A Gain-of-Function Mutation in OsIAA11 Affects Lateral Root Development in Rice

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ABSTRACT Lateral roots are important to plants for the uptake of nutrients and water. Several members of the Aux/IAA family have been shown to play crucial roles in lateral root development. Here, a member of the rice Aux/IAA family genes, OsIAA11 (LOC_Os03g43400), was isolated from a rice mutant defective in lateral root development. The gain-of-function mutation in OsIAA11 strictly blocks the initiation of lateral root primordia, but it does not affect crown root development. The expression of OsIAA11 is defined in root tips, lateral root caps, steles, and lateral root primordia. The auxin reporter DR5–GUS (β-glucuronidase) was expressed at lower levels in the mutant than in wild-type, indicating that OsIAA11 is involved in auxin signaling in root caps. The transcript abundance of both OsPIN1b and OsPIN10a was diminished in root tips of the Osiaa11 mutant. Taken together, the results indicate that the gain-of-function mutation in OsIAA11 caused the inhibition of lateral root development in rice.

Key words: Oryza sativa L.; OsIAA11; lateral root; auxin signaling.

INTRODUCTION

Lateral roots (LR) are the most important root tissue for the uptake of nutrients and water because of their abundance in root systems. Lateral root development under the control of auxin signaling has been reviewed in reference to the dicot model plant Arabidopsis (reviewed in Peret et al., 2009; Benkova and Bielach, 2010). Compared with Arabidopsis, understanding of LR development is limited in monocot plants, including rice, a model cereal plant.

Root development requires the establishment of an auxin response gradient with a maximum at the root tip (Sabatini et al., 1999). Initiation of lateral root primordia (LRP) requires the establishment of an auxin response gradient with local accumulation in root pericycle cells at the basal meristem (De Smet et al., 2007). A ‘down-umbrella’ auxin distribution model has been proposed for root tips of Arabidopsis whereby auxin is carried to the root cap from the shoot through the stele and then is refluxed into the stele through the lateral root cap and epidermis. Auxin transport facilitators play key roles in maintaining this root tip auxin gradient, including auxin efflux carriers PINs (Bilou et al., 2005).

Lateral root primordia are initiated from the pericycle cells adjacent to protoxylem in Arabidopsis (Laskowski et al., 1995). Pericycle cell specification to founder cells adjacent to the protoxylem is dependent on the localized activation of auxin responses (De Smet et al., 2007; Dubrovsky et al., 2008), suggesting that the oscillating auxin response in xylem cells at the basal meristem is the signal that promotes pericycle cell specification. Pulses of auxin signals prime the adjacent pericycle cells, enabling them to become founder cells for LRP development. On the other hand, Dubrovsky et al. (2008) showed that auxin acts as a local morphogenetic trigger, specifying lateral root founder cells. That is, pericycle cells of the mature root can become founder cells to form lateral root primordia by local auxin signaling. Although the basic model of lateral root development is believed to be similar between Arabidopsis and rice, understanding of the mechanisms of auxin signaling in LR development in rice is limited.

In Arabidopsis, many gain-of-function mutants of Aux/IAAs genes have been characterized and found to be related to lateral root formation. Five Aux/IAA genes for lateral root formation have been reported, including IAA3 (Tian and Reed, 1999), IAA14 (Fukaki et al., 2002), IAA18 (Uehara et al., 2008), IAA19 (Tatematsu et al., 2004), and IAA28 (Rogg et al., 2001). It has been demonstrated that IAA14 interacts with ARF7 and ARF19 (auxin response factor) to inhibit lateral root

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initiation (Okushima et al., 2007). In rice, the first identified Aux/IAA that functions in lateral root development is OsIAA3 (LOC_Os12g40900, now named OsIAA31). Transgenic plants with overexpression of the stabilizing OsIAA3 (Osiaa3) gene showed auxin insensitivity, abnormal shoot and root gravitropism, and a defect in lateral root initiation (Nakamura et al., 2006). Overexpression of OsIAA1 in rice (Song et al., 2009b) resulted in the reduced inhibition of root elongation responsive to exogenous auxin and decreased plant height. These results indicated that OsIAA3 and OsIAA1 have pleiotropic effects on plant growth and development as well as LR development.

Aux/IAAs regulate auxin responses through the interaction with ARFs (Weijers et al., 2005). Aux/IAA proteins have four highly conserved domains. They interact with ARF proteins through domains III and IV. ARFs bind auxin responsive elements (AuxREs) (Ulmasov et al., 1997); thus, Aux/IAAs modulate auxin-regulated gene expression by interacting with ARFs. Domain I in Aux/IAA has an ERF-associated amphiphilic repression (EAR) motif and it can repress ARFs at the transcriptional level (Tiwari et al., 2004). Domain II in the Aux/IAA protein is related to the instability of the protein (Ramos et al., 2001). In this study, we demonstrate that OsIAA11 regulates lateral root initiation in rice.

RESULTS
Isolation and Characterization of the Osiaa11 Mutant
We named the mutant Osiaa11 after cloning the mutated gene Osiaa11 using a map-based cloning strategy, the OsIAA11 (LOC_Os03g43400) named by Jain et al. (2006). The library of an M2 generation of ethyl methane sulfonate (EMS)-mutagenized rice seeds (Indica, Kasalath) was screened for mutants defective in lateral root development. One mutant that failed to develop lateral roots was isolated. The mutant showed longer and thicker roots, but no significant differences in the number of crown roots at seedling stages between the mutant and the wild-type (WT) plants (Figure 1A and 1B). The primary root length and root diameter of 7-day-old mutants increased about 10 and 20%, respectively, compared to WT (Figure 1 and Table 1). The mutant also showed a reduced response to gravity (Supplemental Figure 1A). A population derived from a cross between Osiaa11 mutant and Nipponbare (Japonica cultivar) was used to clone the gene for the mutated traits using a map-based cloning strategy. The mutated gene was mapped on the short arm of chromosome 3 between the simple sequence repeat (SSR) markers, RM6266 and RMS626, within the BAC clone OSJNBb0069P02 using 103 F2 mutants (Figure 2A). Two genes of the Aux/IAA family, OsIAA11 and OsIAA12 (LOC_Os03g43400 and LOC_Os03g43410; Jain et al., 2006), were found within the mapped region. Sequence analysis of genomic DNA fragments of the two genes from WT plant and the mutant showed that a point mutation occurred in the domain II of OsIAA11, resulting in a change from

Cloning and Characterization of OsIAA11
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Figure 1. Morphology of Mutant Seedlings.
(A) Phenotype of 7-day-old seedlings of wild-type (left), homozygous mutant (center), and heterozygous mutant (right). Bar = 2 cm.
(B) Roots of 21-day-old plants of wild-type (left) and homozygous mutant (right). Bar = 4 cm.
(C) Longitudinal sections of wild-type (n = 10) (left) and mutant (n = 20) (right) primary roots, 1–2.5 cm from the root tip. e, endodermis; p, pericycle; c, cortex. Bars = 25 µm.
(D, E) Cross-sections of the wild-type (D) and the mutant (E) primary roots, 1–2.5 cm from the root tip, showing methylene blue staining (n = 10). Bars = 50 µm.
C to T, and consequently a change of a proline to a leucine residue at position 106 (Pro106Leu). Comparison of genomic DNA and cDNA of OsIAA11 indicates that OsIAA11 encodes a protein with 233-amino acid residues, including five exons and four introns (Figure 2A).

To confirm the point mutation in OsIAA11, a dCAPS (derived Cleaved Amplified Polymorphic Sequence) marker was developed based on the point mutation. DNA fragments from the mutant and WT plants were digested using restriction enzyme, Nael, and polymorphic bands were detected between the mutant and the WT (Figure 2B). To further confirm that the mutated traits are caused by the point mutation at domain II of OsIAA11, we constructed transgenic plants that harbor the mutated gene in rice culture solution; 7-day-old seedlings were used in the measurement of lateral root numbers, primary root length, and diameters. 21-day-old seedlings were used in the measurement of number and length of adventitious roots. Results were shown as average ± SD (n = 10). * p < 0.05, ** p < 0.01 (Student’s t-test).

Table 1. Root Parameters of the Wild-Type and Osiaa11 Mutant.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type</th>
<th>Homozygous mutant</th>
<th>Heterozygous mutant</th>
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<tr>
<td>Number of lateral roots</td>
<td>191.5 ± 18.9</td>
<td>0</td>
<td>67.3 ± 11.5**</td>
</tr>
<tr>
<td>Length of primary root (cm)</td>
<td>13.4 ± 0.7</td>
<td>14.5 ± 0.4**</td>
<td>14.1 ± 0.6*</td>
</tr>
<tr>
<td>Diameter of primary root (µm)</td>
<td>207.9 ± 14.5</td>
<td>246.6 ± 13.1**</td>
<td>23.0 ± 1.8</td>
</tr>
<tr>
<td>Diameter of stele (µm)</td>
<td>52.2 ± 2.9</td>
<td>65.1 ± 4.4**</td>
<td>23.0 ± 1.8</td>
</tr>
<tr>
<td>Number of adventitious roots</td>
<td>23.4 ± 2.5</td>
<td>24.2 ± 1.6</td>
<td>23.0 ± 1.8</td>
</tr>
<tr>
<td>Length of adventitious roots</td>
<td>19.8 ± 0.7</td>
<td>29.5 ± 2.3**</td>
<td>22.8 ± 0.8**</td>
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Isolating a Intragenic Suppressor of Osiaa11 Mutant

The library of an M2 generation of ethyl methane sulfonate (EMS) mutagenized Osiaa11 seeds was used to screen for suppressors of Osiaa11. A mutant with partial rescue of lateral roots in the Osiaa11 background was isolated. Sequence analysis showed that the partial rescue of lateral roots was caused by a point mutation in the domain III of IAA11, leading to a change from A to T, and consequently a change of a glutamine to a leucine at position 154 (Gln154Leu) (Supplemental Figure 2 and Table 5). It has been reported that Aux/IAA proteins interact with ARF proteins through domains III and IV (Ulmasov et al., 1997).

The mutated amino acid in domain III is not highly conserved among OsIAAs (Jain et al., 2006), which may explain that the point mutation only partially suppressed the Osiaa11 mutant phenotypes. This result also suggests that function loss of OsIAA11 may not cause obvious developmental defects due to functional redundancy among the IAA proteins.

Expression Pattern of OsIAA11

Quantitative reverse transcription PCR (RT–PCR) analysis showed that OsIAA11 is highly expressed in roots and stem-base (SB), but not in leaf, stem, and panicle (Figure 3A), which is consistent with previous reports (Jain et al., 2006; Song et al., 2009a). To further determine the expression patterns of OsIAA11, transgenic plants harboring GUS reporter driven by the promoter of OsIAA11 (OsIAA11p:GUS) were developed. GUS staining in roots showed that OsIAA11 was expressed in the root tip, lateral root cap, stele, lateral root primordia, and lateral root (Figure 3B–3G).

Auxin Signaling Is Altered in the Root Tip of the Osiaa11 Mutant

Auxin sensitivity in the Osiaa11 mutant was investigated. After treatments with NAA (naphthaleneacetic acid) at 0.1 µM for 7 d, the Osiaa11 mutant showed strong resistance to exogenous NAA compared to WT in a root elongation assay, yet lateral roots were not induced in the mutant (Figure 4A). The exogenous auxin treatment induced the proliferation of root hairs toward the root tips of WT; however, no visible induction of root hairs was observed in the Osiaa11 mutant (Figure 4B and 4C).

<table>
<thead>
<tr>
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<th>Wild-type</th>
<th>Homozygous mutant</th>
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<tr>
<td>Seedlings were grown at 30 °C/22 °C in rice culture solution; 7-day-old seedlings were used in the measurement of lateral root numbers, primary root length, and diameters. 21-day-old seedlings were used in the measurement of number and length of adventitious roots. Results were shown as average ± SD (n = 10). * p &lt; 0.05, ** p &lt; 0.01 (Student’s t-test).</td>
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DRS:GUS is widely used for visualizing auxin response maxima (Sabatini et al., 1999; Scarpella et al., 2003). To investigate auxin signaling in the Osiaa11 mutant, the DRS:GUS construct was transformed into WT plants and Osiaa11 mutants. In WT roots, GUS staining was observed in the distal region of the root, root cap, and stele. In contrast, in the Osiaa11 mutant, GUS staining was observed in the stele and apical meristem but not in the root cap (Figure 4E and 4F). Cross-sections of the primary root at lateral root primordia emergence region showed that the GUS staining in the stele was similar between the Osiaa11 mutant and WT. In addition, the cell structure of the stele of Osiaa11 mutant was normal compared to WT (Figure 4G–4J).

Auxin Transporter Genes Are Down-Regulated in the Root Tip of Osiaa11 Mutant

PIN proteins (PIN-formed) as auxin efflux carriers mediate auxin acropetal flow to root tips through the central vasculature and basipetal flow through the lateral root cap and epidermis (Galweiler et al., 1998; Muller et al., 1998; Marchant et al., 1999; Friml et al., 2002a, 2002b; Bilou et al., 2005). OsPIN1b is expressed in the stele and root cap, whereas OsPIN10a is expressed in the lateral root cap (Wang et al., 2009).
determine whether auxin signaling is altered in the root tip of Osiaa11, the expression levels of OsPIN1b and OsPIN10a were analyzed in the root tips of Osiaa11 mutant and WT using qRT-PCR analysis. The results showed that transcript abundance of OsPIN1b and OsPIN10a was decreased in the root tips of Osiaa11 mutant compared to that of WT (Figure 5A). To confirm the results, transgenic plants harboring OsPIN1b:GUS and OsPIN10a:GUS were developed (Wang et al., 2009). GUS staining in root tips of the T2 plants in the Osiaa11 background were reduced compared with that of WT, which was consistent with our results from qRT-PCR analysis (Figure 5B–5E).

DISCUSSION

Although several members of the Aux/IAA family in Arabidopsis have been functionally characterized for lateral root formation (Tian and Reed, 1999; Rogg et al., 2001; Fukaki et al., 2002; Tate-matsu et al., 2004; Uehara et al., 2008), limited information is available on cereal crops, including rice. In the current study, the gain-of-function mutant of OsIAA11 caused by a point mutation in the conserved core sequence GWPPV of domain II was characterized in rice. The mutation of OsIAA11 results in a defect in lateral root primordial initiation but does not affect crown root development. In Arabidopsis, IAA3 (Tian and Reed, 1999), IAA14 (Fukaki et al., 2002), IAA18 (Uehara et al., 2008), IAA19 (Tatematsu et al., 2004), and IAA28 (Rogg et al., 2001) have been reported to be involved in auxin regulation on lateral root
development. All of the Aux/IAA gain-of-function mutants harbor a mutation in the conserved core sequence GWPPV of domain II. The conserved core sequence GWPPV is important for instability of Aux/IAA proteins (Ramos et al., 2001; Tan et al., 2007). Thus, the mutation in the domain II of OsIAA11 is most likely to cause stabilization of OsIAA11 protein, resulting in gain-of-function phenotypes of OsIAA11.

Domain III in AUX/IAA proteins is crucial for the interaction between IAAs and ARFs (Ulamov et al., 1997). Rouse et al. (1998) reported that the mutation in domain III can partially rescue the phenotypes of arx3-1 mutant caused by gain-of-function mutations in IAA17. A partial revertant of Osiaa11 (Osiaa11R-1) mutant plants caused by a point mutation in domain III was also found in our study. Osiaa11R-1 mutant provides additional evidence that the Osiaa11 is a gain-of-function mutant. The partial rescue of the iaa11-dependent phenotype also suggests that the point mutation in domain III leads to a partial loss-of-function of OsIAA11. In Arabidopsis, T-DNA insertion mutants and intragenic suppressors of gain-of-function mutants have subtle or indiscernible phenotypes (Rouse et al., 1998; Fukaki et al., 2002; Overvoorde et al., 2005; Uehara et al., 2008). It is generally believed that functions of IAAs are highly redundant. Our data suggest that it is likely that other Aux/IAA genes have overlapping functions with OsIAA11.

The Gain-of-Function Mutation in OsIAA11 Strictly Blocks the Initiation of Lateral Root Primordia

Osiaa11 mutant showed a defect in pericycle cell division (Figure 1C–1E). Sectional examination revealed that the cells composing the radial structure of the root, including the stele, endodermis, cortex, and epidermis, are normal in Osiaa11 mutant. Given this result and the auxin signaling alteration in the root caps of the mutant (Figures 4 and 5), it is likely that the defect in lateral root primordia initiation in Osiaa11 is caused by impairment of auxin signaling required to establish founder cells at the basal meristem in the Osiaa11 mutant.

In rice, overexpression of the stabilized form of OsIAA3 (IAA31) led to reduced lateral root and crown root formation (Nakamura et al., 2006); however, the number of crown roots did not change in Osiaa11 (Figure 1 and Table 1), indicating
that OsIAA11 specifically blocks LRP initiation. In Arabidopsis, several mutants defective in lateral root formation to different extent caused by gain-of-function mutations in Aux/IAAs have been reported. AtIAA14 appeared to be the prominent Aux/IAA in lateral root development because gain-of-function mutations in AtIAA14 resulted in a lack of lateral roots, fewer root hairs, and reduced gravitropism of shoots and roots (Fukaki et al., 2002). OsIAA11 is of the closest homolog of AtIAA14 based on protein sequences (Jain et al., 2006), but no changes in root hairs and shoot growth were observed in OsIAA11 mutant. Furthermore, OsIAA11 is a semi-dominant mutant for lateral root development. This suggests that the auxin regulation pathway mediated by AtIAA14 and OsIAA11 appeared to be different. In fact, rice lateral root primordium is initiated from both pericycle cells at the phloem pole and endodermis, which differs from Arabidopsis, in which lateral root primordium is initiated from pericycle cells adjacent to the protoxylem (Laskowski et al., 1995). This difference may give rise to different auxin signaling regulation mediated by Aux/IAAs for lateral root primordium formation in rice and Arabidopsis.

**METHODS**

**Plant Growth Conditions and Hormone Treatments**

Hydroponic experiments were conducted using normal rice culture solution (Yoshida, 1976). Rice plants were grown in a greenhouse at a photosynthetic photon flux density of approximately 200 μmol photons m⁻² s⁻¹ with a 12-h light (30 °C)/12-h dark (22 °C) photoperiod. For auxin treatments, culture solution was supplemented with various concentrations of NAA (Sigma-Aldrich, St Louis, MO, USA). Root phenotypes were photographed using a digital camera (Cannon EOS 5D, Japan) or a stereomicroscope (LEICA MZFLIII, Germany).

**Histological Observation**

Lateral root primordium sections from wild-type and mutant seedlings were prepared. Staining, dehydration, clearing, infiltration, and embedding were performed according to Liu et al. (2005). The microtome sections (LEICA EM UC6, Germany) were mounted on glass slides for imaging using a microscope (Nikon 90i, Japan).

**Mapping and Cloning of OsIAA11**

For gene cloning, an F₂ population was developed from a cross between the mutant and a japonica cultivar, Nipponbare. Preliminary mapping was performed with SSR (Simple Sequence Repeat) markers distributed throughout the rice genome. OsIAA11 was mapped on chromosome 3 flanked by two SSR markers, RM6266 and RM5626, using 103 F₂ mutants. Gene annotation analysis of the mapped region was carried out using a database (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice). To confirm the mutation of OsIAA11, a dCAPS marker was developed according to the procedure described at http://helix.wustl.edu/dcaps/dcaps.html. PCR products were digested by Nael according to the manufacturer’s instructions. Primers for dCAPS marker development are listed in Supplemental Table 2.

**Isolation of Intragenic Suppressor of OsIAA11**

Ethyl methanesulfonate (EMS)-mutagenized seeds of the OsIAA11 mutant from 5 000 lines were used for screening for suppressors of OsIAA11. Rice plants were grown in the normal rice culture solution (Yoshida et al., 1976) for 10 d. One OsIAA11 suppressor was isolated (designated OsIAA11R-1). Sequence analysis revealed an additional nucleotide substitution in domain III of OsIAA11.

**Quantitative RT–PCR**

Total RNA was extracted using an RNeasy plant mini kit (Qiangene, Germany) according to the procedures recommended by the manufacturer. First-strand cDNA was synthesized from total RNA using SuperScript III reverse transcriptase. Quantitative RT–PCR was performed using SYBR Premix Ex Taq™ (Perfect Real Time) (TaKaRa Biotechnology, Dalian Co., Ltd) on the LightCycler®480 machine (Roche) following the manufacturer’s instructions. The amplification program was performed at 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 15 s. Triplicate technical replicates and two biological replicates were performed for qRT–PCR analysis. The relative quantification of each sample was determined by normalization to the amount of actin cDNA detected in the same sample. The relative expression level was calculated by the formula $2^{\frac{\Delta\Delta Ct}{Ct}}$. Statistical analysis of data was performed with Student’s t-test for mean comparisons between wild-type and mutant plants using an algorithm incorporated...
into the Excel software program (Microsoft). The primers for qRT-PCR analyses are listed in Supplemental Table 3.

Vector Construction and Transformation

To construct the IAA11p:GUS vector, a 4853-bp fragment containing the first intron was amplified using PCR and inserted into the SALL-Xbal site of pCAMBIA1300 mentioned above, which contains the iaa11 genomic sequence. The DR5 promoter GUS construct was generated as reported previously (Scarpella et al., 2003). The above constructs containing the first intron was amplified using PCR and inserted into the Sall–XbaI site of pCAMBIA1300 mentioned above, which contains the iaa11 genomic sequence. The DR5 promoter GUS construct was generated as reported previously (Chen et al., 2003). All primers used above are listed in Supplemental Table 4.

GUS Staining

Histochemical GUS analysis was performed as described by Liu et al. (2005). Transgenic plant samples were incubated with GUS staining solution (100 mmol L^{-1} NaH_{2}PO_{4} buffer (pH 7.0), 0.5% Triton X-100, 0.5 mg ml^{-1} X-Gluc, and 20% methanol). After staining, the tissue samples were mounted on slides and photographed (LEICA MZFLIII, Germany).

Statistical Analysis of Data

The data were analyzed using Excel software (Microsoft) for average values, standard deviations (SD), and Student's t-test results.

SUPPLEMENTARY DATA

Supplementary Data are available at Molecular Plant Online.

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