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ORIGINAL RESEARCH

A Dominant Mutation in ARL2 Causes Impaired Adventitious Root Development in Rice

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Abstract Adventitious roots are vital for water and nutrient assimilation by cereal crops because they comprise the bulk of the fibrous root system. We isolated and analyzed a rice mutant, adventitious rootless 2 (arl2), which failed to initiate adventitious root primordia during early development. Its seminal root produced fewer lateral roots than from the wild type. This mutant also exhibited pleiotropic phenotypes of longer and thicker seminal roots, a different morphology for the first leaf, delayed heading, and a greater tiller angle. Physiological experiments showed that exogenous auxin and ethylene could rescue adventitious root growth, a response opposite that for two previously reported mutants, arl1 and gnom1. Activity in the auxin signal pathway and the polar auxin transport system was normal for arl2. Compared with the wild type, arl2 plants showed enhanced sensitivity to ethephon but decreased sensitivity to $AgNO_3$, an inhibitor of ethylene. Genetics analysis demonstrated that this mutant is controlled by a single dominant gene; ARL2 was mapped within a 100-kb interval on the short arm of chromosome 2.

Keywords Rice (*Oryza sativa*) · Adventitious root · Mutant · *Arl2* · Auxin · Ethylene

Abbreviations

AR	Adventitious root
EMS	Ethyl methanesulfonate
LR	Lateral root
NPA	*N*-1-Naphthylphthalamic acid
PAT	Polar auxin transport
SL	Shoot length
SRL	Seminal root length
WT	Wild type

Unlike a tap root system in dicots, for which seminal and lateral roots (SR and LR) are the major components, stem-borne adventitious roots (AR) constitute the bulk of the fibrous root system in many cereal crops (Lorbiecke and Sauter 1999). In rice (*Oryza sativa* L.), AR play an important role in anchoring plants within the soil while also enabling the uptake of nutrients and water (Bleecker et al. 1986; Liu et al. 2009).

Adventitious root formation encompasses seven stages: (1) establishment of initial cells; (2) appearance of the epidermis–endodermis, central cylinder, and root cap initials; (3) differentiation of that epidermis and endodermis; (4) differentiation of the cortex; (5) fundamental organization; (6) onset of cell elongation and vacuolation (establishment of AR primordia); and (7) emergence of the crown root (Itoh et al. 2005). The mechanism for this developmental process has been elucidated primarily through methods of forward genetics. In dicotyledonous species, that genetic control and hormonal regulation have been well documented by studying several *Arabidopsis* mutants with impaired rooting (Luschnig et al. 1998; Kerk

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et al. 2000; Fukaki et al. 2002). However, because such roots rarely form even under normal growing conditions in that species, knowledge of root architecture has been limited to that of its seminal and lateral roots. Moreover, although many rice mutants have now been characterized with AR defects, our understanding of that mechanism is still unclear for cereal crops. The first reported AR-deficient mutants have included *arl1* (*crl1*) (Inukai et al. 2005; Liu et al. 2005), *crl2* (Inukai et al. 2001), *crl3* (Kitomi et al. 2008a), and *crl4* (*gnom1*) (Kitomi et al. 2008b; Liu et al. 2009). All of these are recessive and lethal, although the phenotype is distinctive. For example, AR formation in *crl1* is suppressed in the first stage due to abnormal periclinal divisions (Inukai et al. 2001). For the *crl2* mutant, development of AR is blocked in the sixth stage when cell elongation and vacuolation are inhibited (Inukai et al. 2001). Whereas some AR-impaired mutants show normal lateral root growth, others are defective in the number, length, or density of LR. Some mutants also manifest a degree of agravitropism, a phenomenon that suggests an intricate network controls AR formation.

From mutational analysis, researchers have learned that phytohormone activities are closely related to appropriate root development, including that of AR (Swarup et al. 2002; Okushima et al. 2005). For example, the auxin and ethylene signaling pathways are putatively required in cereal crops, as demonstrated by exogenous treatment with those hormones, which leads to the induction of ectopic AR formation (Swarup et al. 2002; McDonald and Visser 2003; Stepanova et al. 2005). However, auxin may also function at an earlier stage of AR development because it is a determinant of the polarity of initial cells (Geldner et al. 2003; Inukai et al. 2005). In contrast, ethylene probably affects the later stages (Lorbiecke and Sauter 1999; Inada et al. 2002). Transmission of an auxin signal is controlled by the interaction between AUX/IAA and ARF (auxin response factor) proteins (Weijers et al. 2005). Likewise, some LOB (lateral organ boundary) proteins, including Crl1 (ARL1) in rice and RTCS in maize (*Zea mays* L.), play a critical role in the formation of AR primordia (Liu et al. 2005; Taramino et al. 2007). They are controlled by an ARF protein that is mediated by an endogenous auxin response. Endogenous transporters of auxin may also affect AR because that hormone is synthesized at the shoot apical meristem or in young leaves but functions at the basal portion of shoots to form AR (Pickett et al. 1990). The number of adventitious roots in rice is decreased if PIN1 (PIN-FORMED 1), an auxin efflux carrier component, is knocked down by RNA interference (Xu et al. 2005). GNOM in *Arabidopsis* regulates the cellular distribution and trafficking of PIN1 (Geldner et al. 2003), whereas in rice *gnom1* mutants, some members of the PIN family alter their transcript levels where AR primordia are being initiated (Liu et al. 2009).

Studies with mutants have shown that ethylene is also required for root formation (Alonso et al. 2003). Two receptors, ETR1 and ERS, influence the activity of a CTR1 GTPase *in vitro* and block the ethylene signal pathway (Clark et al. 1998). Ethylene is also a factor in AR development in rice (Lorbiecke and Sauter 1999), tomato (Phatak et al. 1981), and mung bean (Robbins et al. 1985). For example, when rice plants are growing under normal flooded conditions, only the primordia at the basal node can produce AR because of the high correlation with the accumulation of ethylene at that site (Lorbiecke and Sauter 1999; Le et al. 2001). Furthermore, ethylene induction can cause the epidermal cells around AR primordia to die via the programmed cell death pathway (Inada et al. 2002). Finally, cross talk has been demonstrated in which auxin controls the rate of ethylene biosynthesis while the latter regulates the activity of AUX1 and IAA transport (Alonso et al. 2003; Stepanova et al. 2005; Swarup et al. 2007).

We have now isolated a rice dominant mutant, *adventitious rootless2* (*arl2*), in which the development of adventitious and lateral roots is impaired while the seminal root thickens and lengthens. Unlike previously reported mutants, exogenous auxin and ethylene can restore AR growth in *arl2*, suggesting that auxin and/or ethylene biosynthesis or the signaling pathway may be distorted. Plants of *arl2* are also more sensitive to ethylene compared with wild-type (WT) rice. In addition to these root abnormalities, the mutant has an enlarged tiller angle and delayed heading. Our statistics analysis revealed that a single locus is responsible for that dominant phenotype. Using an F_2 population and a positional cloning strategy, we mapped *ARL2* within a 100-kb interval on the short arm of chromosome 2.

Materials and Methods

Screening for Mutants with Impaired Adventitious Root Development

The rice *arl2* mutant was screened from an EMS (ethyl methanesulfonate, a chemical mutagen) mutant library of *indica* variety Kasalath. After WT seeds were soaked in water for 5 h, 1% EMS was added for 12 h more. We acquired 2600 M_2 lines, from which 40 seeds per line were soaked in water for 24 h in the dark at 30°C. Germinated seeds were sown on floating nets and grown at 25°C in a nutrient solution of 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 $FeCl_3$,

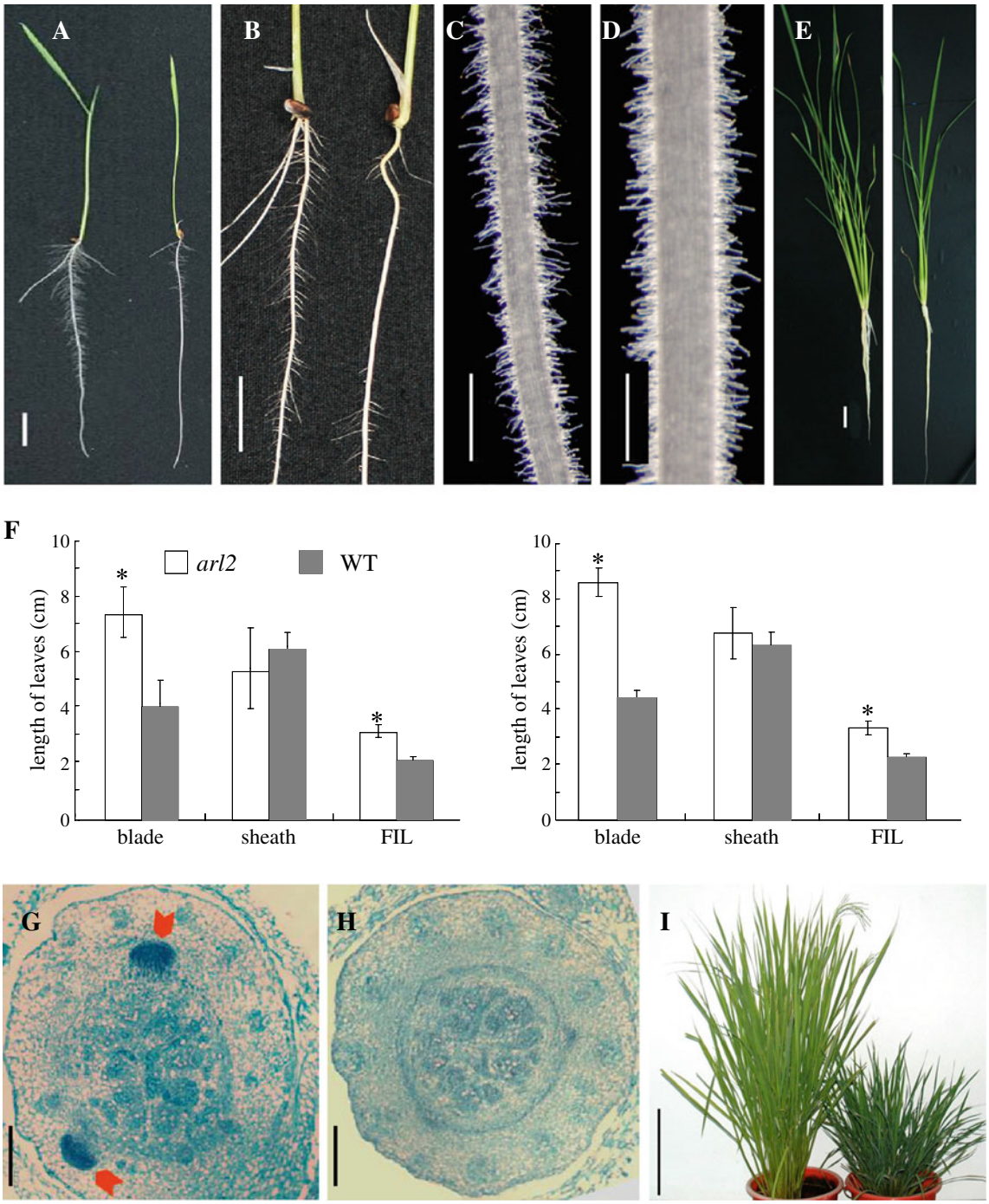

Fig. 1 Phenotypes of WT and *arl2* mutant rice. **a**, **b** 5-day-old seedlings (**a**) and enlarged pictures of root (**b**); WT is on *left*, mutant on *right*. *Bars*=2 cm. **c**, **d** Width of seminal roots from WT (*left*) and *arl2* (*right*). *Bars*=1 mm. **e** 50-day-old WT (*left*) and *arl2* (*right*) plants grown in hydroponic system. *Bar*=5 cm. **f** Size of first leaf (including leaf blade and leaf sheath) and first incomplete leaf (*FIL*). Data were obtained from 1-week-old (*left*) and 2-week-old (*right*) seedlings. Significant differences are marked by *asterisks* (5 plants per line, *t* test, $P<0.05$). **g**, **h** Cross sections of stem base from 5-day-old WT (**g**) and *arl2* (**h**) plants. *Arrows* indicate adventitious root primordium. *Bars*=100 μm. **i** Growth of WT (*left*) and *arl2* (*right*) plants in pots. *Bar*=30 cm

0.070 mM citric acid, and 0.152 mM $ZnSO_4$. One-week-old hydroponic seedlings were then investigated for root morphology. Of these, only one line, *arl2*, lacked AR; it was selected for further experiments.

Growing Conditions and Phenotype Identification

Seedlings of WT rice and mutants were hydroponically reared at pH 5.0 in growth chambers (30°C, 12-h photoperiod from

Table 1 Growth of *arl2* plants relative to other rice genotypes at different developmental stages

DAG			7 days[a]			20 days		50 days
Genotype	"Kasalath"	*arl2*	"Nipponbare"	F_1[b]	"Kasalath"	*arl2*	"Kasalath"	*arl2*
SL(cm)	9.38±1.02[c]	7.79±1.33[d]	9.24±0.82	7.66±1.48[d]	45.01±3.55	28.90±3.97[d]	88.16±7.45	69.41±4.90[d]
SRL(cm)	11.94±0.66	12.52±0.47[d]	10.25±0.61	13.84±0.73[d]	19.83±1.77	25.26±3.51[d]	ND	ND
ARN	2.43±0.46	0[d]	3.12±0.23	0[d]	22.38±4.75	9.75±2.04[d]	74.00±7.17	31.25±2.55[d]
LRN	146.88±24.15	48.75±13.31[d]	192.38±37.36	63.51±10.49[d]	423.54±78.72	182.63±63.27[d]	927.50±165.50	670.17±137.23[d]
TN	0	0	0	0	0	0	4.75±0.89	3.38±0.52[d]
FSW (g)	ND	ND	ND	ND	ND	ND	12.44±1.36	5.88±0.45[d]
FRW (g)	ND	ND	ND	ND	ND	ND	2.41±0.14	1.36±0.10[d]

SL shoot length, *SRL* seminal root length, *ARN* adventitious root number, *LRN* lateral root number, *TN* tiller number, *FSW* fresh shoot weight, *FRW* fresh root weight, *ND* not determined

[a] Measurements were made on hydroponically grown plants (5 per line) at 7 to 50 days after germination (DAG)

[b] F_1 is the heterozygote of *arl2* and "Nipponbare"

[c] Data are presented as means ± SD

[d] Within each row and for each date indicates a significant difference at $P<0.05$, by *t* test

200 μmol photons m^{-2} s^{-1}, and approximately 60% relative humidity). They were photographed at 5 days after germination (DAG). The density of lateral roots was determined with WinRHIZO version 3.9 (Regent Instrument Inc., Quebec, Canada), while the width of the seminal roots was measured on a stereoscopic microscope (Meyer, USA). To observe the AR primordia, we fixed the stem bases from 5-day-old seedlings in FAA for 24 h at 4°C and then dehydrated them in a graded ethanol series. Those samples were embedded in paraffin, and then sectioned to 8 μm thick with a rotary microtome (Microm HM325, Walldorf, Germany). To monitor growth over the entire experimental period, we placed the mutant and WT plants in greenhouse pots, where they were held until maturity. Tiller angle was measured according to the method previously described by Jin et al. (2008).

Plant Hormone Physiological Assay

To analyze the effects of auxin, we exogenously applied a gradient concentration of NAA and 2,4-D to seedlings of *arl1*, *arl2*, *gnom1*, and the corresponding WT. Both *arl1* and *gnom1* were derived from *japonica* "Zhonghua 11," while *arl2* was obtained from *indica* "Kasalath." At 7 DAG, the seedlings were treated with 0.5 μM NPA (*N*-1-naphthylphthalamic acid), an auxin transport inhibitor. To investigate the influence of ethylene on mutant growth, we added an ethylene precursor, ACC (1-aminocyclopropane-1-carboxylate), plus a gradient concentration of ethephon to the hydroponic solution for 7 days. To evaluate their sensitivity to ethylene, plants were exposed to 10 or 15 μM ethephon and ACC for 5 or 10 days before their seminal root lengths (SRLs) were measured. We also used 1 μM $AgNO_3$, an inhibitor of ethylene perception, to examine the sensitivity of that signal. The relative growth rate of SRL (root length under treatment/root length under normal conditions) was calculated at 5, 10, 15, and 20 days after the addition of $AgNO_3$.

RT-PCR Analysis

Using the TRIzol method, we extracted total RNA from the 7-day-old stem bases of *arl1*, *arl2*, *gnom1*, and WT seedlings,

Table 2 Agronomic traits of *arl2* mutants and WT plants grown in pots

	HD	SL (cm)	TN	SNP	SSR (%)	SW (g)	TA
WT[a]	62.7±1.6	110.33±5.86	75.67±6.35	123±24.65	79.4±2.5	15.27±0.12	22.3±3.4
arl2	74.2±1.3[b]	60.67±3.21[b]	76.67±9.50	45±10.22[b]	56.2±3.9[b]	15.08±0.27	61.5±5.7[b]

HD heading date, *SL* shoot length, *TN* tiller number, *SNP* seed number per panicle, *SSR* seed setting rate, *SW* seed weight (g/1,000 seeds), *TA* tiller angle

[a] Three plants were measured per line; data are presented as means ± SD

[b] Within each column indicates a significant difference at $P<0.05$, by *t* test

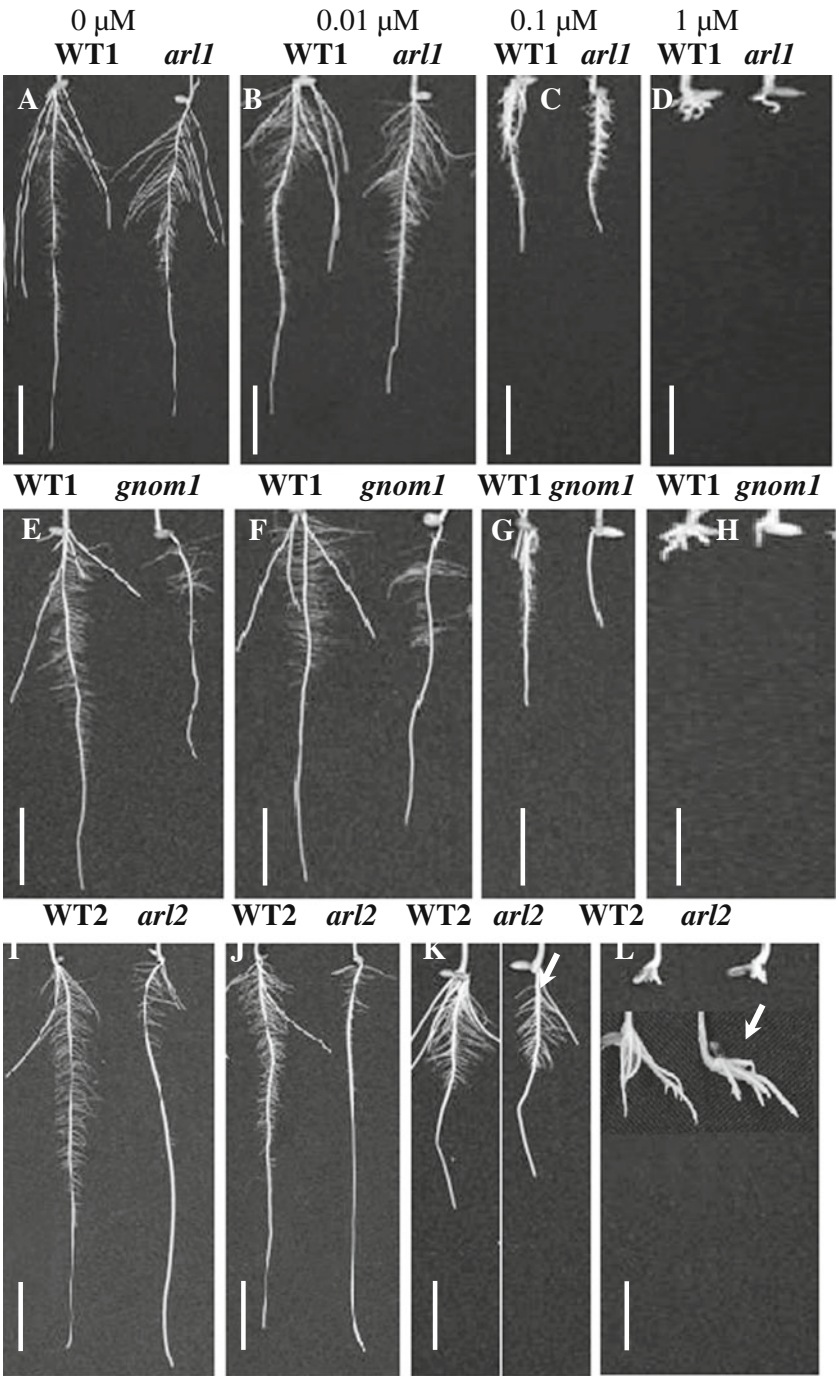

Fig. 2 Effects of NAA on mutant rice at 7 days after germination. **a**–**d** Mock control (**a**) and exogenous addition of 0.01 (**b**), 0.1 (**c**), or 1 μM (**d**) NAA to *arl1*. WT1 ("Zhonghua 11") is on *left*, *arl1* on *right*. **e**–**h** WT1 and *gnom1* treated with various concentrations of auxin. **i**–**l** WT2 ("Kasalath") and *arl2* under NAA treatment. Photo of shoot and root junction is enlargement of *lower part of* **l**, and *arrow* indicates AR formation. *Bars*=2 cm

which had been treated with 1 μM α-NAA for 4 h prior to harvesting. RNA samples (5 μg) were reverse-transcribed with M-MLV reverse transcriptase (Promega, CA, USA) and an oligo (dT) 18, according to the manufacturer's protocol. RT-PCR was performed with gene-specific primers (forward: 5'-GAAGAAGAAGCGGCGGCCAAGATGAC-3', and reverse: 5'-CTTACGAGCGGTTAAGGTAAGCGTAGGCGA-3'). PCR conditions were 94°C for 5 min; followed by 32 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; and then a final extension of 72°C for 5 min. Actin cDNA was amplified for 28 cycles as our control. The products were then analyzed on 1.2% agarose gels. Quantitative RT-PCR analysis was performed with a SYBR Premix Ex TaqTM (Perfect Real Time) Kit (TaKaRa Biomedicals, Tokyo, Japan) on a LightCycler480 machine (Roche Diagnostics, Basel, Switzerland), according to the manufacturer's instructions.

Genetics Analysis

To investigate inheritance of the impaired-root phenotype, we crossed *arl2* to *japonica* "Nipponbare." The AR were measured from 10 F_1 seedlings that had been cultured in water for 7 days. The segregation ratio for AR formation in the F_2 generation was determined at the seedling stage.

Mapping of ARL2

F_2 plants from the cross that showed normal root architecture were harvested. *ARL2* was primarily mapped with simple sequence repeats (SSRs) via bulked segregation analysis. To identify the molecular markers linked to *ARL2* and to improve the efficiency of marker screening, we used four samples to screen polymorphic markers that were dispersed on 12 chromosomes. Those samples included DNA from an F_1 seedling, the two parental lines, and a mix of DNA from 30 F_2 WT plants. PCR was performed in a 10-μL volume containing 15 ng ml^{-1} of genomic DNA, 2 mM of each primer, 0.2 mM dNTPs, 2.5 mM Mg^{2+}, 1 μL of PCR buffer, and 0.4 units of rTaq (Takara, Japan). Products were analyzed on 8% polyacrylamide gels. If the marker was linked with *ARL2*, the mixture of F_2 would have a distorted segregation to "Kasalath." Otherwise, the bands similar to F_1, in which "Kasalath" and "Nipponbare" had identical segregation, would demonstrate that the marker was far from *ARL2*. Finally, the WT plants were separately identified as possibly being recombinants based on the approximate marker.

To develop PCR-based markers, we compared the "Nipponbare" contig sequences with the *indica* variety 93–11 sequence. Their differences were chosen for developing sequence tagged site (STS) markers. CAPS and dCAPS markers were made according to different cleavage sites (or were introduced as a new site) by restriction endonuclease, as described at http://helix.wustl.edu/dcaps/dcaps.html. In all, 620 individuals with the same root architecture as "Kasalath" in the F_2 population were used for finely mapping the interval.

Results

Isolation of the *Arl2* Mutant and Morphological Analysis

One mutant rice line lacked adventitious roots in the early developmental stages. Denoted as *arl2*, it was isolated from 2600 M_2 lines generated from indica "Kasalath." Although such roots eventually emerged by 10 DAG, the total number from the mutant was still <50% of that from the WT (Fig. 1a, b; Table 1). Besides that impairment, *arl2* exhibited other defects, including fewer lateral roots (Fig. 1b; Table 1). However, the seminal root from the mutant was much thicker and somewhat longer than that of the WT ($P<0.05$) when grown hydroponically (Fig. 1c–e; Table 1). Aerial parts of the *arl2* plants were shorter, and they showed delayed development (Fig. 1a, e; Table 1). In addition, the first incomplete leaf (FIL) and the leaf blade from the first true leaf (FTL) were significantly larger from the mutant than from the WT (Fig. 1f), indicating that *ARL2* affects many aspects of rice development.

To determine at which stage AR formation is blocked in *arl2*, we cross-sectioned the basal part of the stems from 5-day-old seedlings. Their AR primordia were apparently differentiated, but root formation was hindered in the mutant (Fig. 1g, h), suggesting that this impairment occurs before the primordia are initiated in the sixth stage.

At maturity, pot-grown *arl2* plants were shorter than the WT (Fig. 1i; Table 2). They also had greater tiller angles, and their heading date was about 12 days later than for the WT. However, compared with their performance when reared hydroponically (Fig. 1e), the total number of tillers at maturity was similar for pot-grown mutant and WT

Table 3 Effects of auxin and its inhibitor on WT and *arl2* growth parameters

	Variety	SL	SRL	ARN	LRN
0.1 μM NAA	"Kasalath"	7.3±0.2[a]	3.3±0.2	4.0±1.0	66.7±7.1
	arl2	5.6±0.5[b]	3.3±0.6	1.3±0.6[b]	32.0±7.0[b]
	"Zhonghua 11"	6.2±0.3	2.7±0.2	4.5±0.9	85.3±4.2
	arl1	6.7±1.2	2.6±0.4	0.0±0.0[b]	56.6±2.4[b]
	gnom1	3.5±2.0[b]	1.7±0.9[b]	0.0±0.0[b]	11.3±6.6[b]
1 μM NAA	"Kasalath"	3.1±0.1	0.8±0.1	5.0±0.0	0.0±0.0
	arl2	3.0±0.1	0.9±0.0	5.3±0.6	0.0±0.0
	"Zhonghua 11"	4.5±0.3	0.7±0.1	5.3±0.4	0.0±0.0
	arl1	3.3±0.1[b]	0.8±0.1	0.0±0.0[b]	0.0±0.0
	gnom1	3.2±0.4[b]	0.7±0.1	0.0±0.0[b]	0.0±0.0
0.5 μM NPA	"Kasalath"	5.3±1.3	6.7±2.4	0.0±0.0	38.4±19.1
	arl2	4.6±2.1[b]	7.2±2.4	0.0±0.0	0.0±0.0[b]

[a] Data were obtained after 7 days of treatment

[b] Within each column and for each treatment indicates a significant difference at $P<0.05$, by t test

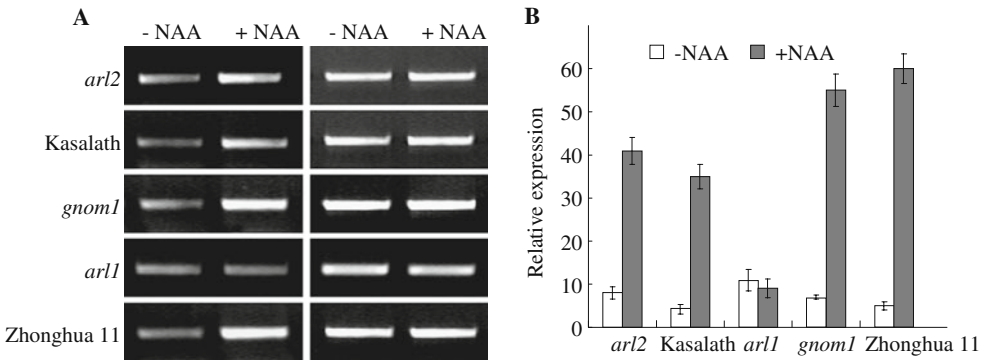

Fig. 3 Expression of *ARL1* in three rice mutants. **a** *Left panels* show expression of *ARL1* after NAA treatment; *Actin* is on *right*. **b** Quantitative RT-PCR of *ARL1* in mutant and WT rice

plants. Finally, *arl2* plants produced fewer seeds per panicle and had much lower seed setting rates than did the WT.

Genetics Analysis

F_1 hybrids between *arl2* and WT "Nipponbare" showed defects in their AR formation and LR development (Table 1), suggesting that this is a dominant mutation. In the F_2 generation, we randomly investigated 119 plants, of which 84 lacked AR while 35 were normal. This segregation fitted well to a ratio of 3:1, with a χ^2 value of 1.235 ($\chi_{0.05,\ 1}^2$=3.84), indicating that the mutation is controlled by a single Mendelian gene.

Analysis of Auxin Response

To evaluate their response to different concentrations of auxin, we grew *arl1*, *arl2*, *gnom1*, and the corresponding WT with 2,4-D and NAA for 7 days. Because both chemicals had similar effects on *arl2* (data not shown), we selected NAA for further experiments. Under equivalent auxin levels, *arl2* showed the same trend as WT (Fig. 2; Table 3). However, when exposed to 1 μM NAA, adventitious roots were induced in nearly the same numbers by both the WT and *arl2* (Fig. 2l), but not in *arl1* or *gnom1* (Fig. 2a–h; Table 3). Furthermore, unlike with *arl1* and *gnom1*, *arl2* formed such roots even at 0.1 μM NAA (Fig. 2i–l; Table 3). Therefore, these results suggest that AR defects in *arl2* are very different from those in either *arl1* or *gnom1*.

Because ARL1 affects AR formation through the auxin response pathway, we checked its expression in different mutants. The same normal response was found with *arl2* and *gnom1* because that gene was induced by NAA in both mutants similar to that found with the WT (Fig. 3a, b). Likewise, because GNOM1 functions in polar auxin transport (PAT) to influence the development of adventitious roots, we used 0.5 μM NPA to monitor PAT in *arl2*. Similar responses were noted between *arl2* and "Kasalath," including values for seminal root length, shoot length, and AR numbers (Table 3), suggesting that PAT is normal in *arl2*. This demonstrated, therefore, that *arl2* is not defective in either its auxin response or auxin transport. Furthermore,

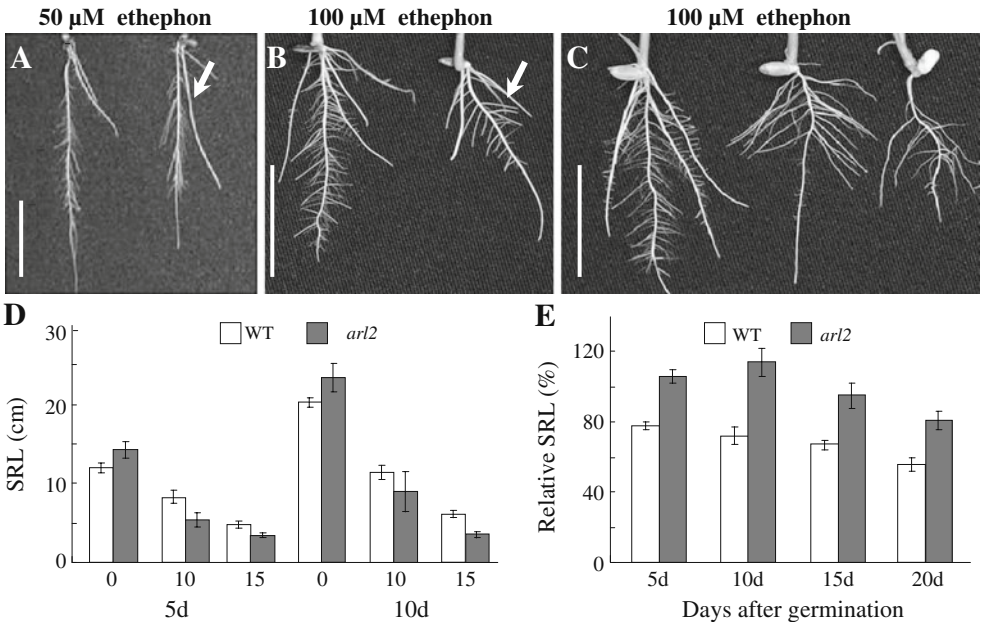

Fig. 4 Effect of ethylene and its inhibitor on rice mutants. **a**, **b** 50 (**a**) or 100 μM ethephon (**b**) were added to WT (*left*) and *arl2* (*right*) plants for 7 days. *Arrows* show rescued AR. *Bars*=2 cm. **c** "Zhonghua 11" (*left*), *arl1* (*middle*), and *gnom1* (*right*) were treated with 100 μM ethephon for 7 days. *Bar*=2 cm. **d** Lengths of seminal roots after plants were treated with 10 or 15 μM ethephon for 5 or 10 days. **e** Relative growth rate of seminal roots after treatment with ethylene inhibitor, 1 μM $AgNO_3$, for 5, 10, 15, or 20 days. Relative SRL=root length under treatment/root length under normal conditions

Table 4 Effects of ethephon on WT and *arl2* growth parameters

	Variety	SL	SRL	ARN	LRN
50 μM	"Kasalath"	10.7±1.4[a]	5.7±0.5	3.0±0.0	131.0±8.2
	arl2	7.2±0.6[b]	4.5±0.6	0.8±0.6[b]	55.0±12.1[b]
	"Zhonghua 11"	10.4±0.3	4.3±0.8	2.3±0.8	142.6±22.5
	arl1	9.8±1.3	4.5±0.4	0.0±0.0[b]	122.8±23.3
	gnom1	4.4±1.2[b]	2.7±0.8[b]	0.0±0.0[b]	24.3±5.6[b]
100 μM	"Kasalath"	10.5±1.7	4.3±0.5	5.3±1.3	95.5±15.1
	arl2	6.6±2.5[b]	3.8±0.2[b]	3.5±1.1[b]	30.8±6.8[b]
	"Zhonghua 11"	10.2±1.6	4.0±0.2	4.1±1.0	117.3±10.4
	arl1	10.1±2.2	3.6±0.4	0.0±0.0[b]	37.3±13.2[b]
	gnom1	3.6±1.2[b]	1.8±0.5[b]	0.0±0.0[b]	23.6±3.7[b]

SL shoot length, *SRL* seminal root length, *ARN* adventitious root number, *LRN* lateral root number

[a] Measurements were made on hydroponically grown plants (5 per line) at 7 DAG. Data are presented as means ± SD

[b] Within each column and for each treatment indicates a significant difference at $P<0.05$, by *t* test

because 7 days of treatment with exogenous NAA enabled plants to restore their AR growth, we can conclude that this delay in root production is related to auxin homeostasis, especially at sites where the AR primordia are initiated.

Sensitivity of *Arl2* to Ethylene and Its Inhibitor

To evaluate the effects of ethylene on *arl2* expression, we applied various concentrations of ethephon to our seedlings. Although AR formation was restored in *arl2* under 50 μM ethephon (Fig. 4a; Table 4), that of *arl1* and *gnom1* was not reestablished even at a level of 100 μM (Fig. 4b, c; Table 4), indicating that the ethylene signal is defective in *arl2*. To determine whether this response could be altered, we treated seedlings with 10 or 15 μM ethephon and found an increase in sensitivity by *arl2*. When treated for 5 or 10 days, elongation of the seminal roots was more intensely inhibited in *arl2* than in the WT (Fig. 4d). That is, those SRs from the mutant averaged 2 cm shorter than from the WT when plants were exposed to 15 μM ethephon for 10 days, compared with performances under normal growing conditions (Fig. 4d).

Another precursor, ACC, had similar effects (Fig. S1). To compare plant sensitivity to an ethylene inhibitor, we added 1 μM $AgNO_3$ to the nutrient solution for 20 days. Relative lengths of SRs (root length under treatment/root length under normal conditions) were significantly higher in *arl2* than in the WT (Fig. 4e). Seminal roots from the WT were only 56.3±2.8% of their normal length after 20 days of treatment versus 81±1.6% of normal for treated *arl2*. This implied that sensitivity to $AgNO_3$ may be decreased in that mutant.

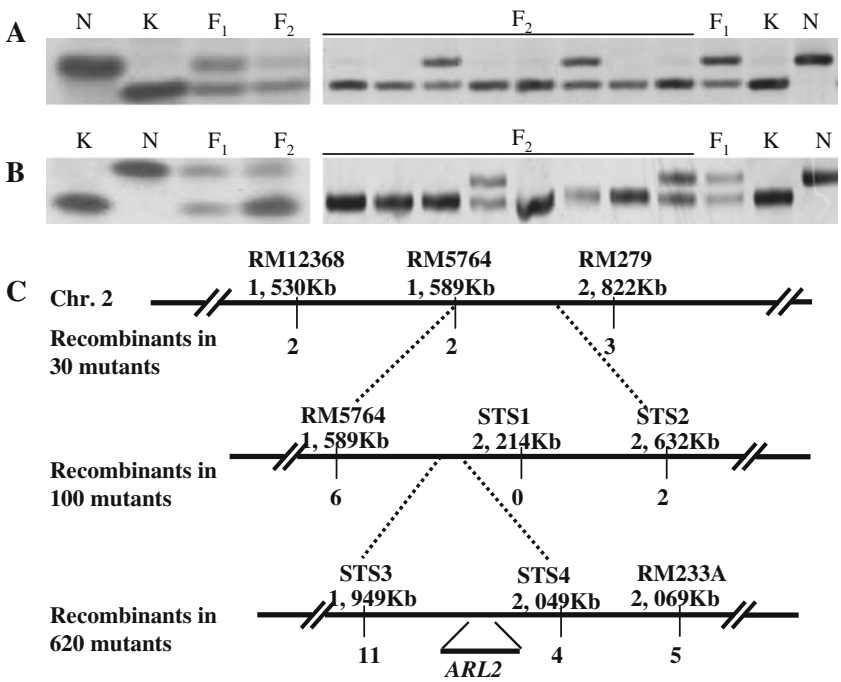

Fig. 5 Linkage map for *ARL2*. **a** RM5764 marker for screening packed (*left*) and unpacked F_2 populations (*right*). *N* "Nipponbare", *K arl2* mutant with "Kasalath" genetic background. **b** RM279 marker. **c** Linkage analysis for *ARL2* on chromosome 2. Numbers of recombinant plants are indicated *below* the map

Genetically Mapping of *ARL2*

To finely map *ARL2*, we used F_2 plants from a cross between the *arl2* mutant (*indica*) and "Nipponbare" (*japonica*). Because *arl2* is a dominant mutant, 30 F_2 plants having the same root architecture as "Kasalath" were harvested for extracting their genomic DNAs, which were then packed to improve the efficiency of marker screening via bulked segregation analysis. This mixture of F_2 rendered a distorted segregation to "Kasalath" at RM5764 and RM279 on chromosome 2 (Fig. 5a, b), demonstrating a rough linkage relationship on the short arm that was further confirmed by separate analysis.

For fine mapping, we developed five STS markers (STS1 to STS5; Fig. 5c) and used 620 individuals with the same root architecture as "Kasalath" in the F_2 population. Here, *ARL2* was located between molecular markers STS4 and STS5, covering an interval of 100 kb (Fig. 5c).

Discussion

The capacity to form adventitious roots is one of the most important agronomic traits in rice, affecting crop yields by assimilating nutrients and water. Here, we used EMS treatment to isolate a new mutant, *arl2*, defective in its production of adventitious roots. Our analysis showed that AR primordia are blocked at an early stage. Other abnormalities included fewer lateral roots, a thicker and somewhat longer seminal root, a larger first leaf, delayed heading, and a greater tiller angle. This indicated that *ARL2* affects overall development in rice. Nevertheless, those plants survived and produced normal seeds, unlike previously described AR-impaired mutants that were nearly all lethal. Therefore, this *arl2* material is an ideal tool for investigating the pathway of AR formation.

Auxin plays a crucial role in root growth. Earlier researchers have described two genes associated with this process—ARL1, involved in the auxin signal pathway, and GNOM1, which functions in polar auxin transport. Unlike with those mutants, impaired AR growth can be rescued in *arl2* by exogenous NAA, providing evidence that auxin-related metabolism is defective in that mutant. However, given that it has normal response to NAA and can be regularly induced by NAA as well, the *arl2* might have normal auxin signaling pathway as WT. Meanwhile, PAT in *arl2* is not likely to be disrupted because sensitivity to the auxin transport inhibitor NPA is the same for those plants as for the WT. That is, the mechanism by which ARL2 affects AR formation differs from that in ARL1 and GNOM1. One possible explanation is that the level of endogenous auxin at the site of AR initiation is too low to promote AR primordia formation in the early stages. This theory will require further study.

It is also possible that ARL2 plays a role in ethylene signaling or biosynthesis. To a certain degree, exogenous ethylene can substitute for auxin in rescuing AR formation in *arl2*. That mutant is also more sensitive than the WT to ethephon but less sensitive to the inhibitor of ethylene. This suggests that ARL2 is involved in the pathways for ethylene signaling or biosynthesis. Therefore, regardless of the pathway, we can assume that cross talk between auxin and ethylene affects AR formation in rice. Because ethylene can upregulate auxin biosynthesis in *Arabidopsis* (Ruzicka et al. 2007; Swarup et al. 2007), a similar ethylene-related mechanism might be found with ARL2. Therefore, defects in adventitious roots in *arl2* might be caused by downregulated auxin biosynthesis, which is mediated by the hindrance of the ethylene signal.

Our genetics analysis revealed that, unlike other AR-impaired mutants, *arl2* is controlled by a single dominant gene. Mapping showed that *ARL2* is located within a 100-kb interval on the short arm of chromosome 2. A search of the TIGR Rice Genome Annotation Database (www.tigr.org) has produced several candidate genes in the ARL2 locus, but none has previously been reported. Thus, we believe that *ARL2* is a new gene, and its characterization will enable us to understand better the mechanisms for ethylene- and auxin-mediated regulation of adventitious root formation.

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References

Alonso JM, Stepanova AN, Solano R, Wisman E, Ferrari S, Ausubel FM, Ecker JR (2003) Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in *Arabidopsis*. Proc Natl Acad Sci 100:2992–2997

Bleecker AB, Schuette JL, Kende H (1986) Anatomical analysis of growth and developmental patterns in the internode of deepwater rice. Planta 169:490–497

Clark KL, Larsen PB, Wang X, Chang C (1998) Association of the *Arabidopsis* CTR1 Raf-like kinase with the ETR1 and ERS ethylene receptors. Proc Natl Acad Sci USA 95:5401–5406

Fukaki H, Tameda S, Masuda H, Tasaka M (2002) Lateral root formation is blocked by a gain-of-function mutation in the SOLITARY-ROOT/IAA14 gene of *Arabidopsis*. Plant J 9:153–168

Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jurgens G (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. Cell 112:219–230

Inada N, Sakai A, Kuroiwa H, Kuroiwa T (2002) Three-dimensional progression of programmed death in the rice coleoptile. Int Rev Cytol 218:221–258

Inukai Y, Miwa M, Nagato Y, Kitano H, Yamauchi A (2001) RRL1, RRL2 and CRL2 loci regulating root elongation in rice. Breed Sci 51:231–239

Inukai Y, Sakamoto T, Ueguchi-Tanaka M, Shibata Y, Gomi K, Umemura I, Hasegawa Y, Ashikari M, Kitano H, Matsuoka M (2005) Crown rootless1, which is essential for crown root formation in rice, 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, Nagato Y (2005) Rice plant development: from zygote to spikelet. Plant Cell Physiol 46:23–47

Jin J, Huang W, Gao JP, Yang J, Shi M, Zhu MZ, Luo D, Lin HX (2008) Genetic control of rice plant architecture under domestication. Nat Genet 40:1365–1369

Kerk NM, Jiang K, Feldman LJ (2000) Auxin metabolism in the root apical meristem. Plant Physiol 122:925–932

Kitomi Y, Kitano H, Inukai Y (2008a) Mapping of the CROWN ROOTLESS3 gene, *CRL3*, in rice. Rice Genet Newslett 24:31–33

Kitomi Y, Ogawa A, Kitano H, Inukai Y (2008b) CRL4 regulates crown root formation through auxin transport in rice. Plant Root 2:19–28

Le J, Vandenbussche F, van der Straeten D, Verbelen JP (2001) In the early response of *Arabidopsis* roots to ethylene, cell elongation is up and down regulated and uncoupled from differentiation. Plant Physiol 125:519–522

Liu H, Wang S, Yu XB, Yu J, He X, Zhang S, Shou H, Wu P (2005) ARL1, a LOB-domain protein required for adventitious root formation in rice. Plant J 43:47–56

Liu S, Wang J, Wang L, Wang X, Xue Y, Wu P, Shou H (2009) Adventitious root formation in rice requires OsGNOM1 and is mediated by the OsPINs family. Cell Res 9:1110–1119

Lorbiecke R, Sauter M (1999) Adventitious root growth and cell cycle induction in deepwater rice. Plant Physiol 119:21–29

Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. Genes Dev 12:2175–2187

McDonald MP, Visser EJW (2003) A study of the interaction between auxin and ethylene in wild type and transgenic ethylene insensitive tobacco during adventitious root formation induced by stagnant root zone conditions. Plant Biol 5:550–556

Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: unique and overlapping functions of ARF7 and ARF19. Plant Cell 7:444–463

Phatak SC, Jaworski CA, Liptay A (1981) Flowering and adventitious root growth of tomato cultivars as influenced by ethephon. Hort Science 16:181–182

Pickett FB, Wilson AK, Estelle M (1990) The *aux1* mutation of *Arabidopsis* confers both auxin and ethylene resistance. Plant Physiol 94:1462–1466

Robbins JA, Reid MS, Paul JL, Rost TL (1985) The effect of ethylene on adventitious root formation in mung bean (*Vigna radiate*) cuttings. J Plant Growth Regul 4:147–157

Ruzicka K, Ljung K, Vanneste S, Podhorska R, Beeckman T, Friml J, Benkova E (2007) Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell 19:2197–2212

Stepanova AN, Hoyt JM, Hamilton AA, Alonso JM (2005) A link between ethylene and auxin uncovered by the characterization of two root-specific ethylene-insensitive mutants in *Arabidopsis*. Plant Cell 17:2230–2242

Swarup R, Parry G, Graham N, Allen T, Bennett M (2002) Auxin crosstalk: integration of signalling pathways to control plant development. Plant Mol Biol 49:411–426

Swarup R, Perry P, Hagenbeek D, van der Straeten D, Beemster GS, Sandberg G, Bhalerao R, Ljung K, Bennett MJ (2007) Ethylene upregulates auxin biosynthesis in *Arabidopsis* seedlings to enhance inhibition of root cell elongation. Plant Cell 19:2186–2196

Taramino G, Sauer M, Stauffer JL, Multani D, Niu X, Sakai H, Hochholdinger F (2007) 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 50:649–659

Weijers D, Benkova E, Jager KE, Schlereth A, Hamann T, Kientz M, Wilmoth JC, Reed JW, Jurgens G (2005) Developmental specificity of auxin response by pairs of ARF and aux/IAA transcriptional regulators. EMBO J 24:1874–1885

Xu M, Zhu L, Shou H, Wu P (2005) A *PIN1* family gene, *OsPIN1*, involved in auxin-dependent adventitious root emergence and tillering in rice. Plant Cell Physiol 46:1674–1681