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Short Communication

Involvement of *ARM2* in the Uptake of Indole-3-butyric Acid in Rice (*Oryza sativa* L.) Roots

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Auxin influx carriers are involved in auxin transport and plant development. Here we show that the mutant of rice (*Oryza sativa* L. ssp. *indica* cv IR8) *arm2* is defective in the uptake of the naturally occurring auxin indole-3-butyric acid (IBA). The acropetal and basipetal transport of IBA is reduced in *arm2* roots compared with wild type. In contrast, *arm2* roots are normal with respect to uptake and transport of indole-3-acetic acid (IAA). Furthermore, *arm2* roots are resistant to IBA but respond normally to IAA. The mutant analysis of *arm2* indicates the presence of an influx carrier system for IBA in rice roots.

Keywords: Auxin influx carrier — Auxin transport — IAA — IBA — *Oryza sativa* L. — Rice root

Abbreviation: IBA, indole-3-butyric acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; NAA, 1-naphthaleneacetic acid; NOA, 1-naphthoxyacetic acid.

Indole-3-butyric acid (IBA) has been shown to be a natural auxin in a number of plant species including monocot, maize (*Zea mays* L.) (Ludwig-Müller and Epstein 1991), and dicot, *Arabidopsis* (Epstein and Ludwig-Müller 1993, Ludwig-Müller et al. 1993), but its physiological roles in plant growth and development have not yet been well established (Ludwig-Müller 2000, Poupart and Waddell 2000, Zolman et al. 2000). Indole-3-acetic acid (IAA), another natural auxin, is mobilized in plants using specialized transport components composed of influx and efflux carriers. Several genes responsible for influx and efflux carriers of IAA have been identified in *Arabidopsis* including *AUX1* (Bennett et al. 1996), *AtPIN1* (Gälweiler et al. 1998), *AtPIN2/EIR1/AGR1/WAV6* (Chen et al. 1998, Luschnig et al. 1998, Müller et al. 1998, Utsuno et al. 1998), *AtPIN3* (Friml et al. 2002a), *AtPIN4* (Friml et al. 2002b) and *AtPIN7* (Friml et al. 2003). However, genes responsible for IBA transport have not been identified. In roots, IAA is transported acropetally from root base to root tip and basipetally backward from the root tip to the base (Tsurumi and Ohwaki 1978, Estelle 1996, Muday and DeLong 2001). The acropetal transport of IAA is involved in lateral root formation (Reed et al. 1998, Bhalerao et al. 2002). The basipetal transport of IAA is involved in root gravitropism (Rashotte et al. 2000) as well as lateral root formation (Casimiro et al. 2001). In the present study, we show that the novel mutant of rice (*Oryza sativa* L. cv. IR8) *arm2* is defective in the uptake and transport of IBA in roots, although it exhibits normal IAA uptake. *arm2* was originally isolated as a 2,4-dichlorophenoxyacetic acid (2,4-D)-resistant mutant and it displays a reduced number of lateral roots and impaired xylem development (Chhun et al. 2003).

If auxin uptake were facilitated by carrier proteins, application of unlabeled auxin would competitively decrease the accumulation of radioactive auxin in roots (Delbarre et al. 1996). As shown in Fig. 1, uptake of [^{3}H]IAA in wild-type roots was interfered with by the presence of unlabeled IAA. The inhibitory effect of unlabeled IAA exhibited a dose-dependent relationship, in which the higher the concentrations

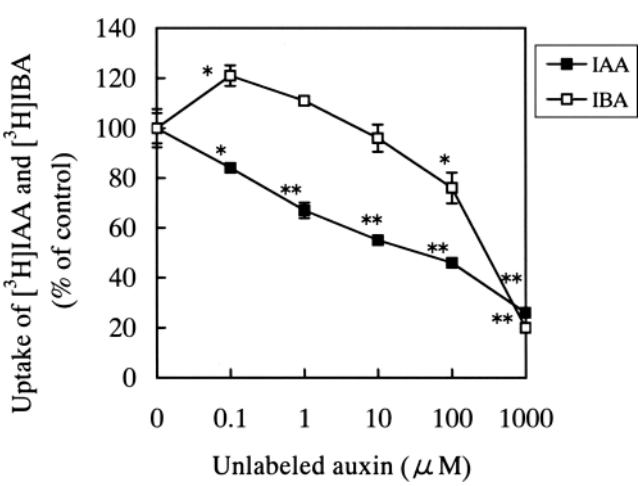

Fig. 1 Effects of unlabeled auxin on the uptake of [^{3}H]IAA and [^{3}H]IBA in 1 cm rice (*Oryza sativa* L. cv IR8) root tips. Ten root tips were incubated with 10 mM MES buffer (pH 5.7) containing 10 nM [^{3}H]IAA or 10 nM [^{3}H]IBA without (control) or with the respective unlabeled auxin for 30 min. Accumulation of labeled auxin in roots is expressed as a percentage relative to control. Data are averages for 30 roots (± SE) with three independent experiments. * and ** indicate significantly different from control at 5 and 1%, respectively.

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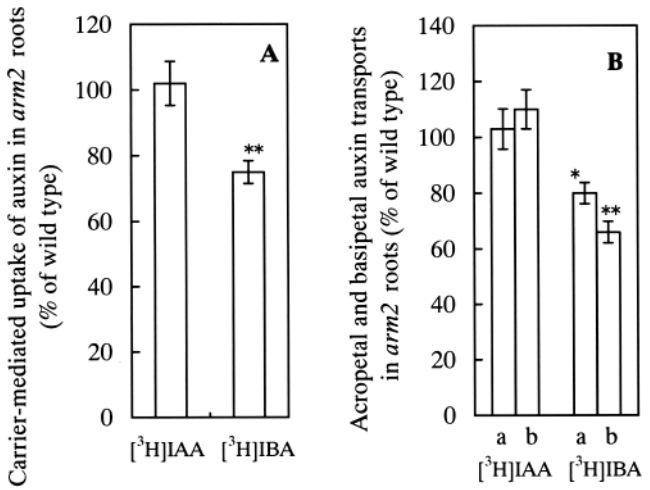

Fig. 2 Uptake (A) and transport (B) of [^{3}H]IAA and [^{3}H]IBA in *arm2* roots compared with wild-type IR8. (A) The carrier-mediated uptake of labeled auxin, the difference between radioactivity accumulation in the presence and absence of 1 mM unlabeled auxin, is expressed as a percentage relative to wild type. (B) Acropetal (a) and basipetal (b) transport of labeled auxin in *arm2* roots is expressed as a percentage relative to wild type. A root segment of 1.6 cm in length was placed on a small filter paper supplemented with 0.2 μM labeled auxin. After 1 h transport, the radioactivity of the distal 3 mm end of root segments was counted. Data are averages for 30 roots (± SE) with three independent experiments. * and ** indicate significantly different from wild type at 5 and 1%, respectively.

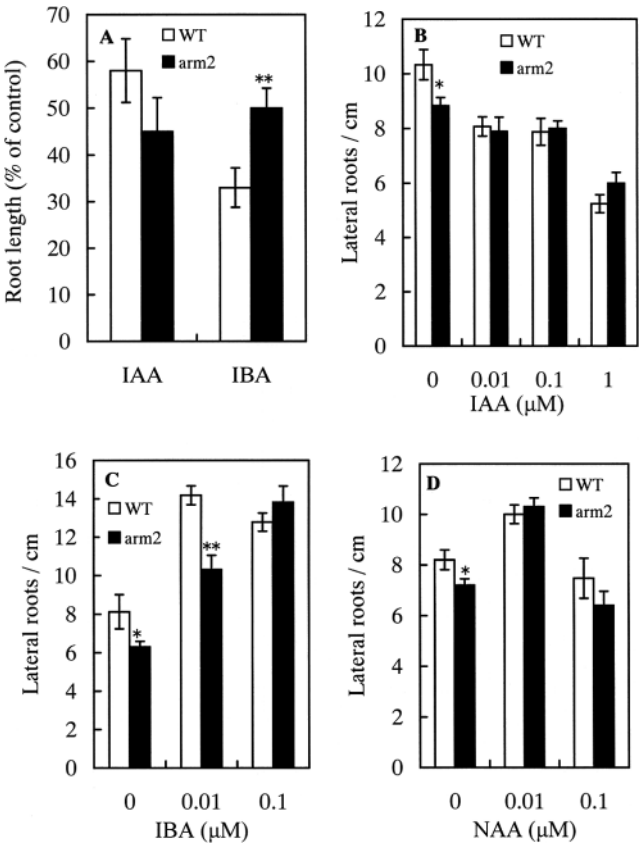

Fig. 3 Effects of auxin on root elongation (A) and lateral root formation (B–D) in wild-type IR8 and *arm2*. (A) Rice seedlings were grown without auxin (control) or with 1 μM IAA and 0.1 μM IBA for 7 d in the light. Root length is expressed as a percentage relative to control. (B–D) Rice seedlings were grown in the absence or presence of IAA (B), IBA (C) and NAA (D) for 7 d in the light. The density of lateral roots was obtained by dividing the number of lateral roots by the length of the seminal root. Data are averages for 20 or 30 roots (± SE) with two or three independent experiments, respectively. * and ** indicate significantly different from wild type at 5 and 1%, respectively.

of unlabeled IAA applied, the less [^{3}H]IAA accumulated in roots. In contrast, a low concentration of unlabeled IBA slightly but significantly ($P < 0.05$) increased accumulation of [^{3}H]IBA in roots. If unlabeled auxin could competitively inhibit the efflux of labeled auxin, application of unlabeled auxin would increase the accumulation of labeled auxin in cells (Delbarre et al. 1996). This may be the most reasonable explanation for the enhanced accumulation of [^{3}H]IBA in the presence of 0.1 μM unlabeled IBA. However, increasing the concentration of unlabeled IBA to >0.1 μM decreased the uptake of labeled IBA in a similar manner to that of IAA (Fig. 1). Furthermore, the uptake of both labeled auxins was reduced to a similar level by 1 mM unlabeled auxin. These results indicate that the uptake of IAA and IBA in rice roots is facilitated by carrier proteins.

Since the uptake of two auxins was greatly reduced in the presence of 1 mM unlabeled auxin, carrier-mediated uptake for auxin was determined from the difference between radioactivity accumulation with and without 1 mM unlabeled auxin. The carrier-mediated uptake of [^{3}H]IAA in *arm2* roots was similar to that in wild-type roots, while that of [^{3}H]IBA in *arm2* roots was significantly ($P < 0.01$) less compared with wild-type roots (Fig. 2A). As reduced auxin influx in *arm2* roots is expected to perturb polar auxin transport, we measured auxin transport in wild-type and *arm2* root segments. As shown in Fig. 2B, both acropetal and basipetal transport of [^{3}H]IBA in *arm2* roots was less compared with wild type, whereas the transport of [^{3}H]IAA in *arm2* roots was normal, as expected from the uptake experiments.

To confirm the difference between the effects of IAA and IBA on *arm2* roots, we also examined the response of *arm2* and wild-type roots to the two auxins in the root elongation assay and lateral root formation. Rice seedlings were grown in auxin solution under continuous white light for 7 d at 25°C. Auxin solution was refreshed every day or every 2 d to prevent reduction of the auxin concentration. Data were presented as the percentage of root length on water culture supplemented with auxin relative to root length on auxin-free medium. As shown in Fig. 3A, *arm2* roots showed a significant resistance to 0.1 μM IBA ($P < 0.01$) but not to 1 μM IAA. These results are consistent with the fact that *arm2* roots are defective in IBA uptake but normal for IAA uptake (Fig. 2A), implying that ARM2 function is specifically involved in IBA uptake.

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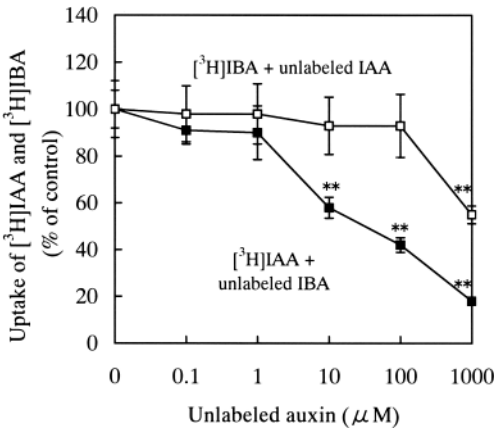

Fig. 4 Competition for auxin uptake between IAA and IBA in wild-type IR8 roots. The competitive reduction in [^{3}H]IAA uptake by unlabeled IBA was compared with that in [^{3}H]IBA uptake by unlabeled IAA. Auxin uptake is expressed as a percentage relative to control (without unlabeled auxin). Data are averages for 30 roots (± SE) with three independent experiments. * and ** indicate significantly different from control at 5 and 1%, respectively.

We further examined the effect of auxin on lateral root formation. It is noteworthy that the density of lateral roots in *arm2* is significantly ($P < 0.05$) lower than that in wild type (Fig. 3B–D). Treatment with IAA did not promote lateral root formation but slightly inhibited it, and the IAA-induced inhibition was pronounced when the IAA concentration reached 1 μM (Fig. 3B), being consistent with our previous observation (Chhun et al. 2004). In contrast to IAA treatment, IBA stimulated lateral root emergence in both mutant and wild-type seedlings and the density of lateral root in *arm2* recovered to the wild-type level in the presence of 0.1 μM IBA (Fig. 3C). The artificial auxin 1-naphthaleneacetic acid (NAA) has been shown to enter into cells through diffusion (Delbarre et al. 1996). To learn more whether the diffusible auxin NAA is able to bypass the *arm2* lesion in lateral root formation, we examined the effect of NAA. As shown in Fig. 3D, application of NAA at a physiological concentration was able to restore the lateral root formation in *arm2* seedlings to the wild-type level, which is consistent with normal NAA uptake in *arm2* roots (Chhun et al. 2003).

Taken together, all of these results suggest that *arm2* roots have a defect in IBA uptake and that the IBA influx system is at least in part different from the IAA influx carrier. To determine the specific nature of the respective carrier uptake, we examined the effect of IAA on IBA uptake and vice versa. As shown in Fig. 4, the uptake of [^{3}H]IAA was inhibited by unlabeled IBA in the range from 10 to 1,000 μM, whereas IAA-induced inhibition of [^{3}H]IBA uptake was observed only at 1,000 μM IAA. The weak effect of unlabeled IAA on [^{3}H]IBA uptake (Fig. 4) is in contrast to its strong effect on [^{3}H]IAA uptake (Fig. 1). We also examined the effect of 1-naphthoxyacetic acid (NOA), which is known to inhibit AUX1 function (Parry et al. 2001). Accumulation of [^{3}H]IAA and [^{3}H]IBA in roots was reduced to about 60% in the presence of 100 μM NOA, suggesting that NOA competes with IAA and IBA.

We previously reported that *arm2* is defective in 2,4-D influx, implying that the artificial auxin 2,4-D is incorporated into cells through the IBA influx system in rice roots. In contrast to our results, 2,4-D has been suggested to enter cells through the IAA influx system (Delbarre et al. 1996). We thus examined the effect of 2,4-D on the uptake of IAA and IBA in rice roots. Application of 1 mM 2,4-D blocked the accumulation of [^{3}H]IAA and [^{3}H]IBA to 63 and 48% of control, respectively, suggesting that 2,4-D competes with IBA as well as IAA before entering cells.

Since the uptake of both [^{3}H]IAA and [^{3}H]IBA was reduced by the presence of the respective unlabeled auxin (Fig. 1), the uptake of two auxins is mediated by carrier proteins in rice roots. We present several lines of evidence using the *arm2* mutant to show the specific carrier system for IBA, which is different from that for IAA. First, the carrier-mediated uptake of [^{3}H]IBA was less in *arm2* roots compared with wild type, but that of [^{3}H]IAA in *arm2* was normal (Fig. 2A). Secondly, the acropetal and basipetal transport of IBA in *arm2* roots was less compared with wild type, but that of IAA in *arm2* was normal (Fig. 2B). Thirdly, *arm2* roots showed resistance to IBA but not to IAA and NAA in a root elongation assay (Fig. 3A and Chhun et al. 2003). Finally, *arm2* seedlings had fewer lateral roots than wild-type seedlings, and application of the permeable auxin NAA was able to restore the density of lateral roots in the mutant seedlings to the wild-type level (Fig. 3D). These results indicate that *arm2* mutant roots are defective in IBA uptake although they are normal for IAA uptake, indicating that IAA and IBA require a respective influx carrier for entering root cells. Our results are consistent with the report by Rashotte et al. (2003), who have reported that IBA transport in *Arabidopsis* does not require the activity of the IAA efflux carrier EIR1 nor the IAA influx carrier AUX1, whereas IAA transport requires both of these proteins. The characteristics of *ARM2* are in striking contrast to those of the *Arabidopsis AUX1* gene, which plays an important role for IAA uptake and root gravitropism (Marchant et al. 1999). *arm2* roots exhibited a normal gravitropic response as did the wild type (Chhun et al. 2003), suggesting that ARM2 is not involved in the root gravity response.

In the present study, we have shown that the novel rice mutant *arm2* exhibits reduced IBA uptake and transport, whereas those of IAA are normal. To our knowledge, this is the first report indicating the presence of an IBA influx carrier in plants by mutant analysis. Although the physiological roles of IBA influx and transport in roots have not yet been established, it is interesting to note that the *arm2* mutant shows reduced lateral root formation (Fig. 3B–D) and impaired xylem development in roots (Chhun et al. 2003). Based on these morphological abnormalities and physiological defect, the carrier mediating IBA influx may be required for normal growth in rice root.

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Materials and Methods

The mutant *arm2* was originally isolated as a 2,4-D-resistant mutant from *indica* type rice cv. IR8 (Chhun et al. 2003). Germinated seeds of the mutant and wild type were placed on a floating net and grown hydroponically in a 900 ml plastic container under continuous white fluorescent light at 25°C, as described before (Chhun et al. 2003). To evaluate influx of IAA and IBA, we used root tips of the respective genotype obtained from 3-day-old seedlings grown in water. 5-[^{3}H]IAA (specific activity 740 MBq μmol^{-1}) and ring-[^{3}H]IBA (specific activity 740 MBq μmol^{-1}) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MD, USA). Ten root tips of 1 cm in length were placed in a 2.6 cm Petri dish containing 10 mM MES buffer (pH 5.7) and 10 nM [^{3}H]IAA (4.70 kBq ml^{-1}) or 10 nM [^{3}H]IBA (4.70 kBq ml^{-1}) with or without the respective unlabeled auxin. After 30 min incubation, the root tips were carefully washed with fresh MES buffer and divided into two groups. Before counting the radioactivity of root tips, the surface water was carefully removed by filter paper and the fresh weight of five roots was measured. To compare auxin accumulation in *arm2* roots with wild type, radioactivity accumulation per mm^2 surface area of root segment was determined as previously described (Chhun et al. 2003).

Both acropetal and basipetal auxin transport in roots was measured by the method of Okada et al. (1991) with some modifications. A root segment was placed on a small filter paper including labeled auxin solution, and the radioactivity of the distal end of segments was measured. To keep the contact of root tip with the donor filter paper, we used decapitated root segments, whose length was 1.6 cm but decapitated by 1 mm, for measuring basipetal transport. For acropetal transport, intact root tips of 1.6 cm in length were used. A root segment was placed in a 1.5 ml Eppendorf plastic tube on a small filter paper (about 1.8 mm in diameter) wetted with 1.7 μl of MES buffer (pH 5.7) supplemented with 0.2 μM [^{3}H]IAA (130.2 kBq ml^{-1}) or 0.2 μM [^{3}H]IBA (130.2 kBq ml^{-1}) in the normal orientation for basipetal transport or in the inverted orientation for acropetal transport. The cap of the plastic tube was closed during transport to maintain humid conditions. After 1 h transport at room temperature, a 3 mm segment was cut from the basal end (for basipetal transport) or from the apical end (for acropetal transport). Five 3 mm root segments were combined and the radioactivity was counted. The transport activity was expressed as the percentage relative to wild-type roots.

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