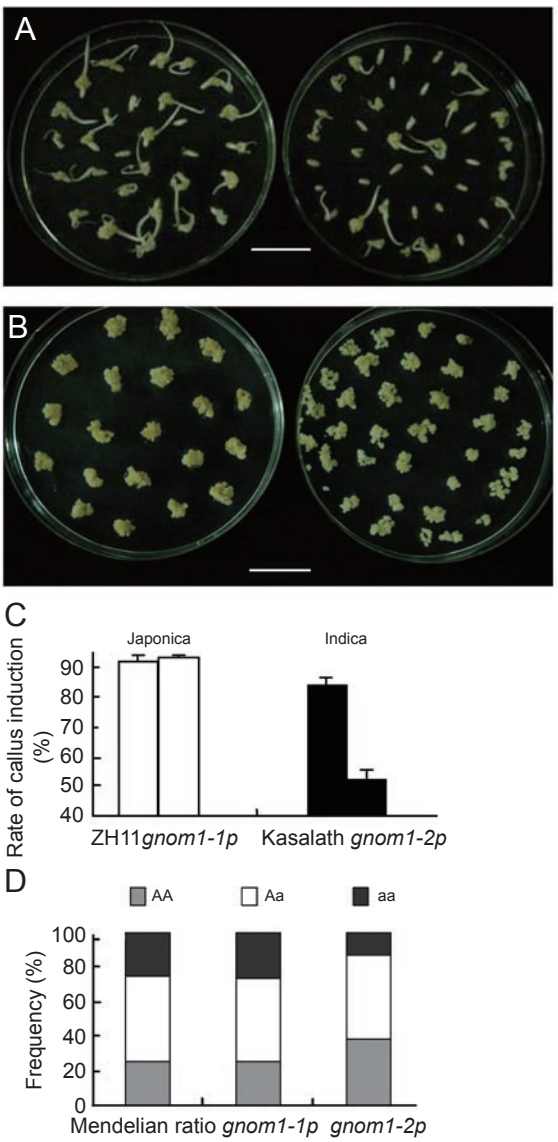

Figure 3 The callus characteristics of indica genotype, Kasalath and *gnom1-2*. **(A)** Calli induction from Kasalath WT seeds (left) and from mixed seeds produced by *gnom1-2* heterozygotic plants (right). Bars = 2 cm. **(B)** Morphology of Kasalath calli (left) and homozygous *gnom1-2* calli (selected by molecular marker C2, right). Bars = 2 cm. **(C)** Rate of callus induction for WT seeds or mixed seeds produced by heterozygous plants, on japonica or indica background. *gnom1-1p* and *gnom1-2p* denote seeds produced by the *gnom1-1* and *gnom1-2* heterozygous populations, respectively. **(D)** The genotype frequency of calli induced from seeds produced by *gnom1-1* and *gnom1-2* heterozygous populations.

er, the phytohormones α-naphthalene acetic acid (NAA) and penetrable 2,4-D did not restore AR development in mutants (Supplementary information, Figure S2).

Suppression of *OsPIN1b* in WT rice resulted in reduced numbers of AR and increased sensitivity to NPA [13]. However, semi-quantitative RT-PCR analysis showed that the *OsPIN1b* transcript level in the basal portion of the shoots where the AR primordia are initiated was almost identical to that of the WT plants (Figure 5A). To investigate whether *OsGNOM1* regulates the ex-

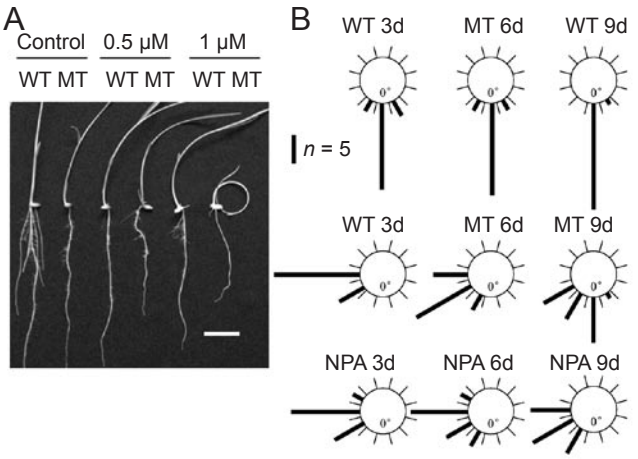

Figure 4 The effects of NPA on WT and *gnom1-1*. **(A)** WT and *gnom1-1* treated with NPA, and the solvent DMSO as control. Bar = 2 cm. **(B)** The root gravitropism response of WT, *gnom1-1* mutant (MT) and WT treated with 1 μM NPA for 3, 6 and 9 days.

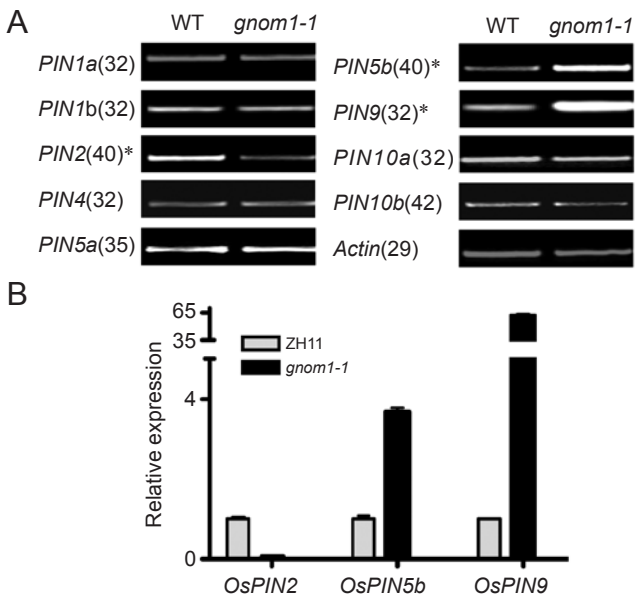

Figure 5 The expression of *OsPIN*s in WT and *gnom1-1*. **(A)** The expression of nine *OsPIN* family members, determined by semi-quantitative RT-PCR analysis of WT and *gnom1-1* stem bases; actin served as an internal control. The differentially expressed genes are marked with *, and the PCR cycles are indicated in the brackets. **(B)** Quantitative RT-PCR of three genes (*OsPIN2*, *OsPIN5b* and *OsPIN9*) in WT and *gnom1-1*.

pression of the *PIN* gene family in rice, RT-PCR analysis was used to compare *gnom1* mutants to WT plants in terms of *OsPIN* expression in the basal part of the stems. The results showed that three out of the nine tested *PIN* genes were expressed differentially in the *gnom1* mutants and WT plants (Figure 5A). While the expression of *OsPIN2* was downregulated in the mutants, *OsPIN5b* and *OsPIN9* were both upregulated (Figure 5A). The differential expression of these genes was further confirmed by quantitative RT-PCR analysis (Figure 5B). These results showed that expression of the *PIN* gene family was disrupted in the roots of *gnom1-1* mutants, further supporting the idea that PAT is affected in these plants.

OsGNOM1 expression pattern

Semi-quantitative RT-PCR analysis was performed to investigate the expression pattern of *OsGNOM1* in various tissues. Results showed that *OsGNOM1* was transcribed in all the tissues tested (Figure 6A). Expression of the gene was slightly higher in root and basal internodes than in shoot, panicle or callus, implying that GNOM1 plays important roles in growth and development in rice. The tissue expression pattern of the *OsGNOM1* gene was analyzed using 2 300 bp of its promoter region and 215 bp of its coding region fused to the GUS-coding region. GUS staining of the transgenic plants revealed that *OsGNOM1* is expressed in the vascular tissues of roots (Figure 6B), LRs (Figure 6B), root tips (Figure 6C), anthers and lemma veins (Figure 6D and 6E), and leaves (Figure 6F). Cross-sections of the stem base showed that *OsGNOM1* was also expressed in the AR primordia (Figure 6G). To determine whether phytohormones affected the expression of *OsGNOM1*, semi-quantitative RT-PCR was performed. As shown in Figure 6H, the expression of *OsGNOM1* was not induced by NAA, NPA or 6-benzylaminopurine (6-BA), but it was strongly induced by abscisic acid (ABA) in both WT plants and *gnom1* mutants.

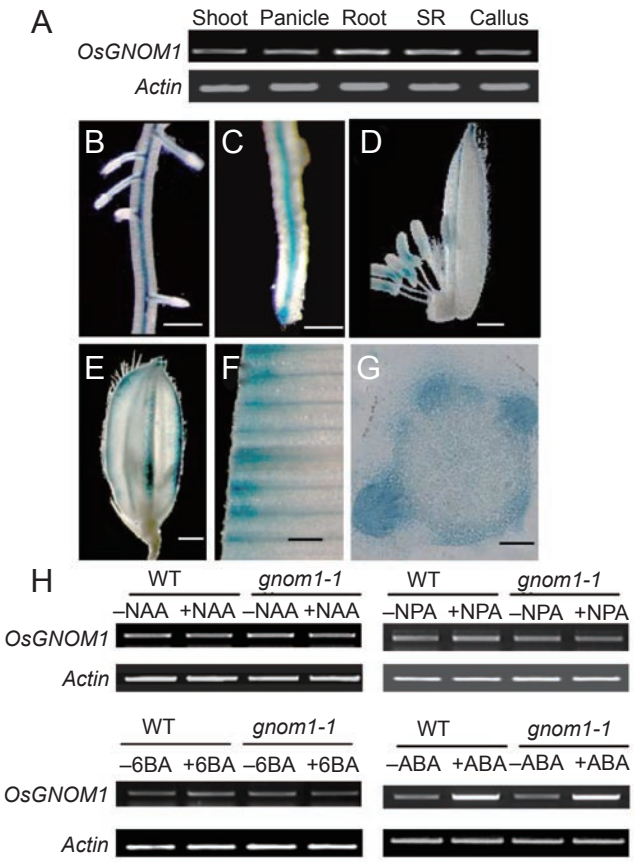

Figure 6 Expression pattern of *OsGNOM1*. **(A)** Semi-quantitative RT-PCR of *OsGNOM1* in various tissues, and actin as a control. **(B-G)** *OsGNOM1* promoter-driven GUS expression in roots and lateral roots **(B)**, root tips **(C)**, anthers **(D)** lemma veins **(E)**, leaves **(F)** and stem-base cross-section **(G)**. Bars in B, C = 200 μm; in D, E = 500 μm; in F, G = 100 μm. **(H)** Semi-quantitative RT-PCR of *OsGNOM1* following various hormone treatments.

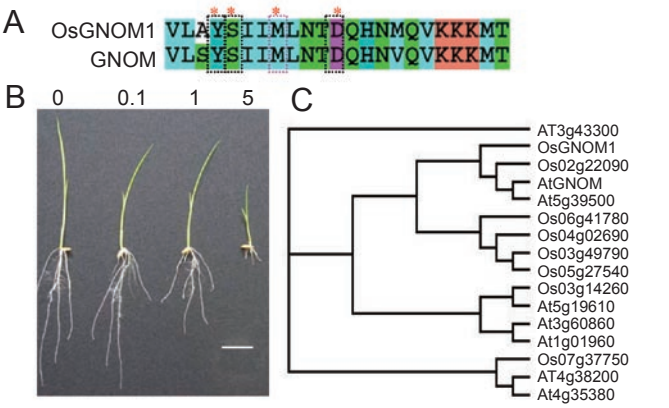

Figure 7 The response of OsGNOM1 to BFA. **(A)** The alignment of OsGNOM1 and GNOM in SEC7 domain conserved amino acids, which predict sensitivity to BFA. The crucial amino acids are marked by *. **(B)** The wild-type seedling response to 0 (only solvent DMSO), 0.1, 1 and 5 μM BFA. Bar = 2 cm. **(C)** Dendrogram of the GNOM family proteins in rice and *Arabidopsis*.

Sensitivity of OsGNOM1 to BFA

Large ARF-GEFs in endosomes can be classified into two groups, according to their response sensitivity to the fungal toxin BFA, which specifically blocks vesicle trafficking. *Arabidopsis* GNOM1 is sensitive to BFA [18, 21]. OsGNOM1 is a closer homolog to the *Arabidopsis* GNOM than to the six other rice GNOM proteins except Os02g22090 (Figure 7C). Four critical amino acids in

Table 2 Effect of BFA on seedling growth

Concentration of BFA (μM)	SL (cm)	SRL (cm)	ARN	LRN
0	9.26 ± 0.75	8.82 ± 0.38	4.80 ± 0.63	85.35 ± 20.48
0.1	8.43 ± 0.31	7.59 ± 0.82	5.1 ± 1.28	112.50 ± 32.76
1	7.35 ± 0.40*	4.97 ± 0.67*	4.5 ± 1.87	30.35 ± 8.24*
5	2.48 ± 0.33*	1.37 ± 0.24*	5.3 ± 0.46	0 ± 0*

[1]BFA, brefeldin A; SL, shoot length; SRL, seminal root length; ARN, adventitious root number; LRN, lateral root number.
[2]*Mean significant difference ($P < 0.05$, *t*-test).
[3]Measurements were obtained from 7-day-old seedlings of the Zhonghua 11 cultivar. Five plants per line were calculated, and data represented as means±SD.

the SEC7 domain that determine the sensitivity to BFA [18] are identical between *Arabidopsis* GNOM and OsGNOM1 (Figure 7A), suggesting that OsGNOM1 might be sensitive to BFA. To determine the effects of BFA on the development of AR and LR, different concentrations of BFA were applied to WT rice seedlings. While the BFA-treated rice seedlings had similar numbers of AR in comparison to control seedlings, the number of LRs decreased with BFA treatments of increasing concentrations (Figure 7B and Table 2). Thus, although BFA inhibited the initiation of LR, BFA application did not phenocopy the effect of OsGNOM1 mutation on AR development.

Discussion

Here, we report that mutants carrying four different mutations in the rice *OsGNOM1* gene display defective AR initiation. *OsGNOM1* proved to be highly homologous to the GNOM protein in *Arabidopsis*. Mutations in *Arabidopsis* GNOM resulted in serious defects in both embryonic and post-embryonic development [34].

Auxin is a major growth-promoting hormone for the initiation of LR primordia in *Arabidopsis* [3]. A previous study revealed that GNOM controlled PAT through polar localization of PIN1 [17], a component of auxin efflux carriers. In addition, some ARF-regulating factors in rice are also related to PAT, such as the ARF-activating protein involved in the localization of AUX1, an auxin influx carrier [35]. All mutant phenotypes observed in *gnom1-1* to *gnom1-4*, including a lack of AR, reduced number of LR, partial agravitropic response and hypersensitivity to NPA, could be explained by disrupted PAT. OsGNOM1 was expressed in a pattern parallel to that of auxin distribution (Figure 6), supporting the hypothesis that OsGNOM1 is involved in PAT. The expression of *OsPINs* was altered in the mutants (Figure 5), including *OsPIN2*, *OsPIN5b* and *OsPIN9* (Figure 5A and 5B), which may also have contributed to the observed phenotype. Moreover, OsPIN1b, OsPIN5b and OsPIN9 are all expressed in the stem base where AR primordia form (Wang *et al.*, unpublished), suggesting that these *PIN* genes are likely involved in the formation of ARs. Since *OsPIN5b* and *OsPIN9* have no corresponding homologous genes in *Arabidopsis*, the function of these genes might explain the difference between the tap root system of *Arabidopsis* and the fibrous root system of rice. These OsPINs function in PAT, either directly controlled by OsGNOM1 or in a feedback loop with auxin [32, 36]. Taken together, our results suggest that OsGNOM1 is required for the initiation of AR primordia, which is mediated by OsPIN family genes.

Due to the one (*gnom1-1*) or two (*1-3* and *1-4*) nucleotide, deletion in the GNOM1 protein-coding sequence, *gnom1-1* to *1-4* mutants cannot produce functional GNOM1 protein. Some strong allelic mutants of *Arabidopsis gnom* showed defects in embryonic development, mainly involving nonsense mutations before or in the central portion of the SEC7 domain [19]. Whereas rice mutants *gnom1-1* to *gnom1-4*did not show any defects in embryonic development, some mutations (*gnom1-4*) occurred prior to the SEC7 domain. In this study, we have mainly focused on screening for mutants with defects in root development. The more severe mutations related to embryonic development may have been missed. It is also possible that OsGNOM1 has little role in the embryonic development in rice, due to the differences in embryonic structure between dicotyledonous plants and cereals.

To date, several mutants affecting root development have been characterized in cereals [37]. Unlike *arl1*, which only affects AR development, mutations in rice *gnom1* not only affect AR development, but also reduce the numbers of LR, suggesting that OsGNOM1 might be involved in the common mechanisms regulating the development of root components.

The fungal toxin BFA was shown to specifically inhibit the membrane trafficking of GNOM in *Arabidopsis* [18]. Thus, application of BFA to the WT *Arabidopsis* plants phenocopied the *gnom* mutants. Interestingly, the function of rice GNOM1 was shown to be partially resistant to BFA, despite predictions that it would be sensitive

due to the presence of conserved amino acids (Figure 7A). An opposite pattern was observed for tobacco Nt-GNL1, sensitive to BFA despite predictions that it would be resistant [38]. BFA application reduced the number of LR (Figure 7B), while it did not affect the number of AR. The discrepancy in BFA sensitivity between rice and *Arabidopsis* might be attributable to differential GNOM protein function in the mono- and dicotyledonous plant species.

Mutation of *OsGNOM1* resulted in a reduced rate of callus induction and changed the characteristics of callus in indica, but not in japonica. It is likely that OsGNOM1 may not function in precisely the same way in indica and japonica rice subspecies.

Identification of the function of the *OsGNOM1* gene in root development demonstrates that some members of the SEC7 domain gene family may play an important role in organ development in plants. In total, eight SEC7 domain genes with various similarities were identified throughout the rice genome (Figure 7C). Further studies to clarify the function of these SEC7 genes utilizing transgenic approaches will greatly enhance our knowledge of the molecular mechanisms underlying rice organ development. Precise monitoring of the expression level and the tissue specificity of OsGNOM1 and its homologs may hold potential for the improvement of crop root architecture, with the ultimate goal of increasing nutrient and water uptake efficiency.

Materials and Methods

Isolation of the mutants

Rice mutant libraries of japonica Zhonghua 11 and indica variety Kasalath were constructed utilizing tissue culture techniques, EMS or ^{60}Co irradiation as described in Table 1. Four mutants (denoted as *gnom1-1* to *gnom1-4*) lacking ARs were identified. *gnom1-1* was from Zhonghua 11, and the *gnom1-2* to *gnom1-4* mutants were generated from Kasalath.

Growth conditions and hormone treatments

Seedlings of WT rice and heterozygous mutants were planted hydroponically at pH 5.0. The hydroponic culture solution contained 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$, 1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 mM $(NH_4)_6Mo_7O_{24}$, 0.019 mM H_3BO_3, 0.155 mM $CuSO_4$, 0.036 mM $FeCl_3$, 0.070 mM citric acid and 0.152 mM $ZnSO_4$ [13]. Rice plants were grown in growth chambers with a 12 h photoperiodic condition (200 μmol photons m^{-2} s^{-1}) and a temperature of 30°C after germination. Humidity was controlled at approximately 60%. To analyze the effects of NPA on the phenotypes, 0.5 μM or 1 μM NPA was applied exogenously. Measurements of LR density were taken with WinRHIZO version 3.9 (Régent Instrument Inc., Québec, Canada). The gravitropic response of the primary root was determined using 20 seedlings grown for 3, 6 and 9 days. BFA dissolved in DMSO was added to the hydroponic solution with a serially diluted concentration gradient.

Mapping and cloning of OsGNOM1

A mapping F_2 population was generated from the crosses between Kasalath and plants heterozygous for the *gnom1-1* mutant. *OsGNOM1* was primarily mapped with SSR and STS markers, which were developed from the sequence difference japonica Nipponbare contig sequences and indica variety 93-11 sequences using 120 F_2 homozygous mutant plants. The mutation was further mapped within a 90-kb region flanking an STS19 marker at a BAC clone (OSJNBa0056E06) on chromosome 3 using 962 F_2 mutant plants. The DNA sequences of candidate genes were amplified from both the *gnom1-1* mutant and WT genomic DNA using three pairs of gene-specific primers (Supplementary information, Table S2), and then cloned into T-vector for sequencing. To confirm the mutation, two dCAPS markers, C1 and C2, were developed according to the sequence described previously [39] at http://helix.wustl.edu/dcaps/dcaps.html. The mutation of *gnom1-3* was confirmed by *Cel* I according to the instructions [40].

A BLAST search was performed to identify the homologs of OsGNOM1. The predicted protein sequences from these homologs were clustered using ClustalW (version 1.74, Thompson, Higgins, Gibson).

Callus survey of the mutants

To calculate the rate of callus induction, calli originating from the indica and japonica backgrounds were induced according to the protocol [41]. A total of 100 seeds from the *gnom1-1* and *gnom1-2* heterozygous populations were divided into three culture dishes, and the experiment was repeated three times. After callus formation, we counted the seeds capable of being induced. The callus was then photographed after subculture. The C1 and C2 dCAPS markers were used to distinguish the homozygous mutant calli from the heterozygous population.

Construction of vectors and plant transformation

For complementation of rice *gnom1-1* mutants, a 4.8-kb cDNA fragment of wild type containing the OsGNOM1 ORF was cloned into a binary vector, modified pCAMBIA 1300 driven by the 35S promoter. The homozygous embryonic calli induced from heterozygous population seeds were identified using dCAPS marker C1 to serve as complementation test recipients. Recipient calli were transformed with the above construct using the *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation system as described [41]. Transgenic plants were selected on medium containing 50 mg/l hygromycin.

For the *OsGNOM1* promoter::GUS construct, a 2.3-kb DNA segment upstream of *OsGNOM1* and 215 bp of the coding region was amplified by PCR and inserted into the 5′ end of the GUS reporter gene in pBI101 [9]. The resulting binary vector was transferred into calli derived from WT Zhonghua 11. All the GUS-related transgenic calli were selected on media containing 200 mg/l geneticin.

Histochemical analysis and GUS assay

Histochemical GUS analysis and methylene blue staining of seminal roots were performed as described [33, 42]. After being stained with X-gluc buffer overnight at 37 °C, shoot bases from 5-day-old seedlings were fixed in FAA for 24 h at 4 °C and then

dehydrated in a graded ethanol series. Dehydrated samples were embedded in paraffin, and then sectioned into 8-μm-thick sections by using a rotary microtome (Microm HM325, Walldorf, Germany).

RT-PCR analysis

Total RNA was extracted from stem bases, roots, shoots and flowers of 7-day-old WT and *gnom1-1* mutant seedlings, using the TRIzol method. Some seedlings were treated by 1 μM α-NAA, 10 μM 6-BA, 10 μM NPA or 100 μM ABA for 4 h to harvest the stem-base samples. Five micrograms of RNA samples were reversely transcribed using M-MLV reverse transcriptase (Promega, CA, USA) and an oligo(dT)18, according to the manufacturer's protocol. RT-PCR was performed using gene-specific primers (Supplementary information, Table S2). Amplification of actin cDNA was performed as a control. The PCR conditions were 94 °C for 5 min followed by 32 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and then a final extension period at 72 °C for 5 min. PCR products were analyzed on 1.2% agarose gels. RT-PCR analysis for the complementation test was performed using 15% PAGE gels to separate PCR products from the wild type and the mutant. Real-time qRT-PCR was performed using the SYBR Premix Ex Taq™ (Perfect Real Time) Kit (TaKaRa Biomedicals, Tokyo, Japan) on a LightCycler480 machine (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

Acknowledgments

This work was supported by the Key Basic Research Special Foundation of China (2005CB20900), the National High Technology Research and Development Program (2007AA021403, 2006AA10Z175), the National Natural Science Foundation of China (30471118 and 30770191) and the Specialized Research Fund for the Doctoral Program of Higher Education (20070335081).

References

1 Kawata S, Yamazaki K, Ishihara K, Shibayama H, Lai KL. Studies on root system formation in rice plants in a paddy. *Proc Crop Sci Soc Jpn* 1963; **32**:163.
2 Scheres B, Benfey PN, Dolan, L. Root development. In: Somerville C, Meyerowitz E, eds. *The Arabidopsis Book*. American Society of Plant Biologists, Rockville, MD 2002:1-19.
3 Casimiro L, Beeckman T, Graham N, *et al.* Dissecting *Arabidopsis* lateral root development. *Trends Plant Sci* 2003; **8**:165-171.
4 Fukaki H, Tasaka M. Hormone interactions during lateral root formation. *Plant Mol Biol* 2009; **69**: 437-449.
5 Lucas M, Godin C, Jay-Allemand C, Laplaze L. Auxin fluxes in the root apex co-regulate gravitropism and lateral root initiation. *J Exp Bot* 2008; **59**:55-66.
6 Hetz W, Hochholdinger F, Schwall M, Feix, G. Isolation and characterization of *rtcs*, a mutant deficient in the formation of nodal roots. *Plant J* 1996; **10**:845-857.
7 Inukai Y, Miwa M, Nagato Y, Kitano H, Yamauchi, A. Characterization of rice mutants deficient in the formation of crown roots. *Breed Sci* 2001; **51**:123-129.
8 Inukai Y, Sakamoto T, Ueguchi-Tanaka M, *et al.* Crown rootless1, which is essential for crown root formation in rice, is a target of an auxin response factor in auxin signaling. *Plant Cell* 2005; **17**:1387-1396.
9 Liu H, Wang S, Yu X, *et al.* ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J* 2005; **43**:47-56.
10 Teale WD, Paponov IA, Palme K. Auxin in action: signalling, transport and the control of plant growth and development. *Nat Rev Mol Cell Biol* 2006; **7**:847-859.
11 Fukaki H, Nakao Y, Okushima Y, Theologis A, Tasaka M. Tissue-specific expression of stabilized SOLITARY-ROOT/IAA14 alters lateral root development in *Arabidopsis*. *Plant J* 2005; **44**:382-395.
12 Liu PP, Montgomery TA, Fahlgren N, *et al.* Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. *Plant J* 2007; **52**:133-146.
13 Xu M, Zhu L, Shou H, Wu P. A *PIN1* family gene, *OsPIN1*, involved in auxin-dependent adventitious root emergence and tillering in rice. *Plant Cell Physiol* 2005; **46**:1674-1681.
14 Taramino G, Sauer M, Stauffer JL, *et al.* The maize (*Zea mays* L.) *RTCS* gene encodes a LOB domain protein that is a key regulator of embryonic seminal and post-embryonic shoot-borne root initiation. *Plant J* 2007; **50**:649-659.
15 Shevell DE, Leu WM, Gillmor CS, *et al.* EMB30 is essential for normal cell division, cell expansion, and cell adhesion in *Arabidopsis* and encodes a protein that has similarity to Sec7. *Cell* 1994; **77**:1051-1062.
16 Busch M, Mayer U, Jurgens G. Molecular analysis of the *Arabidopsis* pattern-formation gene *GNOM*: gene structure and intragenic complementation. *Mol Gen Genet* 1996; **250**:681-691.
17 Steinmann T, Geldner N, Grebe M, *et al.* Coordinated polar localization of auxin efflux carrier PIN1 by GNOM ARF GEF. *Science* 1999; **286**:316-318.
18 Geldner N, Anders N, Wolters H, *et al*. The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* 2003; **112**:219-230.
19 Geldner N, Richter S, Vieten A, *et al.* Partial loss-of-function alleles reveal a role for GNOM in auxin transport-related, post-embryonic development of *Arabidopsis*. *Development* 2004; **131**:389-400.
20 Koizumi K, Sugiyama M, Fukuda H. A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. *Development* 2000; **127**: 3197-3204.
21 Anders N, Jurgens G. Large ARF guanine nucleotide exchange factors in membrane trafficking. *Cell Mol Life Sci* 2008; **21**:3433-3445.
22 Mayer U, Torres Ruiz RA, Berleth T, Misera S, Jurgens G. Mutations affecting body organization in the *Arabidopsis* embryo. *Nature* 1991; **353**:402-407.
23 Shevell DE, Kunkel T, Chua NH. Cell wall alterations in the *Arabidopsis emb30* mutant. *Plant Cell* 2000; **12**:2047-2060.
24 Grebe M, Gadea J, Steinmann T, *et al.* A conserved domain of the *Arabidopsis* GNOM protein mediates subunit interaction and cyclophilin 5 binding. *Plant Cell* 2000; **12**:343-356.
25 Mouratou B, Biou V, Joubert A, *et al.* The domain architecture

of large guanine nucleotide exchange factors for the small GTP-binding protein Arf. *BMC Genomics* 2005; **6**:20.
26 Anders N, Nielsen M, Keicher J, *et al.* Membrane association of the *Arabidopsis* ARF exchange factor GNOM involves interaction of conserved domains. *Plant Cell* 2008; **20**:142-151.
27 Richter S, Geldner N, Schrader J, *et al.* Functional diversification of closely related ARF-GEFs in protein secretion and recycling. *Nature* 2007; **448**:488-492.
28 Teh OK, Moore I. An ARF-GEF acting at the Golgi and in selective endocytosis in polarized plant cells. *Nature* 2007; **448**: 493-496.
29 Muday G.K, Peer WA, Murphy AS. Vesicular cycling mechanisms that control auxin transport polarity. *Trends Plant Sci* 2003; **8**:301-304.
30 Geldner N, Friml J, Stierhof Y, Jurgens G, Palme K. Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* 2001; **413**: 425-428.
31 Friml J, Vieten A, Sauer M, *et al.* Efflux dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* 2003; **426**:147-153.
32 Blilou I, Xu J, Wildwater M, *et al.* The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* 2005; **433**:39-44.
33 Wang H, Taketa S, Miyao A, Hirochika H, Ichii M. Isolation of a novel lateral-rootless mutant in rice (*O. sativa* L.) with reduced sensitivity to auxin. *Plant Sci* 2006; **170**:70-77.
34 Mayer U, Buttner G, Jurgens G. Apical-basal pattern formation in the Arabidopsis embryo: studies on the role of the gnom gene. Development 1993; **117:**149-162.
35 Zhuang X, Jiang J, Li J, *et al.* Over-expression of OsAGAP, an ARF-GAP, interferes with auxin influx, vesicle trafficking and root development. *Plant J*. 2006; **48**:581-591.
36 Paponov IA, Teale WD, Trebar M, Blilou I, Palme K. The PIN auxin efflux facilitators: evolutionary and functional perspectives. *Trends Plant Sci* 2005; **10**:170-177.
37 Hochholdinger F, Park WJ, Sauer M, Woll K. From weeds to crops: genetic analysis of root development in cereals. *Trends Plant Sci* 2004; **1**:42-48.
38 Wang L, Liao FL, Zhu L, Peng XB, Sun MX. NtGNL1 is involved in embryonic cell division patterning, root elongation, and pollen tube growth in tobacco. *New Phytol* 2008; **179**:81-93.
39 Neff MM, Neff JD, Chory J, Pepper AE. dCAPS, a simple technique for the genetic analysis of single nucleotide polymorphisms: experimental applications in *Arabidopsis thaliana* genetics. *Plant J* 1998; **14**:387-392.
40 Till BJ, Burtner C, Comai L, Henikoff S. Mismatch cleavage by single-strand specific nucleases. *Nucleic Acids Res* 2004; **32**:2632-2641.
41 Chen SY, Jin WZ, Wang MY, *et al.* Distribution and characterization of over 1000 T-DNA tags in rice genome. *Plant J* 2003; **36:**105-113.
42 Jefferson RA, Kavanagh TA, Bevan MW. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J* 1987; **6**:3901-3907.

(**Supplementary information** is linked to the online version of the paper on the *Cell Research* website.)