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RESEARCH PAPER

# Interaction between two auxin-resistant mutants and their effects on lateral root formation in rice (*Oryza sativa* L.)

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## Abstract

Since root elongation is very sensitive to auxin, screening for reduced inhibition in root elongation has been an important method for the detection of auxin-resistant mutants. Two recessive auxin-resistant lines of rice (*Oryza sativa* L. ssp. *indica* cv. IR8), *arm1* and *arm2*, have been isolated by screening for resistance to 2,4-dichlorophenoxyacetic acid (2,4-D). *arm1* displays a variety of morphological defects including reduced lateral root formation, increased seminal root elongation, reduced root diameter, and impaired xylem development in roots, while the *arm2* phenotype is almost similar to wild-type IR8 except for a slightly reduced lateral root formation, impaired xylem development in roots and an enhanced plant height. Although the growth of *arm2* roots exhibited a resistance to 2,4-D, it was sensitive to 1-naphthaleneacetic acid (NAA) as the wild type. At the same time, the *arm2* roots showed a reduced [<sup>14</sup>C]2,4-D uptake while uptake of [<sup>3</sup>H]NAA was normal, suggesting that the resistance to 2,4-D of *arm2* roots is due to a defect in 2,4-D uptake. To investigate the possible interaction between *arm1* and *arm2* genes, a double mutant has been constructed. The roots of *arm1 arm2* double mutant were more resistant to 2,4-D and formed fewer lateral roots than those of either single mutant, suggesting that the two genes show synergistic effects with respect to both auxin response and lateral root formation. By contrast, all these mutants displayed the normal gravitropic response in roots, as did the wild-type plants. Taken together, *Arm1* and *Arm2* genes seem to function in different processes in the auxin-response pathways leading to lateral root formation.

Key words: Auxin influx, auxin-resistant mutant, 2,4-dichlorophenoxyacetic acid, gravitropic response, lateral root formation, rice root, root elongation, xylem development.

## Introduction

Auxins are plant hormones that regulate almost every aspect of plant growth and development including cell enlargement and division, lateral branching of shoots and roots, vascular differentiation, gravitropism, and early embryonic development (Davies, 1995; Hobbie, 1998). Roots are important and attractive systems to study auxin action because of their morphological simplicity and well-characterized auxin responses (Hobbie and Estelle, 1995). Since root elongation is sensitive to auxin, screening for reduced auxin sensitivity has been an important approach for the detection of auxin-response mutants (Evans *et al.*, 1994; Lincoln *et al.*, 1990; Pickett *et al.*, 1990; Wilson *et al.*, 1990). In *Arabidopsis*, several auxin-resistant mutants have been isolated and these mutants have proved to be attractive materials in the determination of the mechanism of auxin actions (Gray *et al.*, 1999).

Recently, Hao and Ichii (1999) isolated a dominant auxin-resistant mutant in rice (*Oryza sativa* L. ssp. *japonica* cv. Oochikara) in a screen for 2,4-dichlorophenoxyacetic acid (2,4-D) resistance and named it *Lrt1* (lateral rootless). *Lrt1* also exhibited resistance to synthetic auxin 1-naphthaleneacetic acid (NAA) and natural auxins indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (Chhun *et al.*, 2003). In addition to auxin resistance, *Lrt1* is a lateral rootless mutant showing delayed root gravitropic response and reduced root hairs, suggesting that auxin is required for normal root growth in this process.

In the present study, two non-allelic recessive mutations of a different rice cultivar (*Oryza sativa* L. ssp. *indica* cv.

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IR8), *arm1* and *arm2*, derived from a screen for 2,4-D resistance have been analysed. By crossing the two mutants a double mutant has been constructed that exhibits a greater resistance to 2,4-D in root elongation with fewer lateral roots compared with either single mutant. The uptake of labelled [ $^{14}\text{C}$ ]2,4-D has been investigated in roots of *arm1*, *arm2*, the double mutant, and wild-type IR8. The results show that *Arm1* and *Arm2* genes are involved in different processes of the auxin response pathway required for lateral root formation.

## Materials and methods

### Plant materials

Rice mutant MT16, MT37 and the double mutant derived from a cross between the two mutants were used in this study. MT16 and MT37 were isolated from *indica* type rice cv. IR8 in a screen for 2,4-D resistance. MT16 and MT37 were then characterized and designated as *arm1* and *arm2* (auxin-resistant mutant), respectively.

### Chemicals

Labelled 2,4-dichlorophenoxyacetic acid [2,4-D, ring- $^{14}\text{U}$ ] (specific activity 55mCi mmol $^{-1}$ ) and 1-naphthaleneacetic acid [NAA, 4- $^3\text{H}$ ] (specific activity 25Ci mmol $^{-1}$ ) were purchased from American Radiolabeled Chemicals, Inc. (USA). Scintisol EX-H was from Dojindo Laboratories (Kumamoto, Japan). 2,4-D and NAA were from Nacalai Tesque Inc. (Kyoto, Japan) and Sigma Chemical Co. (St Louis), respectively.

### Mutagenesis and screening

Rice seeds of wild-type IR8 were treated with 1 mM sodium azide for 6 h. Around 62 000 M $_2$  seeds derived from selfing of M $_1$  plants were sterilized with 0.2% Benomyl (Dupont Company, Tokyo, Japan) and germinated in deionized water for 48 h at 25 °C in a glass-covered growth chamber placed in a greenhouse. Germinating seeds were sown on net floats (10×20 cm) in water supplemented with 1  $\mu\text{M}$  2,4-D and grown for 7 d. Putative resistant mutants were transferred to Kimura's B solution (pH 5.0) containing 182  $\mu\text{M}$  (NH $_4$ )SO $_4$ , 274  $\mu\text{M}$  MgSO $_4$ ·7H $_2$ O, 91  $\mu\text{M}$  KNO $_3$ , 90  $\mu\text{M}$  KH $_2$ SO $_4$ , 45  $\mu\text{M}$  K $_2$ SO $_4$ , 176  $\mu\text{M}$  Ca(NO $_3$ ) $_2$ ·4H $_2$ O, and 7  $\mu\text{M}$  FeC $_6$ H $_5$ O $_7$ ·xH $_2$ O for 7 d to allow recovery and then transplanted to pots in a glasshouse to produce M $_3$  seeds. M $_3$  progeny of the selected M $_2$  plants were retested for 2,4-D resistance. At least five mutants were shown to be stably resistant to 2,4-D. From these lines, *arm1* and *arm2* were selected and used in the present study.

### Genetic analysis

To investigate genetic segregation in auxin-resistant mutants, *arm1* and *arm2* were crossed with wild-type IR8. All F $_1$  progeny was tested in Kimura's B supplemented with 1  $\mu\text{M}$  2,4-D for 7 d. The recovered F $_1$  seedlings were transferred to Kimura's B and transplanted to pots. F $_1$  plants were allowed to self-fertilize and F $_2$  segregation was observed by sowing the seeds on 1  $\mu\text{M}$  2,4-D medium. A complementation test was also done by crossing *arm1* with *arm2* to investigate their allelism, and to examine possible genetic interactions between *arm1* and *arm2*.

### Auxin resistance analysis

To evaluate auxin resistance, seedlings were grown in glass cups containing 400 ml water supplemented with or without various concentrations of 2,4-D or NAA. The seedlings were allowed to grow under continuous white fluorescent light (FL20S.W, Toshiba,

Japan) at an irradiance of approximately 67  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at 25 °C for 7 d. Data were presented as the percentage of root length on water culture supplemented with auxin relative to root length on auxin-free medium.

### Histological analysis

Rice seedlings were grown in water under continuous white fluorescent light at 25 °C for 3 d. About 1 cm of root tips was taken from the seminal root and then fixed with acetic acid:ethanol (1:3 v:v) for 24 h. Serial ethanol dehydration was then performed (70, 80, 90, 95, and 100% [twice]) at room temperature for 1 h at each step. Samples were embedded in Technovit 7100 resin. Sections were cut at a thickness of 6  $\mu\text{m}$ , placed on glass slides, dried and observed using a microscope (Olympus BX50-3, Olympus Optical Co., Ltd. Japan).

### Auxin uptake analysis

Rice seedlings were grown for 3 d in water without auxin under continuous white light at 25 °C. Root tips of 1 cm in length were excised from them. Twenty excised root tips were transferred to a 2.6 cm Petri dish containing 1  $\mu\text{M}$  [ $^{14}\text{C}$ ] 2,4-D (2.06 KBq ml $^{-1}$ ) or 1  $\mu\text{M}$  [ $^3\text{H}$ ] NAA (4.7 KBq ml $^{-1}$ ) in 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES) buffer (pH 5.7) and incubated for 30 min with a gentle shaking. After incubation, the root tips were carefully washed twice for 60 s with fresh MES buffer and 20 roots were divided into four groups. The surface water of roots was carefully removed by filter paper and fresh weight of five roots was measured, and then they were soaked overnight in 5 ml liquid scintillation fluid (Scintisol EX-H). Fresh weight of roots did not significantly change during the 30 min incubation. Radioactivity was counted with a scintillation counter (model LS6500, Beckman Instruments, Fullerton, CA). Although 1 cm root tip segments were used in all these auxin uptake experiments, the diameter of mutant root was slightly different from wild-type roots. To find a reasonable method to evaluate auxin uptake in mutant and wild-type roots, the auxin accumulation in roots was expressed in three ways: radioactivity accumulation per 1 cm root segment, per mg fresh weight of root segment and per mm $^2$  surface area of root segment (as shown in Table 4 of the Results).

### Estimation of the surface area of a 1 cm root tip

The surface area of 1 cm root tip was estimated as follows. When the shape of 1 cm root is assumed as a cylinder, the surface area is approximately expressed as:

$$Y = \pi r^2 + 10 \times 2\pi r \quad (1)$$

where  $Y$  (mm $^2$ ) is the surface area of a 1 cm root and  $r$  (mm) is the radius of root. Since the root has a tip, the base area of cylinder is omitted in the equation 1. When the density of root is assumed as 1, the weight of 1 cm root is expressed as:

$$Z = 10 \times \pi r^2 \quad (2)$$

where  $Z$  (mg) is the weight of 1 cm root. From equation 2, equation 1 can be rewritten as:

$$Y = Z/10 + 2(10\pi Z)^{1/2} \quad (3)$$

### Analysis for gravity response in roots

Rice seedlings were grown vertically in plastic cases (10×14×1.5 cm) containing 1% agar under continuous white light at 25 °C for 3 d and then they were rotated by 90°. The gravitropic bending of root tips was measured every 2 h after the reorientation.

### Statistical analysis

The experiments were repeated three to four times and the Student's *t*-test was used for statistical analysis on the difference between mutant and wild-type plants. For analysing genetic segregation, the chi-square test was performed.

## Results

### Isolation of 2,4-D resistant mutants

To investigate molecular mechanism for auxin action, mutants which derived from wild-type IR8 (WT) treated with 1 mM sodium azide were screened. Five 2,4-D resistant lines were isolated. Among them, *arm1* and *arm2* were used in this study. The *arm1* and *arm2* seedlings were able to elongate their roots in Kimura's B medium even in the presence of 1  $\mu$ M 2,4-D, where the growth of wild-type roots was almost completely inhibited.

### Genetic analysis

To determine the genetic basis for auxin resistance in *arm1* and *arm2* mutants, the two lines were crossed to wild-type IR8. The resulting F<sub>1</sub> plants were all auxin-sensitive, and either F<sub>2</sub> segregation was 3:1 (3 sensitive, 1 resistant) (Table 1). Therefore, the resistance to 2,4-D in *arm1* and *arm2* is due to a respective single recessive mutation. To test their allelism, they were crossed with each other. All

F<sub>1</sub> progeny from this cross was sensitive to auxin (Table 1), indicating that the two mutant genes are not allelic. Segregation of F<sub>2</sub> progeny fitted to a 9:3:3:1 ratio (9 wild type, 3 *arm1*, 3 *arm2*, 1 double mutant). The genotype of the double mutant was confirmed by analysing F<sub>3</sub> progeny.

### Morphological analysis

To compare the morphology of mutants, seedlings were grown in water for 7 d. As shown in Table 2, *arm1* seedlings exhibited a greater level of seminal root elongation, fewer lateral roots per plant, less lateral root density, and slightly shorter crown roots compared with wild-type IR8, while no significant difference was observed in root number and plant height. By contrast, *arm2* showed a greater plant height and slightly fewer laterals on both seminal roots (Table 2; Fig. 1) and crown roots (data not shown) compared with the wild type.

In the *arm1 arm2* double mutant, lateral root formation was dramatically reduced compared with parental single mutant plants (Table 2; Fig. 1), suggesting that the two mutant genes provided synergistic effects in inhibiting lateral root formation. By contrast, many other phenotypes of the double mutant plants are similar to parental *arm1* plants in root number, crown root length and plant height. Although the length of the seminal roots of the double mutant is slightly shorter than that of *arm1* and longer than

**Table 1.** Genetic analysis of 2,4-D resistant mutants

To determine genetic segregation in F<sub>1</sub> and F<sub>2</sub> generations, all progeny was grown in 1  $\mu$ M 2,4-D for 7 d to compare with parental wild-type IR8 (WT), *arm1* and *arm2*. The double mutant, isolated from the F<sub>2</sub> population, was confirmed by an F<sub>3</sub> progeny test.

Cross	Auxin response		Expected ratio	$\chi^2$	<i>P</i>
	WT	Mutant			
<i>arm1</i> × WT (F <sub>2</sub> )	231	84	3:1	0.37	0.50–0.75
<i>arm2</i> × WT (F <sub>2</sub> )	176	54	3:1	0.20	0.75–0.90
<i>arm1</i> × <i>arm2</i> (F <sub>1</sub> )	10	0			
	WT	<i>arm1</i>	<i>arm2</i>	<i>arm1arm2</i>	
<i>arm1</i> × <i>arm2</i> (F <sub>2</sub> )	158	55	48	12	9:3:3:1 1.61 0.50–0.75

**Table 2.** Morphological analysis of 7-d-old seedlings of wild-type IR8 (WT), *arm1*, *arm2*, and the double mutant *arm1 arm2*

To evaluate morphological characters, rice seedlings were grown in water culture for 7 d. Data are averages for 10 plants ( $\pm$ SD).

Characteristics	WT	<i>arm1</i>	<i>arm2</i>	<i>arm1 arm2</i>
Seminal root length (cm)	5.75 $\pm$ 0.23	8.25 $\pm$ 0.82*** $\Delta^d$	5.31 $\pm$ 0.89 $\Delta$	7.20 $\pm$ 0.74**
Lateral root number per plant <sup>a</sup>	49.0 $\pm$ 4.13	38.2 $\pm$ 7.95*** $\Delta$	43.0 $\pm$ 5.51*** $\Delta$	24.6 $\pm$ 5.37**
Lateral root density <sup>b</sup>	8.56 $\pm$ 0.74	4.58 $\pm$ 0.62*** $\Delta$	8.02 $\pm$ 1.21 $\Delta$	3.38 $\pm$ 0.50**
Root number	6.10 $\pm$ 0.73	5.80 $\pm$ 0.78	6.00 $\pm$ 0.94	5.20 $\pm$ 0.78*
Crown root length (cm)	4.18 $\pm$ 0.39	3.52 $\pm$ 0.54**	3.70 $\pm$ 0.72	3.36 $\pm$ 0.49**
Plant height (cm)	3.80 $\pm$ 0.57	3.91 $\pm$ 0.44	4.43 $\pm$ 0.35**	4.04 $\pm$ 0.47

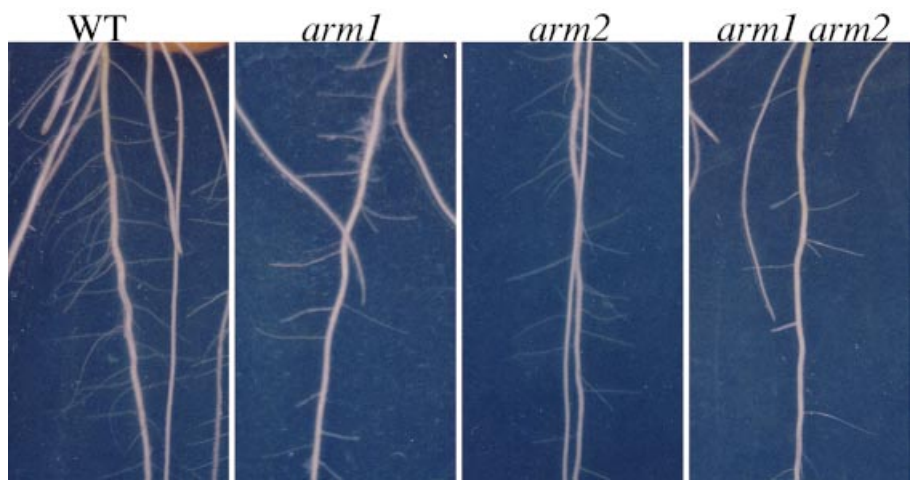
<sup>a</sup> Lateral root number was counted on the seminal root.

<sup>b</sup> Density (lateral root number per cm) was determined by dividing the lateral root number by the seminal root length for each plant.

\* , \*\* Significant difference from wild-type IR8 (WT) at 5% and 1%, respectively.

<sup>d</sup>  $\Delta$  Significant difference from the double mutant at 1%.





**Fig. 1.** Lateral root formation is dramatically reduced in double mutant *arm1 arm2* compared with either single mutant. Rice seedlings were grown in water for 7 d.

**Table 3.** Root structure of 3-d-old seedlings in wild-type IR8 (WT), *arm1*, *arm2*, and the double mutant *arm1 arm2*

Rice seedlings were grown in water culture for 3 d. Cross-sections of roots were obtained at 1 cm from the root tips. Data are averages of five plants ( $\pm$ SD).

Characteristics	WT	<i>arm1</i>	<i>arm2</i>	<i>arm1 arm2</i>
Root diameter ( $\mu$ m)	447.0 $\pm$ 20.5	380.0 $\pm$ 11.7** <sup>a</sup>	433.0 $\pm$ 32.4	368.0 $\pm$ 40.9**
Cortical cell layers	3–4	3–4	3–4	2–3
Cortical cell diameter ( $\mu$ m)	20.6 $\pm$ 1.9	14.7 $\pm$ 1.8*	20.1 $\pm$ 0.5	20.8 $\pm$ 0.1
Protoxylem diameter ( $\mu$ m)	15.4 $\pm$ 1.6	9.7 $\pm$ 1.4**	9.7 $\pm$ 2.0**	8.3 $\pm$ 1.6**
Metaxylem diameter ( $\mu$ m)	35.0 $\pm$ 3.9	21.0 $\pm$ 3.0**	23.3 $\pm$ 1.8**	21.8 $\pm$ 0.2**
Stele diameter ( $\mu$ m)	130.3 $\pm$ 4.6	98.7 $\pm$ 2.3**	126.0 $\pm$ 3.6	126.7 $\pm$ 8.1

<sup>a</sup> \*, \*\* Significant difference from wild-type IR8 (WT) at 5% and 1%, respectively.

**Table 4.** Reduced [<sup>14</sup>C]2,4-D uptake in 1 cm root tips of *arm2* and the double mutant *arm1 arm2*

Rice seedlings were grown in water for 3 d and their 1 cm root tips were incubated with 1  $\mu$ M [<sup>14</sup>C]2,4-D for 30 min. Radioactivity accumulation was expressed as the percentage relative to wild-type IR8 (WT) in three ways: radioactivity accumulation per 1 cm root tip, per mg fresh weight, and per mm<sup>2</sup> root surface area. Relative fresh weight (FW), root radius and root surface area of 1 cm root tips are also shown. Data are averages of three experiments with four replications ( $\pm$ SD).

Line	FW (%)	Root radius (%)	Root surface area (%)	Radioactivity cm <sup>-1</sup> (%)	Radioactivity mg <sup>-1</sup> (%)	Radioactivity mm <sup>-2</sup> (%)
WT	100 $\pm$ 8.2	100 $\pm$ 2.8	100 $\pm$ 16.7	100 $\pm$ 11.3	100 $\pm$ 1.4	100 $\pm$ 6.4
<i>arm1</i>	80 $\pm$ 5.6*** <sup>a</sup>	88 $\pm$ 3.2**	90 $\pm$ 5.4* <sup>a</sup>	91 $\pm$ 4.4*	114 $\pm$ 10.7	102 $\pm$ 7.0
<i>arm2</i>	98 $\pm$ 7.0	102 $\pm$ 3.0	98 $\pm$ 6.7	79 $\pm$ 4.2**	81 $\pm$ 6.2**	81 $\pm$ 6.1**
<i>arm1 arm2</i>	84 $\pm$ 7.1**	87 $\pm$ 4.0**	91 $\pm$ 7.0*	72 $\pm$ 4.7**	80 $\pm$ 11.7*	80 $\pm$ 6.4**

<sup>a</sup> \*, \*\* Significantly different from WT at 5% and 1% levels, respectively.

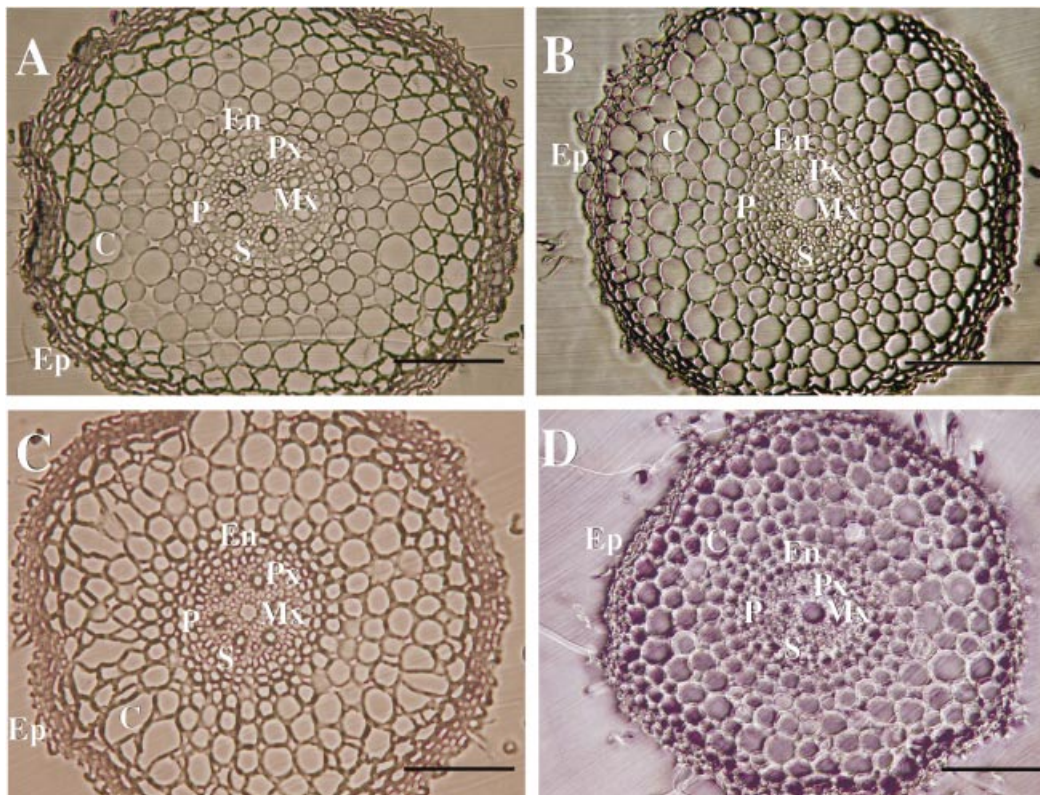
*arm2*, the length of double mutant roots is closer to *arm1* roots compared with *arm2* roots.

Root structure was also compared by analysing their cross-sections at about 9 mm from the root tips (Table 3; Fig. 2). The diameters of *arm1* and double mutant roots were narrower compared with the wild type. In *arm1* roots, both the stele diameter and cortical cell diameter were reduced while the number of cortical cell layers was normal. By contrast, in the double mutant, one cell layer was missing from the cortex. The diameters of protoxylem

and metaxylem were narrower in *arm1*, *arm2* and double mutant roots compared with the wild type, suggesting that the two genes may be involved in xylem development in roots.

### Auxin resistance

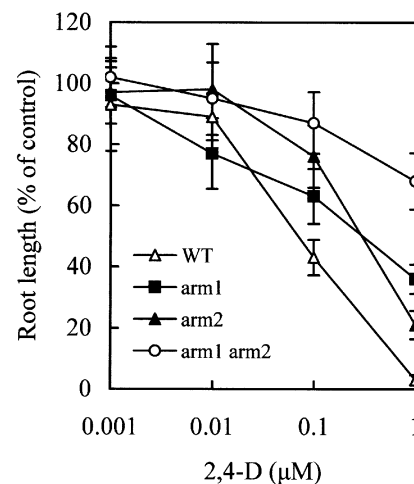
In order to evaluate quantitatively the level of resistance to auxin, the dose–response of root elongation was measured for 2,4-D. Seedlings of *arm1*, *arm2*, the double mutant *arm1 arm2*, and wild-type IR8 were grown with various



**Fig. 2.** Cross-sections of WT (A), *arm1* (B), *arm2* (C), and the double mutant *arm1 arm2* (D) roots. Rice seedlings were grown in water for 3 d and the cross-sections were made about 9 mm from root tips. Ep, epidermis; C, cortex; En, endodermis; P, pericycle; Px, protoxylem; Mx, metaxylem; S, stele. Bars=100  $\mu$ m.

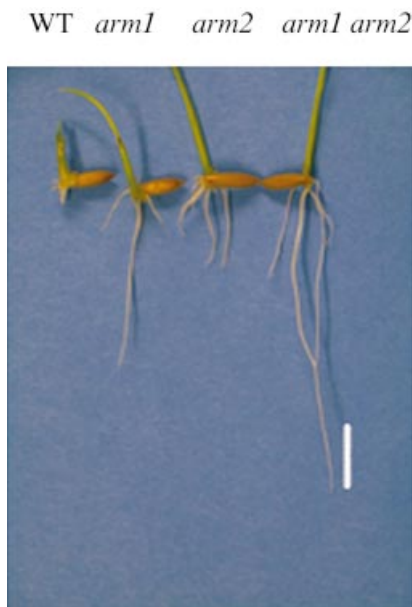
concentrations of 2,4-D for 7 d. The result of the assay is presented in Fig. 3. The roots of *arm2* are more resistant than those of wild-type IR8 in the range from 0.1–1  $\mu$ M, while the response to 2,4-D of *arm1* roots is complex. At 0.01  $\mu$ M 2,4-D, *arm1* roots unexpectedly exhibited a slightly higher sensitivity ( $0.01 < P < 0.05$ ) to auxin compared with the wild type. However, in higher concentrations (0.1–1  $\mu$ M), *arm1* roots were resistant to 2,4-D and showed a greater resistance to 1  $\mu$ M 2,4-D than *arm2* roots. As a result, the dose–response curve for *arm1* roots is crossed with those for wild-type and *arm2* roots. The double mutant roots were more resistant to 2,4-D than parental single mutants and the greater resistance of the double mutant was striking at 1  $\mu$ M 2,4-D ( $P < 0.01$ ) (Figs 3, 4).

In order to compare quantitatively the resistance to auxin, the concentration of 2,4-D which induced a 50% inhibition in root elongation ( $IC_{50}$ ) was calculated.  $IC_{50}$  for *arm1*, *arm2*, and wild-type IR8 was 0.3  $\mu$ M, 0.3  $\mu$ M, and 0.05  $\mu$ M, respectively. The 2,4-D-induced inhibition in the growth of double mutant roots did not reach 50% even at the highest concentration (1  $\mu$ M 2,4-D) (Fig. 3). Although, the  $IC_{50}$  value for *arm1* is the same as *arm2*, the slopes of the dose–response curves for these mutants are different. The slope for *arm2* is similar to that of the wild type, while



**Fig. 3.** Dose–response curves of root elongation in wild-type IR8 (WT), *arm1*, *arm2*, and the double mutant *arm1 arm2* seedlings for 2,4-D. Rice seedlings were grown in water supplemented with various concentrations of 2,4-D in the light at 25  $^{\circ}$ C for 7 d. Data are the percentage of root length on auxin medium relative to root length on auxin-free medium and averages for 10 seedlings ( $\pm$ SD).

the slope for *arm1* is gentler than that of *arm2* as described above.



**Fig. 4.** Resistance to 2,4-D of roots is greatly increased in double mutant *arm1 arm2* compared with the parental single mutant. Rice seedlings, mutants and wild-type IR8 (WT), were grown with 1  $\mu$ M 2,4-D for 7 d. Bar represents 1 cm.

#### Reduced auxin accumulation in *arm2* roots

In order to determine whether or not the resistance phenotype of mutant roots is due to a change in auxin uptake, the accumulation of radioactivity was examined in 1 cm root tips incubated with 1  $\mu$ M [ $^{14}$ C]2,4-D for 30 min using mutants and the wild type. Since the accumulation of radioactivity in root tip segments increased linearly up to 1 h (data not shown), the accumulation for the initial 30 min was measured.

To find the best way to compare auxin uptake between thick roots, as for the wild type, and thin roots as for *arm1*, auxin uptake was expressed in three ways as shown in Table 4; radioactivity accumulation per 1 cm root, per mg fresh weight and per mm<sup>2</sup> surface area. The surface area of root segments was estimated from equation 3. From Table 4, it was concluded that auxin accumulation per mm<sup>2</sup> surface area is the best method of comparing auxin uptake because of the following two reasons. First, since labelled 2,4-D is taken up through the surface of roots, the surface area is the most important factor in short-term experiments. Second, the data expressed per surface area are well correlated to genotypes, suggesting that the *arm2* mutant shows a reduced 2,4-D uptake while the *arm1* mutant shows normal uptake. The reduced auxin accumulation in *arm2* roots and in the double mutant *arm1 arm2* roots was significant for all three methods, suggesting that *arm2* and double mutant roots are defective in 2,4-D uptake.

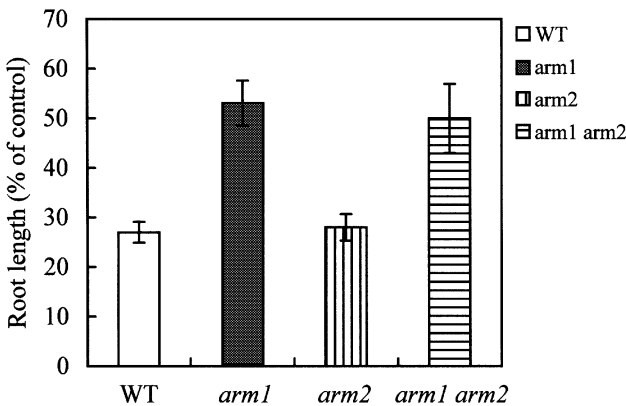
#### Normal uptake of NAA in *arm2* roots

Delbarre *et al.* (1996) reported that an influx auxin carrier facilitates the uptake of 2,4-D, but not NAA which enters

**Table 5.** [ $^3$ H]NAA uptake in 1 cm root tips of WT, *arm1*, *arm2* and the double mutant *arm1 arm2*

Root tips were incubated with 1  $\mu$ M [ $^3$ H]NAA for 30 min. Radioactivity accumulation was expressed as per mm<sup>2</sup> root surface area. Other descriptions are the same as in Table 4.

Line	Radioactivity mm <sup>-2</sup> (%)
WT	100 $\pm$ 8.4
<i>arm1</i>	108 $\pm$ 11.7
<i>arm2</i>	104 $\pm$ 9.0
<i>arm1 arm2</i>	108 $\pm$ 7.3



**Fig. 5.** The growth of *arm2* roots was inhibited by NAA as wild-type IR8. Rice seedlings were grown in water supplemented with 0.1  $\mu$ M NAA for 7 d. Data are the percentage of root length on auxin medium relative to root length on auxin-free medium and averages for 10 seedlings ( $\pm$ SD).

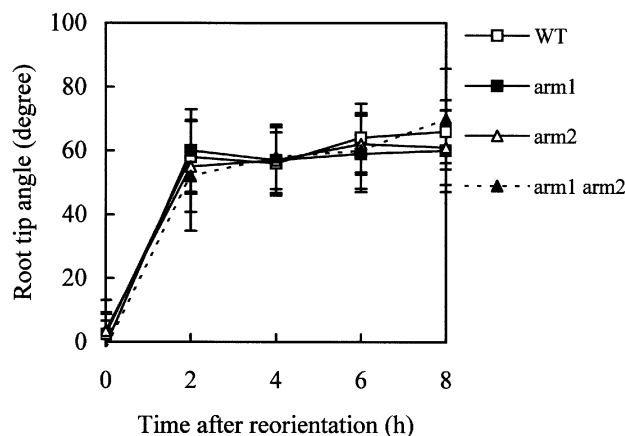
the cells through diffusion. This concept has been supported using *Arabidopsis* roots (Yamamoto and Yamamoto, 1998; Marchant *et al.*, 1999; Rahman *et al.*, 2001). To differentiate carrier uptake from diffusion, the uptake of 1  $\mu$ M [ $^3$ H] NAA was examined. As shown in Table 5, the accumulation of labelled NAA in *arm2* and double mutant roots was similar to the wild type, suggesting that 2,4-D uptake is mediated by an auxin carrier.

Furthermore, the effect of NAA was examined on the growth of roots in *arm2* and wild-type IR8. As shown in Fig. 5, *arm2* roots did not show any resistance to NAA, although the resistance to NAA was observed in *arm1* and double mutant roots. These results are consistent with the idea that *arm2* has a defect in auxin uptake.

#### Normal gravitropic response in the roots of auxin-resistant mutants

Many auxin-resistant mutants have been reported as defective in gravitropic response as well as in lateral root development (Hobbie, 1998). This information prompted an examination of the gravitropic response of the mutants used in this work. Unexpectedly, *arm1*, *arm2* and the double mutant roots bent towards the gravity to the same





**Fig. 6.** Time-course of gravitropic bending of roots in wild-type IR8 (WT), *arm1*, *arm2*, and the double mutant *arm1 arm2* seedlings. Rice seedlings were grown in 1% agar plate in the light for 3 d and then they were rotated by 90° at time 0. No difference was found in gravitropic response of these roots. Data are averages for 8 seedlings ( $\pm$ SD).

extent as the wild-type roots after reorientating the seedlings by 90° (Fig. 6).

## Discussion

Two non-allelic rice mutants, *arm1* and *arm2*, showing a reduced sensitivity to 2,4-D in a root elongation assay have been isolated. Both *arm1* and *arm2* mutants are controlled by a respective single recessive gene (Table 1) and display reduced lateral root formation (Table 2) and impaired xylem development in roots (Table 3; Fig. 2).

In order to investigate whether or not the resistance phenotype of *arm1* and *arm2* mutant roots is due to a change in auxin uptake, the accumulation of radioactivity in 1 cm root tips incubated with 1  $\mu$ M [ $^{14}$ C]2,4-D for 30 min was also examined. Since *arm1* roots are more slender compared with wild-type roots, the radioactivity accumulation data were analysed using the three methods shown in Table 4. It was concluded that auxin accumulation per mm<sup>2</sup> surface area of roots was the best method for comparing auxin uptake, because auxin is taken up through the root surface and the data correlate well with root genotype. The results show that the 2,4-D uptake in *arm2* roots is reduced compared with wild-type IR8, while uptake in *arm1* roots is normal (Table 4). By contrast, the uptake of [ $^3$ H] NAA in *arm2* roots was similar to the wild type (Table 5) and *arm2* roots responded to NAA in the same way as the wild type (Fig. 5). These results suggest that an auxin influx carrier is required for 2,4-D uptake in rice roots and that the *arm2* mutant has a defect in auxin uptake.

In addition, the genetic relationships between *arm1* and *arm2* mutants have been analysed by establishing double mutant plants. The phenotypes of the double mutant were

somewhat complex compared with either parental single mutant (Tables 2, 3; Figs 2, 3). The *arm1 arm2* double mutant was more resistant to 2,4-D and formed fewer lateral roots than either parental single mutant, suggesting that the two mutant genes interact synergistically with respect to both auxin resistance and lateral root formation. Loss of one cortical cell layer in the double mutant roots is possibly due to another synergistic interaction of two genes. Such synergistic interaction was not observed in other phenotypes of the mutants. The diameters of protoxylem and metaxylem were reduced in *arm1* and *arm2* roots compared with the wild type. However, no additive effect was observed in the double mutant. To explain these interactions between *arm1* and *arm2* genes, the hypothesis is presented that both resistance to 2,4-D and reduced lateral root formation in *arm1* are enhanced in the double mutant by reduced auxin uptake in roots, whereas other phenotypes of the double mutant are not much influenced by reduced auxin uptake. This idea suggests that the *Arm2* gene is involved in lateral root formation *in vivo*.

Many auxin-resistant mutants of *Arabidopsis*, *aux1*, *axr1*, *axr4*, and *axr6*, show a defect in gravitropic response as well as in lateral root development (Hobbie and Estelle, 1995; Hobbie *et al.*, 2000; Rahman *et al.* 2001; Marchant *et al.*, 2002). An auxin-resistant mutant of rice, *Lrt1*, also fails to form lateral roots and shows reduced root gravitropic response (Chhun *et al.*, 2003). The tomato mutant *diageotropica* (*dgt*), isolated for a horizontal growth pattern, does not produce lateral roots and exhibits a reduction in auxin sensitivity in the root (Muday *et al.*, 1995). However, rice *arm1*, *arm2* and their double mutant, which have been isolated as auxin-resistant lines, exhibited a normal gravitropic response in roots (Fig. 6). These unexpected results are consistent with the suggestion that the *Arm1* and *Arm2* genes are involved in lateral root formation *in vivo*. An alternative explanation could be that the *arm2* mutant is leaky and unable to inhibit the gravitropic response.

Although auxins are effective in inhibiting root growth and lateral root formation, a higher concentration of auxin is usually required for inducing lateral roots than for root growth inhibition (Zolman *et al.*, 2001; Chhun *et al.*, 2003). Considering this difference, the roles of *Arm1* and *Arm2* genes are hypothesized as follows. If these genes or their products require relatively high concentration of auxin, they could be effective for lateral root formation only *in vivo*. However, when high concentrations of auxin were applied, they could be effective in inhibiting root growth as well. This may explain the results that *arm1* and *arm2* mutations were isolated as resistant mutants to toxic levels of auxin in the root elongation assay and that these mutations inhibit lateral root formation, but not the gravitropic response, in the absence of exogenous auxin.



In rice, no information has so far been reported on the mechanism for reduced sensitivity to inhibitory levels of auxin. In the present study, it has been demonstrated that two genes *arm1* and *arm2* are involved in both reduced 2,4-D sensitivity and poor lateral root formation in rice. It is also suggested that *arm2* has a defect in auxin uptake and that *Arm1* and *Arm2* genes are involved in different processes of the auxin response pathways required for lateral root formation.

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