

A *PIN1* Family Gene, *OsPIN1*, involved in Auxin-dependent Adventitious Root Emergence and Tillering in Rice

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Auxin transport affects a variety of important growth and developmental processes in plants, including the regulation of shoot and root branching. The asymmetrical localization of auxin influx and efflux carriers within the plasma membrane establishes the auxin gradient and facilitates its transport. *REH1*, a rice *EIR1* (*Arabidopsis* ethylene insensitive root 1)-like gene, is a putative auxin efflux carrier. Phylogenetic analysis of 32 members of the *PIN* family, taken from across different species, showed that in terms of evolutionary relationship, *OsPIN1* is closer to the *PIN1* family than to the *PIN2* family. It is, therefore, renamed as *OsPIN1* in this study. *OsPIN1* was expressed in the vascular tissues and root primordial in a manner similar to *AtPIN1*. Adventitious root emergence and development were significantly inhibited in the *OsPIN1* RNA interference (RNAi) transgenic plants, which was similar to the phenotype of NPA (N-1-naphthylphalamic acid, an auxin-transport inhibitor)-treated wild-type plants. α -naphthylacetic acid (α -NAA) treatment was able to rescue the mutated phenotypes occurring in the RNAi plants. Over-expression or suppression of the *OsPIN1* expression through a transgenic approach resulted in changes of tiller numbers and shoot/root ratio. Taken together, these data suggest that *OsPIN1* plays an important role in auxin-dependent adventitious root emergence and tillering.

Keywords: Adventitious root — Auxin transporter — Emergence — *Oryza sativa* L. — PIN-FORMED1 family — Tillering.

Abbreviations: ARF, auxin response factor; FAA, formalin acetic acid-alcohol; GUS, β -glucuronidase; PIN, pin-formed; ORF, open reading frame; NAA, α -naphthylacetic acid; NPA, N-1-naphthylphalamic acid; RNAi, RNA interference.

Introduction

The phytohormone auxin regulates a wide variety of developmental processes of plant growth. This includes changes in growth direction, in shoot and root branching, and in vascular differentiation (Laskowski et al. 1995, Leyser 2001,

Paquette and Benfey 2001, Reinhardt et al. 2000). Auxin is thought to be synthesized in young apical tissues and then transported downward to the maturing stem and the roots by a polar transport system (Crozier et al. 2000). Auxin transportation accomplished by the auxin influx and efflux carrier proteins is strictly directional (Muday and DeLong 2001). It has been reported that the cell-to-cell polar auxin transport could be mediated via the asymmetric localization of auxin-efflux carriers (Friml and Palme 2002a). A number of auxin influx carriers, e.g. the *AUX1* and *LAX* (*AUX1*-like) gene families, and efflux carriers, e.g. the *PIN-FORMED* (*PIN*) family, have been isolated and characterized (Bennett et al. 1996, Friml et al. 2002b, Friml et al. 2002c, Gälweiler et al. 1998, Luschnig et al. 1998). The finding that influx and efflux carriers distribute within the plasma membrane asymmetrically provides direct evidence for the chemiosmotic model of auxin polar transport (i.e. auxin moves along concentration and electrochemical gradients; Gälweiler et al. 1998, Müller et al. 1998, Swarup et al. 2001).

An *Arabidopsis* mutant developed knitting needle-like apices when the gene encoding a putative auxin efflux carrier was interrupted. The mutant was therefore called *pin-formed* or simply *pin1* (Okada et al. 1991). Several other *PIN-FORMED* genes have been recently identified in various plant species, including *Arabidopsis*, *Brassica juncea*, *Medicago truncatula* and the hybrid *Populus* (Ni et al. 2002, Schnabel and Frugoli 2004, Schrader et al. 2003). The *Arabidopsis pin* mutants fail to establish endogenous auxin gradients and show developmental disorders in the shoot (Reinhardt et al. 2003), flower (Vernoux et al. 2000), root (Benková et al. 2003) and embryo (Weijers and Jürgens 2005). The *pin1* mutation in *Arabidopsis* affects leaf formation and the development of the inflorescence. The triple *pin* mutant *pin1 pin3 pin4* displayed defects in primary root development. The *pin1 pin3 pin4 pin7* quadruple mutants showed strong defects in embryonic development (Weijers and Jürgens 2005).

In contrast with the primary root system in *Arabidopsis*, rice produces numerous adventitious roots that are essential for maintaining normal plant development. Application of the polar auxin transport inhibitor N-1-naphthylphalamic acid (NPA) in rice root collars (the junction between root and stem) blocked the initiation and growth of adventitious and lateral roots in rice. This suggested that polar auxin transport was crit-

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ical for the initiation and elongation of adventitious roots (Zhou et al. 2003). Recently, an auxin responsive transcription factor, ARL1, the target of auxin response factor (ARF), was reported to regulate the initiation of adventitious root primordia (Inukai et al. 2005, Liu et al. 2005).

Apically derived auxin is known to inhibit shoot branching by inhibiting the activity of the axillary meristem in *Arabidopsis* (Chatfield et al. 2000). Recent studies indicate that

the distribution of auxin can influence bud development in various stages, from having a relatively minor effect in bud initiation to a major but indirect effect on bud activity. At least two different secondary hormonal messengers have been identified in this auxin signalling pathway (Leyser 2003). Rice plants develop tillers from the axillary buds. The role of auxin in the development of axillary buds in *Arabidopsis* implies that auxin and its signalling may be the key regulator in the development of tillers.

Despite the connection between auxin signalling and the formation and development of adventitious roots and tillers in rice, the molecules involved in auxin polar transport have not yet been identified. EIR1/AGR1/AtPIN2, an *Arabidopsis* root-specific protein, has been shown to act as an efflux carrier (Chen et al. 1998, Luschnig et al. 1998, Müller et al. 1998, Utsuno et al. 1998). In this report, we explore the characterization and functional analysis of the rice homolog of *Arabidopsis* EIR1/AGR1/AtPIN2, OsPIN1 (previously named REH1, GenBank accession no. AF056027). The findings of the study will clarify the role of PIN dependent auxin polar transports in the initiation and development of rice adventitious roots and tillers.

Results

REH1/OsPIN1 encodes a PIN1 family protein

OsPIN1, previously named *REH1*, was proposed as an auxin transporter and a member of the PIN2 protein family (Luschnig et al. 1998). The full-length *REH1* cDNA contains an open reading frame (ORF) of 1,785 nucleotides. The ORF encodes a protein of 595 amino acid residues with a calculated molecular mass of 65 kDa. Alignment between the cDNA and the genomic sequence of *REH1* indicates that the *REH1* gene has five introns (Fig. 1A). The deduced amino acid sequence of *REH1* shows 69% sequence identity with *Arabidopsis AtPIN1* (See supplemental data).

Hydropathy and transmembrane motif analyses of the deduced amino acid sequence revealed that the *REH1* protein contains three characteristic regions, including a hydrophobic region with five transmembrane segments, a predominantly hydrophilic core, followed by another hydrophobic region with five transmembrane segments (Fig. 1B). This is consistent with

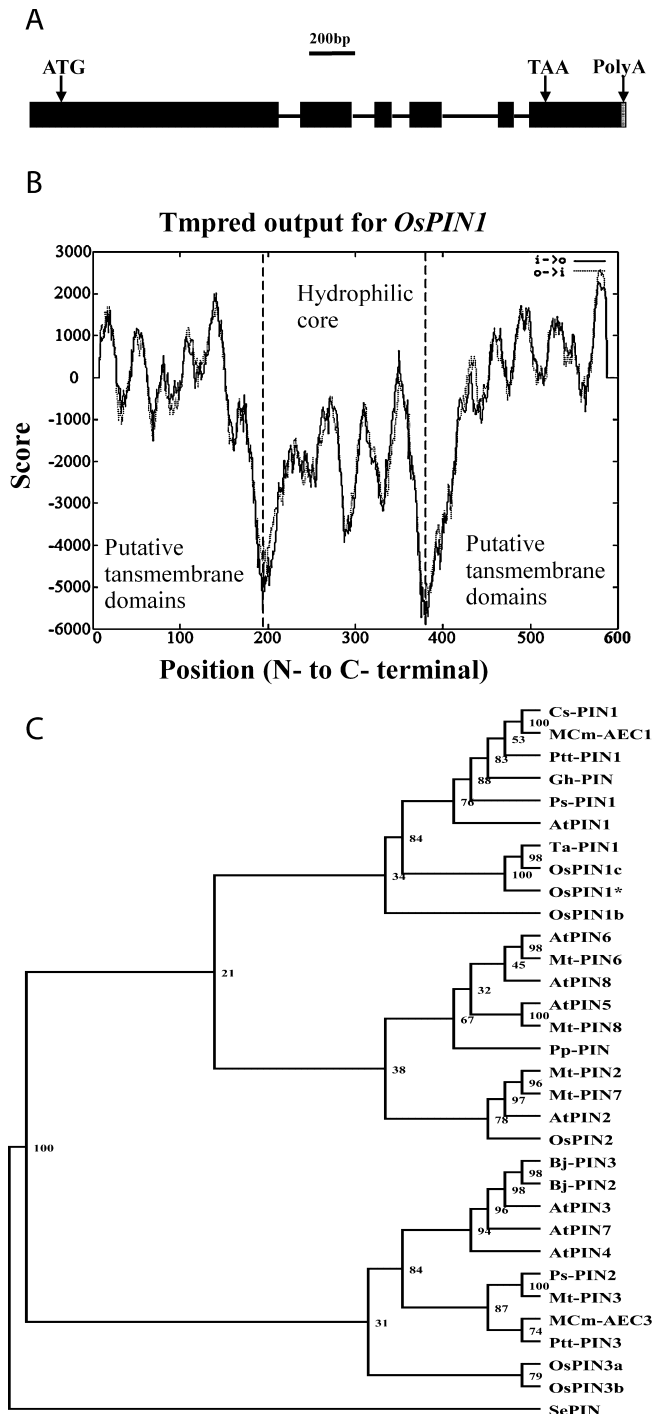


Fig. 1 Gene structure and characterization of *OsPIN1*. (A) Gene structure of *OsPIN1*; bar = 200 bp. (B) The predicted membrane-spanning region and its orientation in the *OsPIN1* protein using Tmpred. (C) Phylogenetic analysis of PIN families of *Arabidopsis thaliana* (At), *Oryza sativa* L. (Os), *Gossypium hirsutum* (Gh), *Triticum aestivum* (Ta), *Cucumis sativus* (Cs), *Momordica charantia* (Mcm), *Populus tremula* x *Populus tremuloides* (Pt), *Pisum sativum* (Ps), *Physcomitrella patens* (Pp), *Brassica juncea* (Bj) and *Medicago truncatula* (Mt). *, *OsPIN1* gene studied. *SEPIN* (gi:57636762) from *Staphylococcus epidermidis* was selected as the outgroup. The phylogeny was constructed using the neighbor-joining method with 100 bootstrap replicates.

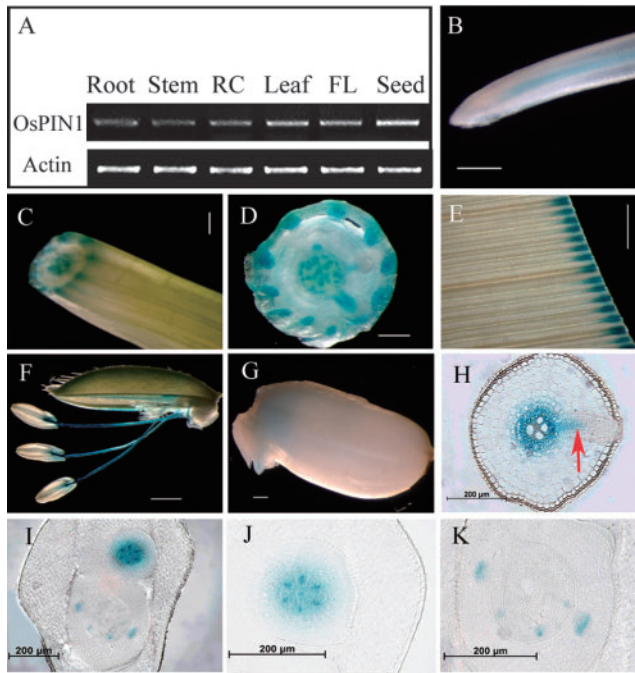


Fig. 2 *OsPIN1* expression in different tissues. (A) Tissue-specific expression pattern revealed by RT-PCR. 28 and 25 running cycles for *OsPIN1* and actin, respectively. RC, root collar, the junction between shoot and root. FL, flower. (B–K) GUS expression of different tissues in p*OsPIN1*-GUS transgenic plants. (B) Adventitious root. (C) Stem. (D) Hand cross-section of the root collar. The arrow points to a primordium of the adventitious root at the first node. (E) Leaf. (F) Flower. (G) Germinated seed. (H) Root cross-section of the segment arrow-pointed in Fig. 1B. Arrow here indicates GUS expression of young lateral root. (I) GUS staining of the germinating seeds. (J–K) Enlarged pictures of Fig. 1I. (B–G) Bar = 0.5 mm. (H–K) Bar = 200 μ m.

the results of Luschnig et al. (1998). Alignment of members of the PIN1 family across different species revealed that the highly conserved portions of the family are uniformly located in the transmembrane domains (Fig. 1B). Phylogenetic analysis of 32 members of PIN family showed that REH1 was evolutionarily closer to the PIN1 family than to the PIN2 family (Fig. 1C). Based on the above result, we renamed REH1 as *OsPIN1*.

Expression pattern of *OsPIN1*

The expression of *OsPIN1* in different tissues was analyzed by reverse transcriptase PCR (RT-PCR) and a β -glucuro-

Fig. 4 Adventitious roots in *OsPIN1* transgenic plants. (A–D) Roots of the transgenic plants underexpressing *OsPIN1* by RNA interference grown under solution culture for 7, 14, 21 and 50 days. (E–H) Roots of wild-type plants grown under solution culture for 7, 14, 21 and 50 days. (I–M) Transverse sections at the first node of the RNAi1 and wild-type (WT) plants sampled at 7 days old (WT, I; Ri, J) and 14 days old (WT, K; Ri, L). Red arrows point to the adventitious root primordial (I, J, L) or mature adventitious root (K).

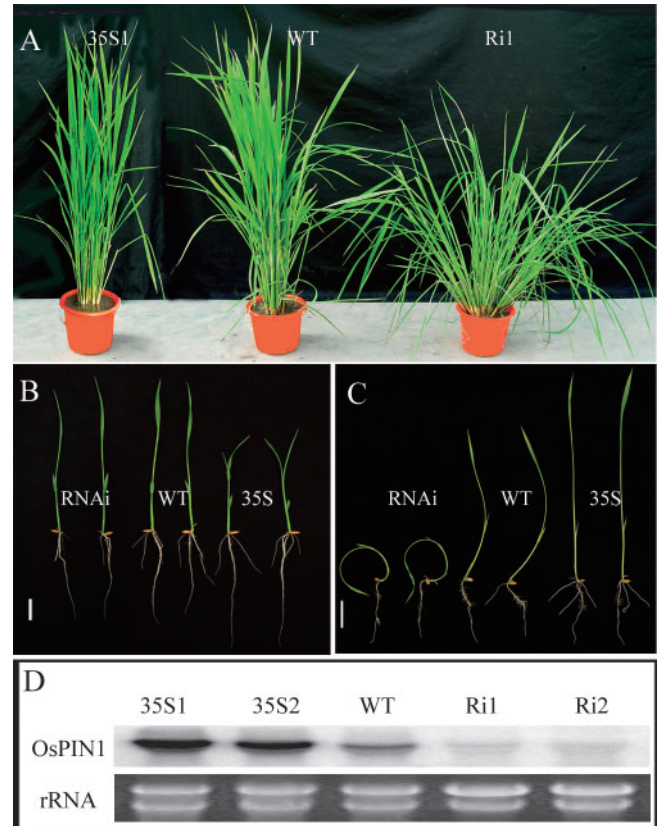


Fig. 3 Phenotypes of *OsPIN1* transgenic plants. (A) The 80-day-old (the maximum tillering stage) 35S-*OsPIN1* (left), wild-type (WT) (middle) and RNAi (right) transgenic plants grown in the greenhouse. (B) and (C) WT, *OsPIN1*, RNAi event1 and event2, and 35S-*OsPIN1* event 1 and event 2 were grown in rice nutrient solution (B) and the solution with 0.5 μ M NPA (C) for 7 days. Bar = 2 cm. (C) Northern blot analysis for *OsPIN1* in the WT, two transgenic lines overexpressing *OsPIN1* (35S1 and 35S2) and two transgenic lines underexpressing *OsPIN1* (Ri1 and Ri2).

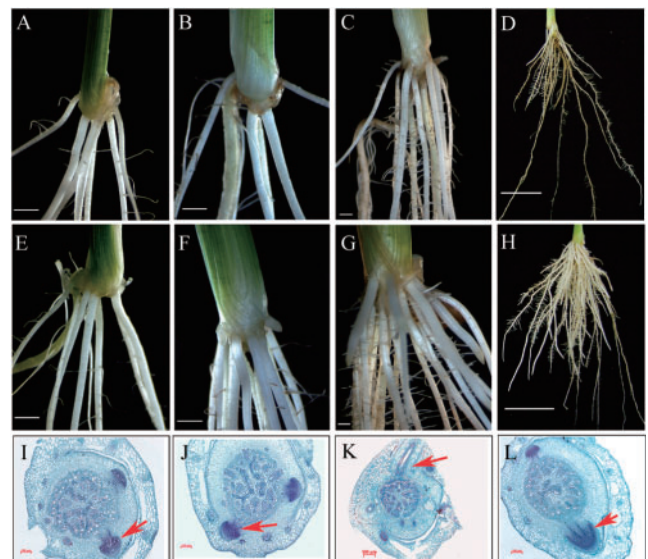


Table 1 Phenotypes of OsPIN1 transgenic and wild-type seedlings

Genotypes	Shoot length (cm)	Primary root length (cm)	Shoot/root ratio	Number of adventitious roots	Number of lateral roots	Number of tillers
WT	30.9 ± 1.5 ^A	10.2 ± 1.1 ^C	3.1 ± 0.3 ^A	13.6 ± 1.1 ^A	146.8 ± 9.8 ^B	24.0 ± 3.8 ^C
RNAi-1	29.5 ± 1.1 ^B	10.1 ± 0.6 ^C	2.9 ± 0.2 ^A	5.7 ± 0.8 ^B	138.7 ± 10.4 ^B	46.2 ± 4.8 ^A
RNAi-2	29.3 ± 1.0 ^B	9.9 ± 0.9 ^C	3.0 ± 0.3 ^A	5.9 ± 0.8 ^B	142.3 ± 9.6 ^B	40.9 ± 5.4 ^B
35S-1	21.7 ± 1.0 ^C	13.6 ± 1.1 ^A	1.6 ± 0.1 ^B	13.5 ± 1.5 ^A	163.0 ± 11.3 ^A	23.7 ± 4.2 ^C
35S-2	22.1 ± 1.2 ^C	12.9 ± 0.9 ^B	1.7 ± 0.1 ^B	13.5 ± 0.6 ^A	157.1 ± 10.1 ^A	23.9 ± 4.5 ^C

Numbers are presented as mean ± SE. The number of observations in each mean is 20. Means in the same column followed by the same letter are not significantly different ($P < 0.05$, LSD test). Date was recorded from 14-d seedlings except tiller number, which was recorded at 80-d after germination.

nidase (GUS) assay of transgenic plants expressing an *OsPIN1* promoter/GUS fusion construct (Fig. 2). RT-PCR analysis showed that *OsPIN1* mRNA was transcribed in all tissues tested. The expression of the gene was higher in leaf, flower and seed than in root, stem and root collar (Fig. 2A). To confirm the expression patterns, a binary vector containing the *GUS* gene driven by the *OsPIN1* promoter was constructed and used for rice transformation. The transgenic plants showed *GUS* expression in the vascular tissues of root, stem, anther, leaf and embryo of the seed (Fig. 2B–G). Cross-sections showed that *GUS* is also expressed in the primordia of adventitious and lateral roots (Fig. 2D, H), suggesting that *OsPIN1* may be involved in root development.

OsPIN1 is involved in polar auxin transport in rice

To investigate the function of *OsPIN1* in rice, transgenic plants overexpressing or underexpressing *OsPIN1* were produced using 35S-driven *OsPIN1* and *OsPIN1* RNA interfering (RNAi) constructs. Two transgenic lines were selected to represent the overexpressing or underexpressing groups. They were designated as 35S1 and 35S2, and RNAi1 and RNAi2, respectively (Fig. 3C). At the seedling stage, 14 days after germination, plant height, primary root length, adventitious root number and lateral root number on the primary root were investigated in wild-type and transgenic plants. The tiller number was counted at the maximum tillering stage (80 days after germination) under field conditions. The results showed that overexpression of the *OsPIN1* gene significantly increased the primary root length and lateral root number, while underex-

Table 2 Roots number of wild-type and OsPIN1 transgenic seedlings with NAA or NPA treatment

Treatments	Genotypes	Number of adventitious roots ^a		
		7-day-old seedlings	14-day-old seedlings	21-day-old seedlings
CK	WT	5.2 ± 0.8 ^{BCD}	12.1 ± 1.1 ^B	22.4 ± 1.0 ^B
	RNAi-1	4.7 ± 0.8 ^{CD}	5.4 ± 0.8 ^{EF}	10.7 ± 1.5 ^F
	RNAi-2	4.8 ± 0.8 ^D	5.5 ± 0.8 ^{EF}	11.1 ± 1.4 ^F
	35S-1	5.1 ± 0.6 ^{BCD}	12.0 ± 1.5 ^B	22.1 ± 1.8 ^{BC}
	35S-2	5.1 ± 1.0 ^{BCD}	11.5 ± 1.7 ^{BC}	22.2 ± 1.7 ^{BC}
NAA ^b	WT	6.9 ± 1.1 ^A	14.3 ± 1.1 ^A	24.6 ± 1.1 ^A
	RNAi-1	5.1 ± 1.1 ^{BCD}	11.8 ± 1.1 ^{BC}	21.4 ± 1.1 ^{CD}
	RNAi-2	4.9 ± 1.0 ^{CD}	11.1 ± 1.1 ^C	20.9 ± 0.9 ^D
	35S-1	7.2 ± 0.9 ^A	14.3 ± 1.5 ^A	24.7 ± 1.5 ^A
	35S-2	7.0 ± 0.8 ^A	14.1 ± 0.8 ^A	24.2 ± 1.4 ^A
NPA ^c	WT	5.3 ± 0.6 ^{BC}	5.9 ± 1.0 ^E	8.7 ± 1.6 ^G
	RNAi-1	4.7 ± 0.9 ^D	4.7 ± 0.9 ^G	NA ^d
	RNAi-2	4.8 ± 0.7 ^{CD}	4.8 ± 0.7 ^{FG}	NA
	35S-1	5.2 ± 1.1 ^{BCD}	7.9 ± 1.4 ^D	12.9 ± 1.6 ^E
	35S-2	5.5 ± 0.8 ^B	7.3 ± 1.1 ^D	12.2 ± 1.7 ^E

Means in the same column followed by the same letter are not significantly different ($P < 0.05$, LSD test).

^a Numbers are presented as mean ± SE. The number of observations in each mean is 20.

^b The seedlings were grown under 0.1 μM NAA treatment after germination.

^c The seedlings were grown under 0.5 μM NPA treatment after 7 d normal cultivation.

^d NA, not available. Seedlings died after 21 d treatment.

pression of *OsPIN1* resulted in a reduced number of adventitious roots and a significantly increased number of tillers (Table 1). Moreover, the tiller angles in the RNAi plants exceeded 30 degrees, which was much higher than the wild-type plants (Fig. 3A).

To verify whether *OsPIN1* is involved in polar auxin transport, seedlings of the wild-type and transgenic plants were exposed to 0.1 μ M α -naphthylacetic acid (α -NAA) and 0.5 μ M NPA. The numbers of adventitious roots at the 7, 14 and 21-day-old seedling stages were recorded (Table 2). Results showed that the suppression of the *OsPIN1* gene inhibited the formation of adventitious roots. Thus the number of adventitious roots in the *OsPIN1* RNA interference (RNAi) transgenic plants was significantly less than that in wild-type plants (Table 2). NPA treatment had a similar effect on the wild-type plants (Table 2), suggesting that underexpression of the *OsPIN1* gene may have affected the auxin transport process. Exogenously applied NAA on the RNAi transgenic plants partially complemented the inhibition effect of the underexpression of *OsPIN1*, confirming that *OsPIN1* is involved in polar auxin transport.

Fig. 3B and C show that the expression level of *OsPIN1* was inversely correlated with the sensitivity of the plants to the NPA treatment. In NPA treatment, the adventitious root numbers in transgenic plants overexpressing *OsPIN1* (35S1 and 35S2) were significantly higher than in wild-type seedlings (Table 2). This suggests that the higher level of *OsPIN1* expression had partially overcome the inhibition effect of the NPA treatment on the formation of adventitious roots. On the other hand, the *OsPIN1* RNAi plants were hypersensitive to the treatment of NPA. Application of NPA to the *OsPIN1* RNAi transgenic seedlings resulted in these seedlings losing their apical dominance in shoot growth (Fig. 3B), and eventually dying after a 14-day treatment (Table 2). These results provided further evidence that *OsPIN1* is involved in polar auxin transport.

OsPIN1 is involved in the emergence of adventitious root primordia

Five adventitious roots were formed at the coleoptile node of 7-day-old seedlings in both the RNAi transgenic and the wild-type plants (Fig. 4A, E). In the second week, numbers of adventitious roots developed from the first node of the wild-type plants, while no new adventitious roots were initiated until the third week in the RNAi transgenic plants (Fig. 4B, F). Moreover, the number of adventitious roots in the RNAi transgenic plants was significantly less than in the wild-type plants (Table 2, Fig. 4C, D, G, H).

To investigate whether the delayed development of the adventitious roots from the first node resulted from the arrest of primordia initiation or primordia emergence, transverse sections of the first node at different time points were examined. Results showed that adventitious root primordia were initiated in 7-day-old seedlings of both wild-type and *OsPIN1* RNAi transgenic plants (Fig. 4 I, J), indicating that the formation of adventitious root primordia at the first node was not affected by

the underexpression of *OsPIN1*. However, in the 14-day-old seedlings, new adventitious roots had developed in the wild-type plants (Fig. 4K), while they were not visible in the *OsPIN1* RNAi transgenic plants. The adventitious root primordia in these RNAi plants stayed at the same size as those in the 7-day-old seedlings (Fig. 4L), indicating that the emergence of adventitious root primordia was arrested in these RNAi seedlings. Itoh et al. (2005) divided the process of adventitious root development into seven stages. Our data showed that the development of adventitious roots in RNAi seedlings ceased at the sixth stage.

Discussion

Phylogenetic analysis showed that the putative auxin efflux carrier, *PIN1*, is a highly conserved gene family, which may play a key role in polar auxin transport (Friml and Palme 2002a). The *PIN1* family often contains a hydrophilic region interconnecting two blocks of transmembrane segments (Palme and Gälweiler 1999). The *OsPIN1* protein contains all of these conserved transmembrane segments. It has been reported that the expression of *AtPIN1* was localized at the basal side of auxin-transport-competent cells within root and shoot vascular tissues (Gälweiler et al. 1998) and narrowed down to provascular cells in germinated seeds (Palme and Gälweiler 1999). Our results showed an expression pattern in *OsPIN1* similar to that in *AtPIN1* (Fig. 2), which confirmed that *OsPIN1* is evolutionarily closer to the *PIN1* than the *PIN2* family as previously reported (Luschnig et al. 1998).

That *OsPIN1* acts as an auxin efflux is confirmed by similar phenotypes in NPA-treated wild-type plants and *OsPIN1* suppression transgenic plants. NPA is a well-characterized inhibitor of the polar auxin transport, which strongly inhibits auxin efflux (Luschnig 2001). NPA treatment of wild-type *Arabidopsis* plants phenocopied the mutant phenotype of *pin1* in *Arabidopsis* (Vernoux et al. 2000). Application of an exogenous auxin to the naked inflorescence apex complemented the defects of flower development in NPA-treated plants or *pin1* mutants (Reinhardt et al. 2000). In this study, the inhibition effect of *OsPIN1* suppression on the development of adventitious root primordia was similar to that of NPA-treatment. Application of exogenous NAA rescued the defective phenotype in RNAi transgenic plants (Table 2). Our data supported that, acting like *PIN1* in *Arabidopsis*, *OsPIN1* is an auxin efflux carrier in rice.

Arabidopsis *PIN1* efflux carriers were shown to express in a specific pattern in the shoot apical meristem. They are involved in the apical-basal axis formation of the embryo (Friml et al. 2003), development of lateral root primordia (Benková et al. 2003), and regulation of cell fate at the periphery of the shoot apical meristem (Vernoux et al. 2000). Adventitious roots and tillers are very important organs in rice. In this study, the transgenic plants that underexpress *OsPIN1* display a significant decrease in adventitious root numbers. The decrease

of adventitious root numbers in *OsPIN1* RNAi plants was caused by the arrest of the primordia emergence. Our data support the hypothesis that auxin and its polar transport are critical for the development of adventitious roots (Zhou et al. 2003).

Unlike the *Arabidopsis PIN1* family, there are three auxin efflux carriers in the rice *PIN1* family (Fig. 1E), *OsPIN1*, *OsPIN1b* and *OsPIN1c*. Studies have shown that *AtPIN1* is essential in the initiation and development of lateral root primordia in *Arabidopsis* (Benková et al. 2003). In the present study, suppression of *OsPIN1* did not affect the development of lateral roots (Table 1). It is possible that the three *OsPIN1* genes in rice are functionally complementary in the controlling of the development of later roots.

Studies in several species has shown that auxin does not act directly in the axillary bud, since apically applied IAA is not transported into inhibited buds (Hall and Hillman 1975, Morris 1977). It must, therefore, act elsewhere to control the synthesis, transport or metabolism of one or more of the secondary messengers, such as cytokinin and some yet unidentified substances (Ward and Leyser 2004). As a non-cell autonomous signal, auxin often interacts with other signalling pathways through its polar transport to regulate plant developmental processes. It has been reported that the endogenously applied auxin affects shoot branching (Rogg et al. 2001, Romano et al. 1991). Transgenic plants with a reduction of *OsPIN1* gene expression display an increase in tiller number as well as till angle. The phenotype may result from the disturbance of auxin-mediated bud inhibition. Further research is needed to clarify the intrinsic reason. The difference in the regulation effect of *OsPIN1* on the tiller primordia compared with the adventitious primordia may be caused by different effects of local auxin concentration in the different parts of the plants. More direct evidence, however, is needed to confirm this hypothesis.

Functional redundancy among PIN proteins has been found in *Arabidopsis* (Benková et al. 2003, Friml et al. 2003). For instance, the *AtPIN1* protein localization was changed in *Atpin3pin4pin7* triple mutants (Blilou et al. 2005). The highly conserved portions of PIN family proteins in the transmembrane domains, may be further evidence that they are, at least in part, functionally redundant. To understand the effect of *OsPIN1* on the expression of other *OsPIN* genes, we analyzed the expressions of other putative auxin efflux carriers *OsPIN1b*, *OsPIN1c*, *OsPIN2* and *OsPIN3a* in *OsPIN1* RNAi transgenic plants. It was found that the expressions of these putative auxin efflux carrier genes were not altered by the underexpression of *OsPIN1* (data not shown), indicating that these auxin efflux carriers may act independently.

In conclusion, our results proved that rice *OsPIN1* acts as a polar auxin transport. The evidence for this conclusion came from the predicted *OsPIN1* protein structure, the preferential expression of *OsPIN1* in vascular tissues, and the alteration of the sensitivities to NAA and NPA in transgenic plants that

over- or underexpressed the *OsPIN1* gene. The finding that *OsPIN1* is involved in auxin-dependent adventitious root emergence and tillering provides a new insight into the function of the *PIN1* family in rice.

Materials and Methods

Structure and phylogenetic analysis

The hydropathy plot analysis was performed with ProtScale (<http://us.expasy.org/tools/protscale.html>). The membrane-spanning regions and their orientation were predicted by the Tmpred method (http://www.ch.embnet.org/software/TMPRED_form.html). The phylogenetic tree was generated based on minimal evolution criterion using the neighbor-joining method with 100 bootstrap. SEPIN (gi:57636762) from *Staphylococcus epidermidis* was selected as the outgroup. Putative *OsPIN1b* (gi:32988390), *OsPIN1c* (gi:51535183), *OsPIN2* (gi:52077371), *OsPIN3a* (gi:55297110) and *OsPIN3b* (gi:50932811) were identified through a TBLASTN search of *Oryza sativa* cDNA sequences in GenBank and named according to the phylogenetic analysis. *AtPIN1* (gi:15219501), *AtPIN2* (gi:42558886), *AtPIN3* (gi:42558887), *AtPIN4* (gi:42558871), *AtPIN5* (gi:42558885), *AtPIN6* (gi:42558888), *AtPIN7* (gi:42558877) and *AtPIN8* (gi:42558880) were from *Arabidopsis thaliana* (thale cress). *Gh-PIN* (gi:25140423), *Ta-PIN1* (gi:42556526), *Cs-PIN1* (gi:25956262), *MCM-AEC1* (gi:33339148) and *MCM-AEC3* (gi:33339152), *Ptt-PIN1* (gi:10441744) and *Ptt-PIN3* (gi:21435938), *Ps-PIN1* (gi:28301753) and *Ps-PIN2*, *Pp-PIN* (gi:55859521), *Bj-PIN2* (gi:15485153) and *Bj-PIN3* (gi:15485155), *Mt-PIN2* (gi:28204657), *Mt-PIN3* (gi:25986775), *Mt-PIN6* (gi:49035694), *Mt-PIN7* (gi:49035696) and *Mt-PIN8* (gi:58339253) were from *Gossypium hirsutum* (upland cotton), *Triticum aestivum* (bread wheat), *Cucumis sativus* (cucumber), *Momordica charantia* (balsam pear), *Populus tremula* x *Populus tremuloides*, (gi:39840930), *Pisum sativum* (pea), *Physcomitrella patens*, *Brassica juncea* and *Medicago truncatula* (barrel medic), respectively.

Construction of vectors and plant transformation

A 2.1 kb promoter region of the *OsPIN1* gene was amplified by PCR using the upper primer 5'-TTTtaggagcagcgttttagcattgttcacg-3' and lower primer 5'-ATAGGTACCGGAGGAGGAGCATTGATTGG-3'. The PCR product was then inserted upstream of the 5' end of the *GUS* gene (*gusA*) in pCAMBIA1391Z to create a P_{OsPIN1} -*GUS* fusion construct vector. For the RNA interfering vector construct, a 219 bp cDNA fragment between positions 1136 and 1354 of the *OsPIN1* cDNA, was amplified using the upper primer 5'-CGAAGGACAGGGAGGACTACGTGG-3' and lower primer 5'-TGTTCCGGTTGCGGATGAG-3'. The second intron of *NIR1* from maize was inserted between the sense and antisense cDNA fragments to form an intron-containing hairpin RNA. It was then inserted into pCAMBIA1301-35S. The resulting construct was named p $OsPIN1$ -RNAi. The *OsPIN1* cDNA was inserted into the binary plant vector pCAMBIA1301-35S. The resulting plasmid was named 35S::*OsPIN1*. The above constructs were transformed into a callus initiated from mature wild-type plant (*Oryza sativa* L ssp. Japonica, Nipponbare) seeds by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation (Chen et al. 2003).

Histochemical analysis and GUS assay

Histochemical GUS analysis was performed as described (Jefferson et al. 1987). Transgenic plant samples were incubated with X-gluc buffer overnight at 37°C. The stained tissues were photographed using a LEICA MZ95 stereomicroscope with a color CCD camera (Leica Instrument, Nussloch, Germany). After being stained,

the tissues were rinsed and fixed in formalin acetic acid-alcohol (FAA) fixation solution at 4°C overnight, embedded in paraffin, and then sectioned. Sections of thickness, 8–10 µm were mounted on slides and photographed using a Zeiss Axiocam HRC digital color camera (Zeiss, Jena, Germany).

RT-PCR analysis

Total RNA from the roots, stems, root-collar, leaves, flowers, and immature seeds of wide-type plants were isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The first-strand cDNA was synthesized by using Superscript II (Invitrogen, Carlsbad, CA, USA) and total RNA was used as the RT-PCR template. RT-PCR was performed using the gene-specific primers 5'-TCTCGCTCGGACATCTACTCCC-3' and 5'-AGTCCTCCTGTCCCTTCGCTC-3'.

RNA gel blotting analysis

RNA gel blotting analysis was performed as described (Church and Gilbert 1984). Briefly, 20 µg of the total RNA isolated using Trizol was separated on 1% agarose gels containing 1× MOPS and 0.66 M formaldehyde, transferred onto a Hybond-N⁺ nylon membrane (Amersham Pharmacia, Piscataway, NJ, USA) and hybridized in a solution containing 0.5M sodium phosphate (PH 7.0), 1mM EDTA, 1% BSA, and 7% SDS for 20h at 65°C. The probe was prepared using a random priming method. After hybridization, the blot was washed twice with a solution containing 0.1× SSC and 0.1% SDS for 5 min at room temperature, followed by two washes of the same solution at 55°C for 20 min. Hybridization was performed with a [α -³²P] dCTP-labeled probe by the random priming method (Sambrook et al. 1989). Hybridized membranes were scanned and analyzed with a Typhoon 1600 PhosphorImager scanner (Molecular Dynamics, Sunnyvale, CA USA).

Seedling growth conditions and hormone treatments

The germinated rice seeds were planted in a hydroponic culture containing a half-complete nutrient solution in the first week and a complete nutrient solution beyond that point. The hydroponic system was kept in the greenhouse with 12 h photoperiodic conditions and 28/24°C thermoperiods (12h/12h), and 80% humidity. The hydroponic solution contained 3.0 mM NH₄NO₃, 1.0 mM CaCl₂, 0.32 mM NaH₂PO₄, 0.51 mM K₂SO₄, 1.65 mM MgSO₄, 3.13 mM MnCl₂, 1.52 mM (NH₄)₆Mo₇O₂₄, 1.5 mM H₃BO₃, 1.5 mM ZnSO₄, 1.6 mM CuSO₄, 35 mM FeCl₃, and 70 mM C₆H₈O₇. The pH of the solution was adjusted to 5.0. Wild-type and T₂ transgenic plants were used for the analyses of the effects of exogenously applied α -NAA at 10⁻⁷ M and NPA at 5×10⁻⁷ M on adventitious root growth. The root collars of wild-type and OsPIN1 transgenic plants were photographed using a LEICA MZ95 digital camera. The lateral root number on the primary root was measured using the scanner-based image-analysis system WinRhizo (Regent Instruments, Quebec, Canada).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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References

- Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G. and Friml, J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* 115: 591–602.
- Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B. and Feldmann, K.A. (1996) *Arabidopsis* AUX1 gene: a permease-like regulator of root gravitropism. *Science* 273: 948–950.
- Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K. and Scheres, B. (2005) The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. *Nature* 433: 39–44.
- Chatfield, S.P., Stirnberg, P., Forde, B.G. and Leyser, O. (2000) Hormonal regulation of bud growth in *Arabidopsis*. *Plant J.* 24: 159–169.
- Chen, R., Hilson, P., Sedbrook, J., Rosen, E., Caspar, T. and Masson, P.H. (1998) The *Arabidopsis thaliana* AGR1/TROPIC 1 gene encodes a component of the polar-auxin-transport efflux carrier. *Proc. Natl Acad. Sci. USA* 95: 15112–15117.
- Chen, S.Y., Jin, W.Z., Wang, M.Y., Zhang, F., Zhou, J., Jia, Q.J., Wu, Y.R., Liu, F.Y. and Wu, P. (2003) Distribution and characterization of over 1000 T-DNA tags in rice genome. *Plant J.* 36: 105–113.
- Church, G.M. and Gilbert, W. (1984) Genomic sequencing. *Proc. Natl Acad. Sci. USA* 81: 1191–1195.
- Crozier, A., Kamiya, Y., Bishop, G. and Yokota, T. (2000) Biosynthesis of hormones and elicitor molecules. In *Biochemistry and Molecular Biology of Plants*. Edit by Buchanan, B.B., Gruisssen, W. and Gones, R.L. pp. 884–894. The American Society of Plant Physiologists, Rockville.
- Friml, J. and Palme, K. (2002a) Polar auxin transport – old questions and new concepts? *Plant Mol. Biol.* 49: 273–284.
- Friml, J., Wisniewska, J., Benková, E., Mendgen, K. and Palme, K. (2002b) Lateral relocation of auxin efflux regulator AtPIN3 mediates tropism in *Arabidopsis*. *Nature* 415: 806–809.
- Friml, J., Benková, E., Blilou, I., Wisniewska, J., Hamann, T., Ljung, K., Woody, S., Sandberg, G., Scheres, B., Jürgens, G. and Palme, K. (2002c) AtPIN4 mediates sink driven auxin gradients and patterning in *Arabidopsis* roots. *Cell* 108: 661–673.
- Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R. and Jürgens, G. (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* 426: 147–153.
- Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A. and Palme, K. (1998) Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. *Science* 282: 2226–2230.
- Hall, S.M. and Hillman, J.R. (1975) Correlative inhibition of lateral bud growth in *Phaseolus vulgaris* L.: timing of bud growth following decapitation. *Planta* 123: 137–143.
- Inukai, Y., Sakamoto, T., Ueguchi-Tanaka, M., Shibata, Y., Gomi, K., Umemura, I., Hasegawa, Y., Ashikari, M., Kitano, H. and Matsuoka, M. (2005) The crown rootless1 gene in rice is essential for crown root formation and is a target of an AUXIN RESPONSE FACTOR in auxin signaling. *Plant Cell* 17: 1387–1396.
- Itoh, J., Nonomura, K., Ikeda, K., Yamaki, S., Inukai, Y., Yamagishi, H., Kitano, H. and Nagato, Y. (2005) Rice plant development: from zygote to spikelet. *Plant Cell Physiol.* 46: 23–47.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β -glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* 6: 3901–3907.
- Laskowski, M.J., Williams, M.E., Nusbaum, C. and Sussex, I.M. (1995) Formation of lateral root meristems is a two-stage process. *Development* 121: 3303–3310.
- Leyser, O. (2001) Auxin signalling: the beginning, the middle and the end. *Curr. Opin. Plant Biol.* 4: 382–386.
- Leyser, O. (2003) Regulation of shoot branching by auxin. *Trends Plant Sci.* 8: 541–545.
- Liu, H.J., Wang, S.F., Yu, X.B., Yu, J., He, X.W., Zhang, S.L., Shou, H.X. and Wu, P. (2005) ARL1, a LOB-domain protein required for adventitious root formation in rice. *Plant J.* 43: 47–56.

- Luschig, C., Gaxiola, R., Grisafi, P. and Fink, G. (1998) EIR1, a root specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev.* 12: 2175–2187.
- Luschig, C. (2001) Auxin transport: why plants like to think BIG. *Curr. Biol.* 11: 831–833.
- Morris, D.A. (1977) Transport of exogenous auxin in two-branched dwarf pea seedlings (*Pisum sativum* L.). *Planta* 136: 91–96.
- Muday, G.K. and DeLong, A. (2001) Polar auxin transport: controlling where and how much. *Trends Plant Sci.* 6: 535–542.
- Müller, A., Guan, C., Gälweiler, L., Tänzler, P., Huijser, P., Marchant, A., Parry, G., Bennett, M., Wisman, E. and Palme, K. (1998) AtPIN2 defines a locus of *Arabidopsis* for root gravitropism control. *EMBO J.* 17: 6903–6911.
- Ni, W.M., Chen, X.Y., Xu, Z.H. and Xue, H.W. (2002) Isolation and functional analysis of a *Brassica juncea* gene encoding a component of auxin efflux carrier. *Cell Res.* 12: 235–245.
- Okada, K., Ueda, J., Komaki, M.K., Bell, C.J. and Shimura, Y. (1991) Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* 3: 677–684.
- Palme, K. and Gälweiler, L. (1999) PIN-pointing the molecular basis of auxin transport. *Curr. Opin. Plant Biol.* 2: 375–381.
- Paquette, A.J. and Benfey, P.N. (2001) Axis formation and polarity in plants. *Curr. Opin. Genet. Dev.* 11: 405–409.
- Reinhardt, D., Mandel, T., and Kuhlemeier, C. (2000) Auxin regulates the initiation and radial position of plant lateral organs. *Plant Cell* 12: 507–519.
- Reinhardt, D., Pesce, E.R., Stieger, P., Mandel, T., Baltensberger, K., Bennett, M., Traas, J., Friml, J. and Kuhlemeier, C. (2003) Regulation of phyllotaxis by polar auxin transport. *Nature.* 426: 255–260.
- Rogg, L.E., Lasswell, J. and Bartel, B. (2001) A gain-of-function mutation in IAA28 suppresses lateral root development. *Plant Cell* 13: 465–480.
- Romano, C.P., Hein, M.B. and Klee, H.J. (1991) Inactivation of auxin in tobacco transformed with the indole acetic acid-lysine synthetase gene of *Pseudomonas savastanoi*. *Genes Dev.* 5: 438–446.
- Sambrook, J., Fritsch, E.T. and Maniatis, T. (1989) *In Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schnabel, E.L. and Frugoli, J. (2004) The PIN and LAX families of auxin transport genes in *Medicago truncatula*. *Mol. Genet. Genomics* 272: 420–432.
- Schrader, J., Baba, K., May, S.T., Palme, K., Bennett, M., Bhalerao, R.P. and Sandberg, G. (2003) Polar auxin transport in the wood-forming tissues of hybrid aspen is under simultaneous control of developmental and environmental signals. *Proc. Natl Acad. Sci. USA* 100: 10096–100101.
- Swarup, R., Friml, J., Marchant, A., Ljung, K., Sandberg, G., Palme, K. and Bennett, M. (2001) Localization of the auxin permease AUX1 suggests two functionally distinct hormone transport pathways operate in the *Arabidopsis* root apex. *Genes Dev.* 15: 2648–2653.
- Utsuno, K., Shikanai, T., Yamada, Y. and Hashimoto, T. (1998) AGR, an agravitropic locus of *Arabidopsis thaliana*, encodes a novel membrane-protein family member. *Plant Cell Physiol.* 39: 1111–1118.
- Vernoux, T., Kronenberger, J., Grandjean, O., Laufs, P. and Traas, J. (2000) PIN-FORMED 1 regulates cell fate at the periphery of the shoot apical meristem. *Development* 127: 5157–5165.
- Ward, S.P. and Leyser, O. (2004) Shoot branching. *Curr. Opin. Plant Biol.* 7: 73–78.
- Weijers, D. and Jürgens, G. (2005) Auxin and embryo axis formation: the end in sight? *Curr. Opin. Plant Biol.* 8: 32–37.
- Zhou, D., Yin, K., Xu, Z. and Xue, H. (2003) Effect of polar auxin transport on rice root development. *Acta Botanica Sinica* 45: 1421–1427.

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