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ARL1, a LOB-domain protein required for adventitious root formation in rice

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Summary

Adventitious roots constitute the bulk of the fibrous root system in cereals. Compared with the current understanding of shoot development, knowledge of the molecular mechanisms of development of the adventitious roots of cereals is limited. We have isolated and characterized a novel gene controlling the initiation of adventitious root primordia in rice (*Oryza sativa* L.). The gene, designated *Adventitious rootless1* (*ARL1*), encodes a protein with a *LATERAL ORGAN BOUNDARIES* (*LOB*) domain. It is expressed in lateral and adventitious root primordia, tiller primordia, vascular tissues, scutellum, and young pedicels. ARL1 is a nuclear protein and can form homodimers. *ARL1* is an auxin- and ethylene-responsive gene, and the expression pattern of *ARL1* in roots parallels auxin distribution. Our findings suggest that ARL1 is an auxin-responsive factor involved in auxin-mediated cell dedifferentiation, and that it promotes the initial cell division in the pericycle cells adjacent to the peripheral vascular cylinder in the stem.

Keywords: *Oryza sativa* L., adventitious root primordia, primordium initiation, LOB domain gene, auxin-responsive factor.

Introduction

Rice, one of the most important food crops worldwide, has a fibrous root system, which consists of one primary root originating from the seed and a mass of adventitious roots formed from the stem during post-embryonic development. The primary root is short-lived, while the adventitious roots grow acropetally along both the main stem and the tillers to keep pace with leaf emergence (Kawata *et al.*, 1963). Thus, the plant's acquisition of water and nutrients during the growing period is mainly dependent on adventitious and lateral roots.

Due to the simplicity of root structure and the availability of root-specific mutants, extensive studies have been conducted on the root development of Arabidopsis (Casimiro *et al.*, 2003; Scheres *et al.*, 2002). The monocot cereals, however, have a complex root structure compared with Arabidopsis. The fundamental difference between the root system in cereals and that of Arabidopsis is the presence of an extensive stem-borne root system in cereals.

A growing number of studies have focused on the characterization of root development in cereals using mutational analysis. Root-specific mutants show defects in one or more components of the root system (Hao and Ichii, 1999; Hetz *et al.*, 1996; Hochholdinger and Feix, 1998; Inukai *et al.*, 2001). It is known that the development of certain types of roots is subject to both general and specific regulation (Hochholdinger *et al.*, 2004). The identities of genes that are involved in these pathways, especially in the initiation of adventitious root primordium in cereals, are still far from clear.

We isolated a mutant defective in adventitious root formation from somatic mutants and designated it *adventitious rootless1* (*arl1*). The mutant completely lacks adventitious roots. Segregation analysis revealed that the mutant phenotype is controlled by a single locus. Using a positional cloning strategy, we cloned the *ARL1* gene responsible for the *arl1* mutant phenotype. We characterized the expression pattern of *ARL1*, and

investigated the effects of various hormones on *ARL1* gene expression.

Results

Isolation and morphological characterization of the arl1 mutant

A tissue culture-derived rice mutant exhibiting a defect in adventitious root formation was identified and designated *arl1*. In contrast to a normal rice plant, *arl1* mutant seedlings lack adventitious roots (Figure 1a). Due to its lack of essential root structure, the homozygous *arl1* mutant cannot grow to maturity (Figure 1b), although at the seedling stage no significant difference was observed in shoot morphology between the wild type and the mutant. Cross sections at the base of the stem of 5-day-old seedlings revealed that the formation of adventitious primordium is impaired in the *arl1* mutant (Figure 1c,d). Adventitious root primordia were induced at the fourth node of 8-week-old wild-type plants by submergence for 24 h (Figure 1e), but were not induced in the *arl1* mutant (Figure 1f). No significant difference in the number of lateral roots was observed between 7-day-old wild type and *arl1* mutant seedlings, indicating that *ARL1* is specifically involved in the initiation of stem-borne root primordia, but not root-borne primordia. The tiller primordia developed earlier in the *arl1* mutants than in the wild-type plants. No normal tillers were observed in *arl1* mutant plants because the plants died due to the lack of adventitious roots (Figure 1b,g,h).

The rice *WUSCHEL*-type homeobox gene *QHB* and the *OsSCR* gene are expressed at the initiation of periclinal division of cells to form adventitious root primordia (Kamiya *et al.*, 2003). We investigated the expression of these two genes in pericycle cells at the stem base region of 7-day-old wild type and *arl1* mutant seedlings. The tissue-specific RNA samples were extracted using laser-capture microdissection (LCM) (Figure 1i). A marker gene, *OsNAS1*, that is strongly expressed in pericycle and not in surrounding tissues under normal growing conditions (Inoue *et al.*, 2003) was used to confirm that pericycle cells were sampled from the wild type and *arl1* mutant. RT-PCR analysis showed that *OsNAS1* was expressed in the two samples, while both *QHB* and *OsSCR* were expressed in the pericycle cells of wild-type plants, but not in those of *arl1* mutant plants (Figure 1j). These results indicate that the initiation of periclinal division of cells to form primordia is impaired in the *arl1* mutant.

Cloning and characterization of ARL1

Genetic analysis of 200 F_2 progeny derived from a cross between a heterozygous *arl1* line and the indica cultivar

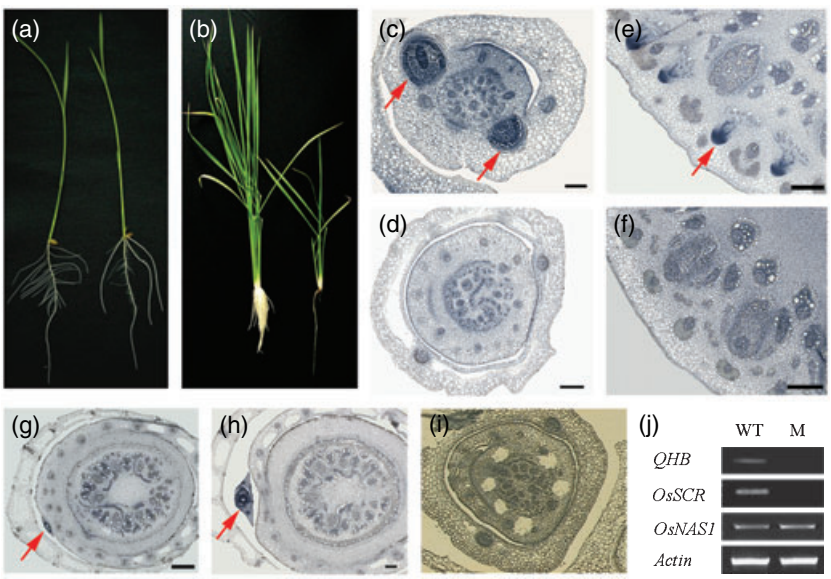

Figure 1. (a) One-week-old seedlings of wild-type plant (right: Zhonghua 11) and *arl1* mutant (left).
(b) Eight-week-old wild-type plant (left: Zhonghua 11) and *arl1* mutant (right).
(c and d) Cross sections through the coleoptilar node of 5-day-old wild type (c) and *arl1* mutant seedling (d). Arrows indicate adventitious root primordia at the coleoptilar node. Bars = 100 μm.
(e and f) Cross sections of the fourth node of 8-week-old wild-type plant (e) and *arl1* mutant plant (f) under submergence for 24 h. Arrowheads indicate adventitious root primordia. Bars = 200 μm.
(g and h) Cross sections of the stem base of 8-week-old wild-type plant (g) and *arl1* mutant plant (h). Arrowheads indicate tiller primordia. Bars = 200 μm.
(i) LCM sampling of pericycle cells adjacent to the peripheral vascular cylinder on cross sections of the stem base.
(j) RT-PCR analysis of expression of *QHB*, *OsNAS1* and *OsSCR* in pericycle cells adjacent to the peripheral vascular cylinder in wild type (WT) and *arl1* mutant (M) plants.

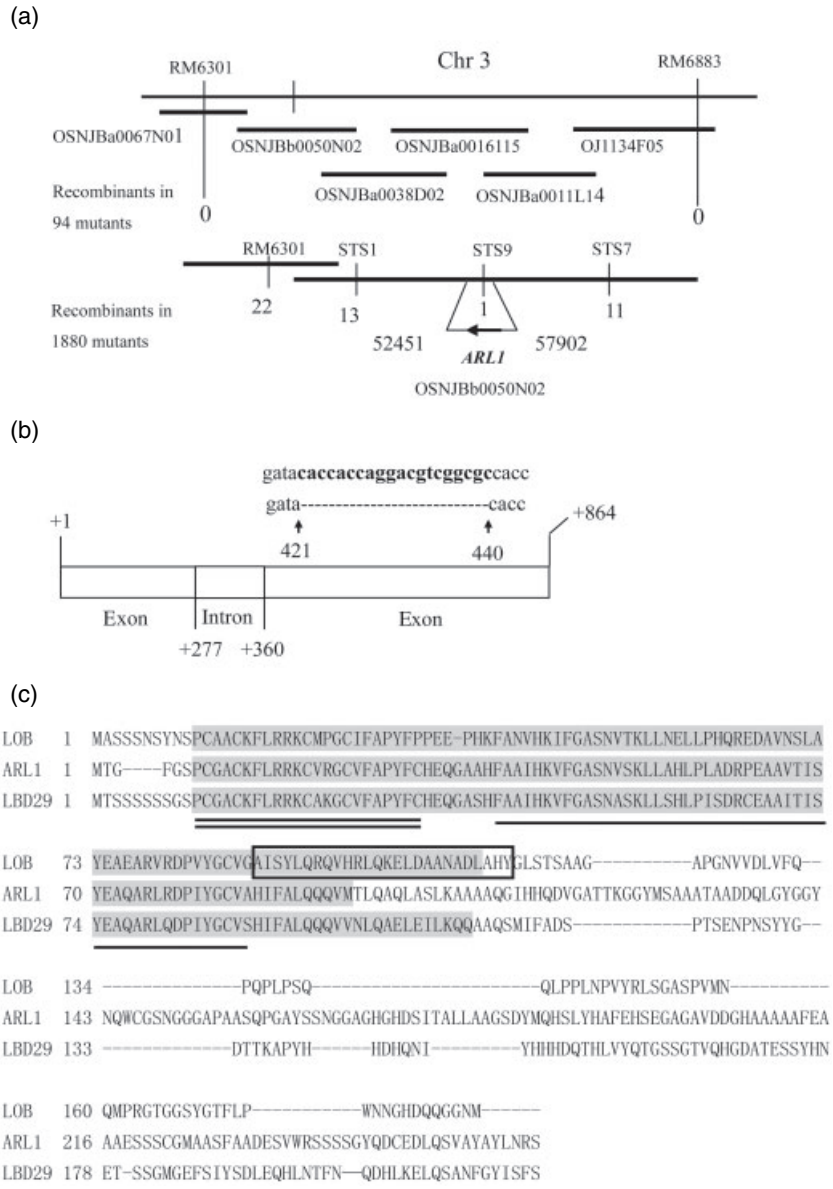

Figure 2. (a) Cloning of *ARL1*. *ARL1* was mapped within the BAC clone OSNJBb0050N02, and was further localized within a 10-kb region from STS9. BAC, bacterial artificial chromosome.
(b) A fragment with 20 bp was deleted at the second exon of *ARL1* from 421 to 440 bp.
(c) Protein sequence alignment between the ARL1 protein and Arabidopsis LOB, LBD29 protein (gene). The LOB domain is highlighted in gray, and conserved C blocks and GAS blocks are underlined with single and double lines, respectively. The predicted coiled-coil is shown with a rectangular box.

Kasalath revealed that *arl1* possessed a recessive mutation at a single nuclear locus. The *ARL1* locus was first mapped to the short arm of chromosome 3 between the markers RM6301 and RM6883 using 94 F_2 mutant plants. Three new sequence-tagged site (STS) markers were developed between RM6301 and RM6883 for fine mapping using 1880 F_2 *arl1* mutant plants among 6500 F_2 plants. The *ARL1* gene was fine-mapped to a 10-kb region near STS9 in a BAC clone (OSNJBb0050N02) (Figure 2a). The 10-kb regions were then amplified from both wild type and *arl1* mutant plants and sequenced. BLAST analysis revealed the presence of an *arl1* mutant-specific 20-bp deletion extending from +421 to +440 bp of a gene encoding a member of the *LATERAL ORGAN BOUNDARIES* (LOB) domain family of plant-specific proteins (Shuai *et al.*, 2002) (Figure 2b). The gene is designated *OsARL1*. BLAST analysis of its amino acid sequence indicated that OsARL1 is highly similar to the Arabidopsis LBD proteins, especially LBD29. Prediction of the secondary structure of the protein was performed. The LOB domain, C block, GAS block, and the predicted coiled-coil structure that are conserved in LOB domain proteins were found in the rice protein (Figure 2c). The results indicate that ARL1 is a member of the plant-specific LOB domain family of proteins.

A full-length 780-bp *OsARL1* cDNA was cloned by reverse transcription (RT)-PCR using RNA prepared from wild-type seedlings. Alignment of the cDNA with the genomic DNA sequences revealed that the gene contains an 84-bp intron and two exons. The 20-bp deletion occurred in the second exon of the gene (Figure 2b). The wild-type *OsARL1* gene

encodes a protein containing 259 amino acids. The 20-bp deletion in the mutated gene results in a frame shift, which produces a prematurely terminated polypeptide of 155 amino acids.

Complementation test for arl1 mutant

Complementation analysis of the *arl1* mutant was performed using *Agrobacterium tumefaciens*-mediated transformation. A 5.4-kb wild-type genomic DNA fragment containing the *ARL1* ORF, 3.7 kb of upstream and 0.8 kb of downstream sequence was cloned into the pCAMBIA 1301 vector for transformation. Twenty independent transgenic lines were obtained with 10–50 sibling plants in each line. Insertion and expression of the transgene were confirmed by RT-PCR (Figure 3b) and Southern blot analysis (Figure 3c). The transgenic *arl1* lines were able to form normal adventitious roots and tillers (Figure 3a), indicating the complete rescue of the mutant phenotype. In addition, the reduction of *ARL1* expression in wild-type plants through RNA interference (RNAi) resulted in a defect in adventitious roots, but not in lateral roots (Figure 3d).

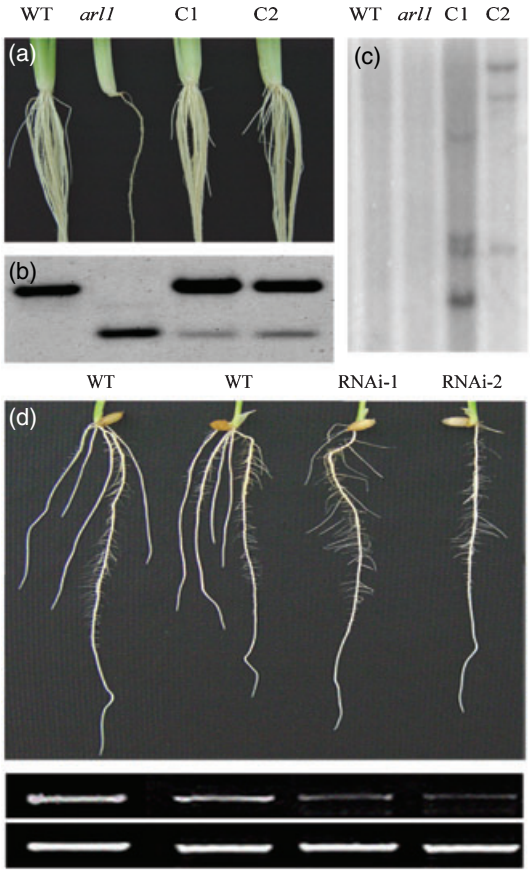

Figure 3. (a) Complementation analysis of the *arl1* mutant. From left to right: wild-type plant, *arl1* mutant, two lines of *arl1* mutant harboring four and three *ARL1* transgene copies, respectively.
(b) PCR analysis using cDNA for *ARL1* in PAGE gel. WT, wild type; *arl1* mutant; C1, complemented line 1; C2, complemented line 2.
(c) Southern blotting analysis using *Hyg* (hygromycin gene) as probe.
(d) Root morphology of wild-type plants (WT), with RNAi of *ARL1*. Indicated at the bottom is expression of *ARL1* and actin gene based on RT-PCR analysis. RT-PCR analysis for 30 running cycles for *ARL1* and 27 cycles for actin.

Subcellular localization and activation domain analysis of ARL1

To examine the subcellular localization of ARL1, *ARL1* was fused in-frame to the N-terminus of *mGFP4* and transiently expressed in onion epidermal cells. The ARL1-GFP green fluorescent signal was detected mainly in nuclei, suggesting that ARL1 is a nuclear protein (Figure 4a). A yeast two-hybrid

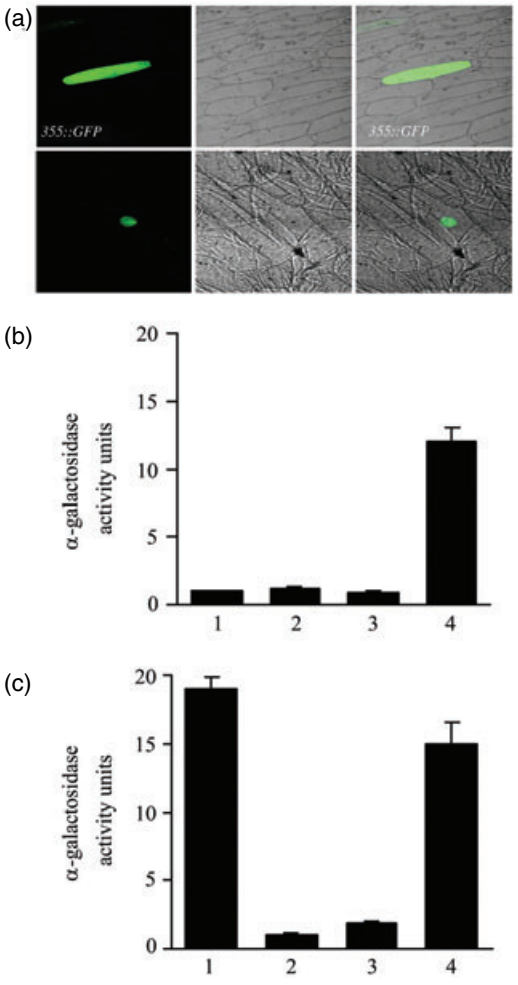

Figure 4. (a) ARL1 protein subcellular localization. Fluorescent microscopy of transiently transformed epidermal onion cells expressing a 35S promoter-driven mGFP4 fusion protein (upper) and the 35S promoter-driven ARL1-mGFP4 fusion protein (lower). The image of mGFP4 green fluorescence (left), the same cells under optic microscope (middle) and their merged image (right).
(b and c) α-Galactosidase activity assays (mU ml^{-1} × cell) in yeast two-hybrid system. (b) 1, BD; 2, BD-ARL1; 3, BD-ARL1(208–259 AA); 4, BD-ARL1(101–259 AA). Activity values are the mean of three measurements. (c) 1, positive control, AD-SV40 + BD-p52; 2, AD-ARL1 + BD; 3, AD-ARL1 + BD-ARL1 (208–259 AA); 4, AD-ARL1 + BD-ARL1.

assay was performed to identify ARL1 protein–protein self-interactions. To define the ARL1-activating domain, a fragment of *ARL1* without the LOB domain [from 101 to 259 amino acid (AA)] and the C-terminal fragment from 208 to 259 AA were also cloned for analysis. These two subclones were named *ARL1*(101–259 AA) and *ARL1*(from 208 to 259 AA). Yeast cells containing the BD-ARL1(101–259 AA) construct had 10-fold higher α-galactosidase activity than those containing BD-ARL1 or BD-ARL1(208–259 AA) (Figure 4b). The results indicate that amino acids 101–259 may act as a transcriptional activator, and that this activation is dependent on the unmasking of a repressor domain (the N-terminal LOB domain). The α-galactosidase activity of the yeast containing the AD-ARL1 and BD-ARL1 constructs was about 10-fold higher than that of the yeast transfected with the AD-ARL1 and BD, or with AD-ARL1 and BD-ARL1(208–259 AA), suggesting that ARL1 may form homodimers (Figure 4c). As the *arl1* mutant lacks the C-terminus of ARL1 due to the 20-bp deletion from 421 to 440 bp and the resultant frame shift, the mutated protein may not possess the functional domain.

Expression pattern of ARL1

To describe the expression pattern of *ARL1* and the sequences required for gene expression, 1.9 kb of upstream DNA and 106 bp of the coding region of the *ARL1* gene were fused to the GUS reporter gene. This chimeric gene cassette was introduced into the wild-type plant via *Agrobacterium*-mediated transformation. Histochemical staining for GUS activity in T_2 plants showed that ARL1 is expressed in lemma tips, lemma veins, pedicels of young panicles, auricles, the bases of leaf blades, the vascular cylinder tissue of primary and lateral roots, root tips, and in the vascular tissue of the anther (Figure 5a–h). GUS staining was observed in adventitious root primordia, scutellum, stele of the primary root, all cells of younger lateral roots except epidermal cells, and lateral root primordium (Figure 5i–m). RNA *in situ* hybridization confirmed that *ARL1* is expressed in tiller primordia and adventitious roots (Figure 5n,p). Five adventitious root primordia, including emerged primordium and initiated primordium, were observed (Figure 5p). The RNA *in situ* hybridization analysis showed that ARL1 is expressed at the beginning of primordium formation (Figure 5p).

Hormonal responses of the arl1 mutant and ARL1 gene

To examine the responses of the *arl1* mutant to hormones, seeds of the wild type and the *arl1* mutant were germinated and grown on 0.8% agar with different concentrations of α-naphthylacetic acid (α-NAA), a synthetic auxin analogue, and ethephon (2-chlorethanephosphoric acid), a chemical precursor of ethylene. Similar to ethylene, ethephon can induce adventitious root growth in rice (Lorbiecke and Sauter, 1999). Treatments with 10^{-6} M α-NAA and

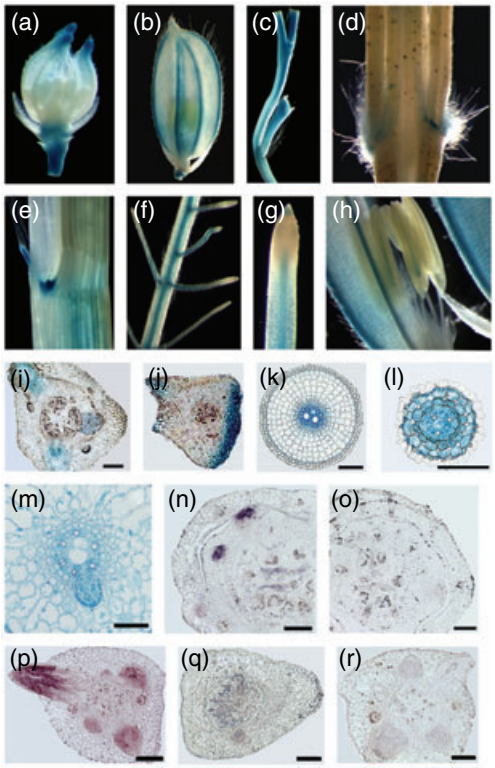

Figure 5. *ALR1* promoter-driven GUS expression in various tissues.
(a–h) (a) Lemma tips. (b) Lemma vein. (c) Pedicel of young panicle. (d) Auricle. (e) Base of leaf blade. (f) Vascular cylinder tissue of primary root and lateral roots. (g) Root tip. (h) Vascular tissue of anther.
(i–m) (i) Cross sections of adventitious root primordia at first node. (j) Scutellum. (k) Vascular cylinder of seminal root. (l) Young lateral root. (m) Lateral root primordium of 7-day-old seedlings. In (k), bar = 100 μM; in (m) and (l), bar = 50 μM; in (i) and (j), bar = 200 μM.
(n–r) RNA *in situ* hybridization of *ARL1* using digoxigenin-labeled RNA probes. Cross sections at shoot base of 28-day-old plants with antisense probe (n) and sense probe (o). The results show the expression of *ARL1* in the primordium of tiller (n). Cross sections at coleoptilar node with antisense probe for 7-day-old wild type (p) and *arl1* mutant (q) seedlings, and with sense probe for 7-day-old wild-type seedling (r). Bar = 200 μM.

1.5×10^{-4} M ethephon significantly enhanced the growth of adventitious roots in wild-type seedlings (Figure 6a–c). However, these treatments did not rescue the *arl1* mutant phenotype. Lateral root growth in both wild type and *arl1* mutant seedlings was repressed by both treatments (Figure 6f–h). Wild type and *arl1* mutant plants responded similarly to N-1-naphthylphthalamic acid (NPA), an inhibitor of auxin polar transport, and to bicyclo(2.2.1)hepta-2,5-diene (NBD), a competitive inhibitor of ethylene activity (Bleecker *et al.*, 1987). Treatment with 10^{-6} M NPA blocked geotropism, while 10 μl l^{-1} NBD dramatically inhibited the elongation of primary roots (Figure 6d,e,i,j). These findings suggest that ARL1 only affects the development of stem-borne primordia, and not the auxin and ethylene pathways.

Using *ARL1*::*GUS* transgenic rice plants, GUS expression was observed throughout the adventitious roots and in the

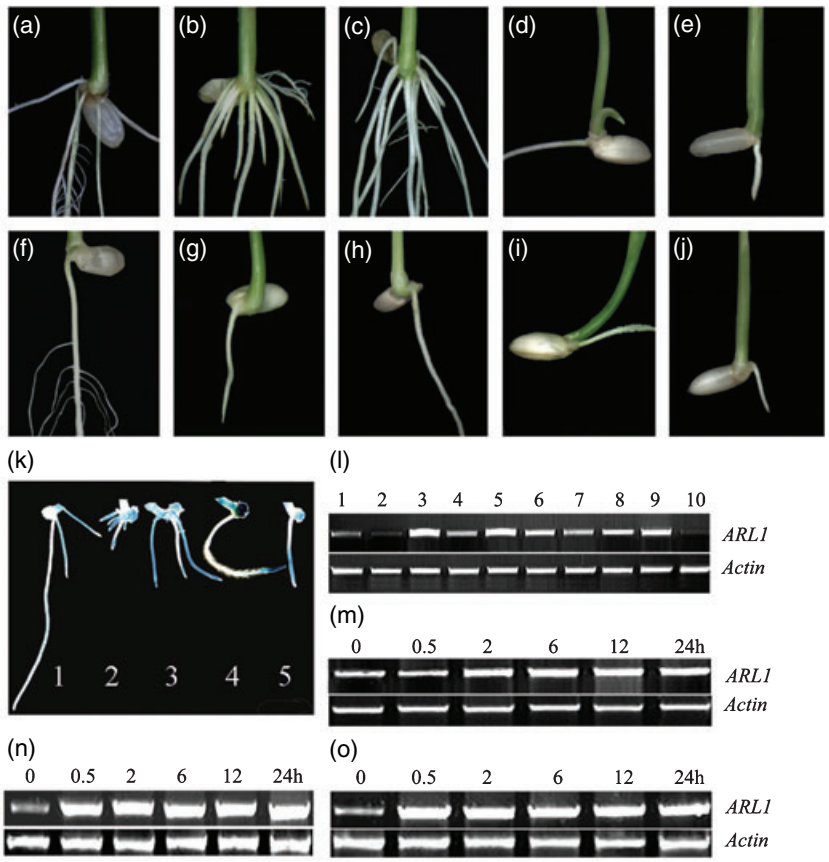

Figure 6. Responses to hormones of wild type and *arl1* mutant plants in term of adventitious root growth.
(a–e) Wild type 10-day-old seedlings. Control (a) treated with 10^{-6} M α-NAA (b), 1.5×10^{-4} M ethyphone (c), 10^{-6} M NPA (d) and 10 μl l^{-1} NBD (e).
(f–j) *arl1* mutant 10-day-old seedlings. Control (f) treated with 10^{-6} M α-NAA (g), 1.5×10^{-4} ethyphone (h), 10^{-6} M NPA (i), and 10 μl l^{-1} NBD (j).
(k) GUS staining of roots under control condition (1), 10^{-6} M α-NAA (2), 1.5×10^{-4} M ethephon (3), 10^{-6} M NPA (4), and 10 μl l^{-1} NBD (5).
(l) RT-PCR analysis of expression of *ARL1* in roots and stem base of wild-type seedlings under control condition, root (1) and stem-base region (2); 10^{-6} M α-NAA, root (3) and stem-base (4); 1.5×10^{-4} M ethephon, root (5) and stem-base region (6); 10^{-6} M NPA, root (7) and stem-base region (8) and 10 μl l^{-1} NBD, root (9) and stem-base (10). mRNA level of actin gene is illustrated at the bottom.
(m–o) Time course experiments with 1.5×10^{-4} M ethyphone (m), 10^{-6} M α-NAA (n) and a mixture of 10^{-6} M α-NAA and 2×10^{-5} M cycloheximide (o) for 4-day-old seedlings of the wild type under solution culture at time points of 0, 0.5, 2, 6, 12, and 24 h. RT-PCR analysis for 28 running cycles for the actin gene and 30 cycles for the *ARL1* gene.

lower parts of primary roots. Intense GUS staining was observed in root tips and scutellum upon treatment with 10^{-6} M NPA (Figure 6k). This might have been caused by auxin accumulation in the root tip and scutellum. The enhanced expression of *ARL1* induced by NPA in the stem base was confirmed by RT-PCR analysis. RT-PCR amplification of *ARL1* showed that *ARL1* expression level is higher in roots than in the stem base region. It can be upregulated in roots by α-NAA, ethephon, and NBD (Figure 6l), indicating that *ARL1* is an auxin- and ethylene-responsive gene. There are three auxin-responsive and two ethylene- responsive elements in the promoter of *ARL1*, including the auxin response factor-specific binding element (AuxRE) TGTCTC (Tiwari *et al.*, 2003) (Table 1). However, alignment of the ARL1 and OsIAA1 (Thakur *et al.*, 2001) amino acid sequences did not reveal conserved Aux/IAA domains in ARL1.

Time course experiments with 10^{-6} M α-NAA and 1.5×10^{-4} M ethephon treatments were performed to assess

Table 1 Hormone-responsive elements exit in the promoter of *ARL1* (http://www.dna.affrc.go.jp/PLACE/signalscan.html)

Factor or site name	Site (strand)	Element
ACGTABREMOTIFA2OSEM	1762 (+) ACGTGKC	ABA-responsive expression
ARFAT	191 (+) TGTCTC	ARF 2
ASF1MOTIFCAMV	1618 (+) TGACG	Auxin and/or salicylic acid 2
DPBFCOREDCDC3	782 (+) ACACNNG	ABA 4
ERELEE4	1897 (–) AWTTCAAA	ERE
GADOWNAT	1762 (+) ACGTGTC	GA-downregulated
GAREAT	512 (–) TAACAAR	GARE
GCCCORE	5 (+) GCCGCC	ERE
RYREPEATBNNAPA	1679 (–) CATGCA	ABA
SEBFCONSSTPR10A	190 (+) YTGTCWC	Auxin response element 2
WRKY71OS	1617 (+) TTGAC	Gibberellin signaling pathway 7

ABA, abscisic acid; ARF, auxin response factor; ERE, ethylene-responsive element; GA, gibberellic acid; GARE, GA-responsive element.

the rapidity of induction of *ARL1* by the hormones. The results showed that the expression of ARL1 was strongly induced by α-NAA within 30 min (Figure 6n), while the induction by ethephon was detectable after 6 h of treatment (Figure 6m). To confirm the direct effect of auxin on the expression of *ARL1*, cycloheximide, an inhibitor of protein synthesis, was used at 2×10^{-5} M together with α-NAA in a time course experiment. The result was similar to that of α-NAA treatment alone (Figure 6o), indicating that the influence of auxin on *ARL1* is direct.

Discussion

Here we report the cloning and characterization of the LOB domain gene *ARL1*, which is required for the initiation of adventitious root primordia in rice. A rice mutant designated *crown rootless1* (*crl1*), with a phenotype similar to that of the *arl1* mutant, has been reported (Inukai *et al.*, 2001). The *crl1* mutant, however, can grow normally to maturation except for a paucity of adventitious roots relative to the wild-type plant. In addition, the number of adventitious roots in *crl1* mutant plants can be significantly increased by treatment with α-NAA at 10^{-6} M. The *arl1* mutant, however, completely lacks adventitious roots during the entire growth period, and this defect cannot be rescued by exogenous auxin.

In rice, the primordia of adventitious roots are initiated from several cells of the pericycle layer adjacent to the peripheral vascular cylinder in the stem (Kaufman, 1959). The development of adventitious root can be divided into 12 successive stages (Kawata and Harada, 1975). At the first stage, the primordia are initiated by the periclinal division of several cells of the pericycle layer, which results in two cell layers, the outer layer and inner layer. The rice *WUSCHEL*-type homeobox gene (*QHB*) and the *OsSCR* gene are expressed in the outer layer at this initiation stage (Kamiya *et al.*, 2003), and can be used as markers for the initiation of adventitious root primordia. We detected neither *QHB* nor *OsSCR* expression in *arl1* at the pericycle layer adjacent to the peripheral vascular cylinder in stem base tissue, although expression of both was seen in wild-type plants (Figure 1j), indicating that the *arl1* mutant is defective in the initiation of the periclinal division of the pericycle cells.

A tBlastn search of the rice genome database with the entire ARL1 amino acid sequence as the query was performed to identify genes related to ARL1. Thirty-four LOB domain genes (OsLBD) were identified (data not shown). All shared varying degrees of similarity in the LOB domain. OsLBD genes can be classified into two classes (class I and class II), similar to the LBD genes in Arabidopsis (Shuai *et al.*, 2002). The analysis of members of multigene families can be particularly difficult, as these genes may be functionally redundant. The discovery of *ARL1* implies that some members of the LOB domain gene family may play an important role in organ development in plants. *LRP1*, *RML1*, *RML2*, and *ALF4-1* are reported to be involved in the development of lateral and adventitious root primordia in Arabidopsis (Celenza *et al.*, 1995; Cheng *et al.*, 1995; Smith and Feodoroff, 1995), whereas no gene functioning only in the formation of adventitious root primordia has been reported so far.

Nuclear localization of the CaMV35S-driven ARL1::mGFP4 fusion protein was observed, while CaMV35S-driven mGFP4 was distributed throughout the cell (Figure 4a). This result indicates that ARL1 is a nuclear protein. A leucine-zipper-like sequence that is conserved in the LOB domain (Iwakawa *et al.*, 2002) is found in ARL1 (Figure 2c). Leucine-zipper-containing proteins can form a scissors-like homodimer that binds the major groove of DNA. Yeast two-hybrid experiments suggest that, as a nuclear protein, ARL1 may homodimerize and activate reporter genes, but also that it has no activating ability by itself due to the repressive activity of the LOB domain (N-terminal 100 AA) (Figure 4c). Our results suggest that ARL1 may act as a transcription factor to regulate the expression of a certain gene(s) as a homodimer or heterodimer and thereby control the initiation of adventitious root primordia.

In Arabidopsis auxin is the major growth-promoting hormone for the initiation of lateral and adventitious root growth. It induces cells in the pericycle and parenchyma to dedifferentiate and enter initial cell divisions. Primordium initiation is controlled by auxin, while the development and emergence of the root primordium are controlled by ethylene (Celenza *et al.*, 1995; De Klerk *et al.*, 1995; Lorbiecke and Sauter, 1999; Pelosi *et al.*, 1995). Neither α-NAA nor ethephon at the optimal concentrations for promoting adventitious root growth could rescue the defect in *arl1*. NPA at 10^{-6} M completely inhibited lateral and adventitious root primordia. A quick and direct response of *ARL1* to α-NAA was observed from the time course experiments with α-NAA alone as well as with a mixture of α-NAA and the protein synthesis inhibitor cycloheximide (Figure 6n,o). These results support the conclusion that *ARL1* is an auxin-responsive gene.

Several root component-specific mutants have been found in cereals. Different root components can be regulated by both common and alternative root type-specific pathways (Hochholdinger *et al.*, 2004). The *arl1* mutant fails to form adventitious roots, but forms normal seminal and lateral roots. ARL1, an auxin-responsive factor, was expressed in a pattern parallel to that of auxin distribution. Based on these facts, we propose that ARL1 is involved in auxin-mediated cell dedifferentiation and promotion of the initial cell division through an interaction with other regulatory protein(s) in the pericycle cells or in the adjacent peripheral vascular cylinder in the stem.

The description of *ARL1*, a key regulator of adventitious root development in rice, provides new insight into the post-embryonic development of adventitious roots in cereal plants. Manipulation of the regulation of ARL1 (and its homologs in other crops) has great potential for increasing the ability of crops to absorb water and nutrients from the soil through improvement in their root architecture, which is required for achieving high yields.

Experimental procedures

Isolation of arl1 mutant

The rice *arl1* mutant was screened from tissue culture plants of the Zhonghua 11 cultivar. A mapping population of 1880 F_2 mutant plants were generated from the crosses between heterozygous plants of the *arl1* mutant and an *indica* variety Kasalath.

Hormone treatments and seedling growth conditions

Seeds of the wild type and *arl1* mutant were individually sowed and germinated on 0.8% agar medium in 100 ml tubes. The medium was supplemented with α-NAA at 10^{-6}, 10^{-7} or 10^{-8} M, ethephon at 10^{-4}, 1.5×10^{-4} or 2×10^{-4} M, NPA, an inhibitor of auxin polar transport, at 10^{-6} M, and NBD, and inhibitor of effects of ethylene, at 10 ml l^{-1}. The tubes were sealed with parafilm and incubated in a growth chamber at 28 ± 1°C under a 12-h photoperiod (200 μmol photons m^{-2} sec^{-1}) regime. The number of adventitious roots was counted at the 10-day-old seedling stage. Time course experiments were conducted using 10^{-6} M α-NAA, 1.5×10^{-4} M ethephon, and 10^{-6} M a-NAA together with an inhibitor of protein synthesis, cycloheximide at 2×10^{-5} M (Calbiochem, San Diego, CA, USA) to treat 4-day-old seedlings of the wild type under solution culture condition at time points of 0.5, 2, 6, 12, and 24 h.

Mapping and cloning of ARL1

ARL1 was mapped primarily with simple sequence length polymorphism (SSLP) and sequence tagged site (STS) markers, using 94 F_2 mutant plants. The *ARL1* locus was finally mapped within a 10-kb region flanking an STS9 marker at a BAC clone (OSNJBb0050N02) on chromosome 3 using 1880 F_2 mutant plants. The candidate gene was amplified from both the *arl1* mutant and wild-type genomic DNA using gene-specific primers for *ARL1* forward (5′-GAAGAAGAAGCGGCGGCCAAGATGAC-3′) and reverse (5′-CTTACGAGCGGTTAAGGTAAGCGTAGGCGA-3′). The PCR products were cloned into T-vector and then sequenced.

Construction of vectors and plant transformation

For complementation of *arl1*, a 5452 bp genomic DNA fragment, containing the full-length *ARL1* gene, and 3747 bp upstream and 841 bp downstream sequence, was cloned into a binary vector, pCambia 1301. The construct was transformed into calli developed from 100 homozygous *arl1* seeds through *A. tumefaciens* (strain EHA105)-mediated transformation system as described (Chen *et al.*, 2003).

A 1.9 kb promoter containing 106 bp of the coding region of the *ARL1* gene was amplified using a PCR approach. It was inserted into the 5′ end of the GUS gene (*β-glucuronidase*) in pCAMBIA1391Z to create a vector of GUS driven by the ARL1 promoter, *ARL1*::GUS. For the RNAi construct, 278 bp of *ARL1* cDNA in antisense orientation, the second intron of *NIR1* in maize and 278 bp of *ARL1* cDNA in sense orientation were inserted into multicloning sites of pCAMBIA1301 (35S). The above constructs were transformed into calli developed from wild-type plant (Zhonghua 11) seeds through *A. tumefaciens* (strain EHA105)-mediated transformation.

Histochemical analysis and GUS assay

Histochemical GUS analysis was performed as described (Jefferson *et al.*, 1987). Transgenic plant samples were incubated with X-gluc overnight at 37°C. After being stained, the tissues were rinsed and fixed in formalin acetic acid ethanol (FAA) fixation solution at 4°C overnight, embedded in Spurr's resin, and then sectioned. Sections (thickness, 5 μm) were mounted on slides and photographed (Zeiss Axioskop, Jena, Germany).

Histology and in situ *hybridization*

Shoot bases of 5-day-old seedlings were fixed with FAA fixation solution at 4°C overnight, dehydrated and embedded in paraffin (Paraplast Plus; Sigma, St Louis, MO, USA). Sections (thickness, 8 μm) were cut with a microtome (Microm HM325; Walldorf, Germany) and stained with hematoxylin. Sections were observed under a bright-field microscope (Zeiss AxioCam HRC).

A 278-bp fragment of the 3′ end of *ARL1* cDNA was subcloned into pBluescript SK^+ vector. The antisense probe was transcribed with T7 RNA polymerase, and the sense probe was transcribed with T3 RNA polymerase. *In situ* hybridization of digoxigenin-UTP-labeled RNA probes was performed as described (Fobert *et al.*, 1994). For *in situ* hybridization of sectioned materials, we performed tissue fixation, embedding, and tissue sectioning as described (Chen *et al.*, 2003).

Subcellular localization of ARL1

The CaMV35S-ARL1-mGFP4 was subcloned into the binary vector PCAMBIA 1301.The CaMV35S ::mGFP4 empty vector (PCAMBIA 1301) was used as control. The resulting construct was sequenced to verify in-frame fusion and used for transient transformation of onion epidermis using a gene gun (Bio-Rad, Hercules, CA, USA). Transformed onion cells were observed under a confocal microscope (Zeiss LSM 510).

Yeast two-hybridization analysis

The Matchmaker yeast two-hybrid system (cat. no. K1615-1; Clontech, Mountain View, CA, USA) was used. The deduced amino acid sequence of *ARL1* was inserted into the vectors pGBKT7 (BD) and pGADT7 (AD). Two subclones with C-terminal 159 amino acid (from 101 to 259 AA) and 52 amino acids (from 208 to 259 AA) of *ARL1* were inserted into pGBKT7 (BD), resulting in fusions with the FGAL4 DNA-binding domain and activation domain. The fusion plasmids, BD-ARL1, AD-ARL1, BD-ARL1(101–259 AA), and BD-ARL1(208–259 AA), respectively, were transformed into yeast stain AH109. Quantitative α-galactosidase assays were performed using the method described in the Clontech protocol. The interaction between the pGBKT7(BD)-p53 and pGADT7(AD)-SV40 large T-antigen (supplied with the Matchmaker system) served as a positive control. The empty pGBKT7 (BD) vector was used as a negative control.

RT-PCR analysis

Total RNA was extracted from stem bases and roots of 7-day-old seedlings of wild type, *arl1* mutant and transgenic plants with RNAi of *ARL1* and used for reverse transcription using Superscript II (Invitrogen, Carlsbad, CA, USA). First-strand cDNA was synthesized from total RNA and used as RT-PCR templates. RT-PCR was performed using gene-specific primers for *ARL1* forward (5′-GAAGAAGAAGCGGCGGCCAAGATGAC-3′) and reverse (5′-CTTACGAGCGGTTAAGGTAAGCGTAGGCGA-3′). Amplification of actin cDNA was performed as a control. The amplification was performed as follows: one cycle of 2 min at 94°C, and 30 cycles of 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. PCR products were analyzed on 1% agarose gels. RT-PCR analysis for complementation test was performed using 15% PAGE gel to separate PCR products from wild type *ARL1* and mutated *arl1* with 20 bp deletion on the gel.

DNA gel blot analysis

Genomic DNA was isolated from transgenic mutant plants harboring genomic *ARL1* gene using the cetyl trimethyl ammonium bromide method (Murray and Thompson, 1980). Five micrograms of genomic DNA was digested with *Sac*I and separated on 0.8% agarose gel. After electrophoresis, the digested DNA was transferred to a Hybond-N^+ Nylon membrane (Amersham Bioscience, Little Chalfont, UK). ^{32}P-dCTP-labeled DNA of Hyg (hygromycin gene) was used as a probe. The blots were hybridized and washed at 65°C under stringent conditions.

Computational analysis

The sequence of the full-length cDNA was analyzed using BLAST and Motifscan (http://hits.isb-sib.ch/cgi-bin/PFSCAN). Sequence alignment was carried out using ClustalW at the European Bioinformatics Institute.

Laser-capture microdissection assay

Stem base tissues of 3-day-old seedlings were fixed in Methacarn solution overnight at 4°C, then dehydrated in graded ethanol series (Shibutani *et al.*, 2002; Takagi *et al.*, 2004) and embedded in low-melting paraffin (Paraplast; Sigma-Aldrich, St. Louis, MO, USA). Preparation of sections (thickness, 7 μm) for LCM using the Pix-Cell II LCM system (Arcturus, Mountain View, CA, USA) was performed as described (Kerk *et al.*, 2003; Nakazono *et al.*, 2003). RNA was extracted and isolated from captured tissues using the Picopure RNA isolation kit (Aruturus) according to the manufacturer's protocol. Total RNA concentrations from the captured pericycle cells at the first node of wild type and *arl1* mutant plants were measured using the Ribogreen reagent (Molecular Probes, Eugene, OR, USA). One hundred nanograms of total RNA from each sample was used for the first amplification with the RiboAmp Kit according to the manufacturer's instruction (http://www.arcturus.com). *OsSCR*, *QHB* and *OsNAS1*-specific primers were designed as follows. *OsSCR*: 5′-ACCGGGCAGAACTCGAATGGCAATCC-3′ and 5′-CGGAGTCGGTGAACGCGGACAACCTC-3′; *QHB*: 5′-ATCCCGGAAGAGACCAAATCAGA-3′ and 5′-TAGGACTAGGCACAGCGACAAGAG-3′; *OsNAS1*: 5′-CTCGCCCGCTTCCCGTACTA-3′ and 5′-ACCGCCAGAACGTCGAACC-3′. PCR conditions for these genes were as follows: 94°C for 4 min, followed by 30 cycles of 94°C for 45 sec, 61°C for 45 sec, and 72°C for 1 min.

Conflict of interest statement

The authors declare that they have no competing financial interests.

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