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OsIAA6, a member of the rice *Aux/IAA* gene family, is involved in drought tolerance and tiller outgrowth

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ABSTRACT

Auxin signaling is a fundamental part of many plant growth processes and stress responses and operates through Aux/IAA protein degradation and the transmission of the signal via auxin response factors (ARFs). A total of 31 *Aux/IAA* genes have been identified in rice (*Oryza sativa*), some of which are induced by drought stress. However, the mechanistic link between *Aux/IAA* expression and drought responses is not well understood. In this study we found that the rice *Aux/IAA* gene *OsIAA6* is highly induced by drought stress and that its overexpression in transgenic rice improved drought tolerance, likely via the regulation of auxin biosynthesis genes. We observed that *OsIAA6* was specifically expressed in the axillary meristem of the basal stem, which is the tissue that gives rise to tillers. A knock-down mutant of *OsIAA6* showed abnormal tiller outgrowth, apparently due to the regulation of the auxin transporter *OsPIN1* and the rice tillering inhibitor *OsTB1*. Our results confirm that the *OsIAA6* gene is involved in drought stress responses and the control of tiller outgrowth.

1. Introduction

The phytohormone auxin modulates plant growth patterns and developmental processes, such as embryogenesis, tropisms, apical dominance, shoot and root development and senescence [1], and also participates in responses to abiotic stresses, including drought, salinity and cold temperatures [2–5]. Auxin homeostasis is tightly regulated by its biosynthesis, via conjugation reactions between active and inactive auxins, by its degradation, and by its translocation throughout the plant, which is controlled by auxin transporters. Auxin accumulation results in the activation of signaling pathways and induction of auxin responsive genes, which are influenced by the actions of the Aux/IAA and ARF (Auxin Response Factors) protein families.

Aux/IAAs are short-lived proteins that accumulate rapidly in response to auxin signaling, and they comprise four highly conserved domains (I–IV) [6]. In the absence of auxin, Aux/IAAs and ARFs dimerize through domains III and IV, located at the C-terminal regions of both proteins, to prevent ARF-mediated transcriptional regulation of early auxin response genes [7,8]. However, in the presence of auxin, Aux/IAAs bind to TIR1/AFB (Transport Inhibitor Response1/Auxin Signaling F-box) and the coupled proteins are then ubiquitinated by the $SCF^{TIR/AFBs}$ complex [9,10]. Subsequently, ubiquitin-conjugated Aux/IAA proteins are degraded via the 26S proteasome, allowing ARF-mediated transcriptional regulation of the early auxin response genes [11,12]. Conserved amino acid sequence (GWPPV) of the functional domain II is responsible for the rapid degradation of Aux/IAA protein [13]. Mutations in this sequence result in degradation resistance and maintain constitutive suppression of ARF activity [14]. Analysis of these *aux/iaa* mutants has permitted new insights into the *Aux/IAA*'s functions in many aspects of plant development and auxin mechanisms of *Arabidopsis thaliana* and rice (*Oryza sativa*) [15–21].

Axillary meristems have essential role in plant architecture and reproduction. In rice, they give rise to tillers at the axils of leaves during vegetative development, but form flowering branches during reproductive development [22]. Rice tillers develop into two different kinds of branches, the basal branch produced from the stem base comprising non-elongated internodes and the aerial branch produced from the upper nodes of elongated internodes

Abbreviations: ABA, abscisic acid; *ABRE*, ABA-responsive elements; ARF, auxin response factor; DIP1, dehydration inducible protein 1; GFP, green fluorescent protein; GUS, beta-glucuronidase; IAA, indole-3-acetic acid; MYB, myeloblastosis transcription factor; MYC, myelocytomatosis transcription factor; NAA, 1-naphthyleneacetic acid; NT, non-treated control; PIN1, pin-formed 1; SCF, Skp, Cullin, F-box containing complex; TB1, teosinte 1; TIR1/AFB, Transport Inhibitor Response1/Auxin Signaling F-Box; YUCCA, flavin monooxygenase; X-GluC, 5-bromo-4-chloro-3-indolyl glucuronide; WT, wild-type.

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[23]. The two stages of tiller development (i.e. bud formation and bud outgrowth) are regulated by auxin. Auxins synthesized in shoot apex basipetally move to suppress tiller bud outgrowth known as apical dominance [24,25]. Accordingly, if the main inflorescence apex of *A. thaliana* or rice is removed to prevent the apical dominance effect, plants accelerate axillary branch or tiller outgrowth, a response that can be suppressed by the addition of exogenous auxin [26,27]. It has been observed that auxin does not enter the lateral buds and that bud outgrowth due to inhibition of polar auxin transport is not enough [28,29], and these observations support the involvement of a second messenger(s), such as cytokinin and strigolactone, in the regulation of bud outgrowth [29]. Indeed, genes required for the biosynthesis of strigolactone and cytokinins are in turn regulated by auxin [29,30]. While strigolactone blocks bud outgrowth, it is stimulated by cytokinin via the regulation of the gene *teosinte branches 1* (*TB1*), which is a key negative regulator of bud outgrowth [31,32]. During early stages of bud development, *TB1* is expressed at high levels and remains high until the bud becomes dormancy. When *TB1* expression declines, the bud forms into a tiller or a branch [31]. Orthologs of *TB1* have been isolated from *A. thaliana* (*BRC1*), rice (*OsTB1*) and sorghum (*Sorghum bicolor*; *SbTB1*) and shown to act as tillering inhibitors [26,33,34].

Auxin is also involved in plant responses to abiotic stresses [35,36]. For example, auxin content is reduced under drought conditions [37] and the transcript expression of genes in the biosynthesis of an auxin indole-3-acetic acid (IAA), as well as some auxin-responsive genes including *Aux*/*IAA*s, is also affected by drought treatment [38,39]. *Aux/IAA* gene numbers 29, 31 and 26 from *A. thaliana*, rice and sorghum, respectively are either suppressed, or are non-responsive to drought stress [3,40–43], while some *Aux/IAA* members (e.g. *AtIAA30* from *Arabidopsis*, *SbIAA8*, *-11*, *-22*, *-23* and *-26* from sorghum, and *OsIAA6*, *-9*, *-18*, *-19* and *-20* from rice) are drought induced [3,42–45]. However, as far as we are aware, no functional analysis of any *Aux/IAA* gene in the context of drought stress has yet been reported. In this current study, *OsIAA6*, a drought-induced *Aux/IAA* gene, was overexpressed in rice and shown to improve tolerance to drought stress, while an *OsIAA6* T-DNA mutant exhibited abnormal tiller outgrowth, suggesting a role for the OsIAA6 protein in both drought stress response and the control of tiller outgrowth.

2. Materials and methods

2.1. *Overexpression of* OsIAA6 *in* PGD1:OsIAA6 *transgenic rice plants*

The predicted coding sequence of *OsIAA6* (GenBank accession number Os01g0741900) was amplified using the high-fidelity DNA polymerase PrimeStar (TaKaRa, Kyoto, Japan) with the primers *OsIAA6*_OX_F and R, before being cloned into the *pENTR/D* (Invitrogen, CA, USA). The coding sequence of *OsIAA6* was then inserted into a rice transformation vector containing the *PGD1* promoter [46] using the Gateway system (Invitrogen, Carlsbad, CA, USA). The resulting plasmid (*PGD1:OsIAA6*; Fig. S1) was introduced into rice (*O. sativa* cv. Nackdong) by *Agrobacterium tumefaciens* (strain LBA4404)-mediated transformation, as previously described [47]. All primer sequences are listed in Table S1.

2.2. *Abiotic stress and auxin treatments*

The expression of *OsIAA6* in wild-type rice plants exposed to different abiotic stress conditions was evaluated using stress treatments as previously described [48]. Briefly, rice seeds (*O. sativa* cv. Nackdong) were germinated on MS-agar medium in a growth chamber at 28 °C for 5 days. Seedlings were then transplanted into the soil and grown for 14 days in the greenhouse prior to abiotic stress treatments. Drought stress was induced by air-drying the seedlings for 2 h at 28 °C and salinity stress was imposed by incubating the seedlings in water containing 400 mM NaCl at 28 °C for 2 h. To evaluate the response of *OsIAA6* to auxin, 6-day old seedlings were grown on solid MS medium containing 100 μM NAA (1-naphthyleneacetic acid) in a growth chamber at 28 °C for 24 h.

2.3. *Measurement of chlorophyll fluorescence*

To evaluate the phenotypes of mature plants exposed to drought conditions, seedlings of transgenic and wild-type plants were initially transplanted into pots (3 cm × 3 cm × 5 cm; 4 plants per pot) containing soil (Bio-media, Kyeongju, Korea) and grown for 5 weeks in the greenhouse at 28–30 °C. Drought stress was induced by withholding water for 5 days, followed by re-watering. Photographs of the plants were taken at different time points (0, 3 and 5 days after drought treatment and +5 days after re-watering). To determine the F_v/F_m values, leaves from the 5-week old plants were harvested, placed in a dark clip (Walz, Effeltrich, Germany), dried for up to 3 h, and measured every 0.5 intervals for 3 h at 28 °C. F_v/F_m values were then measured as previously described [49,50].

2.4. *Protoplast isolation and immunoblot analysis*

In order to generate *cMYC-OsIAA6* sequence, encoding the OsIAA6 protein with a MYC peptide tag, the *OsIAA6* coding sequence was amplified with the gene specific primers *OsIAA6*-tag_F and R containing the restriction enzyme site, B*amH*I and *Not*I and ligated into the *pE3n* vector [51]. The *cMYC-OsIAA6* sequence was then inserted into a rice transformation vector containing the *GOS2* promoter [52] to drive constitutive expression, using the Gateway system (Invitrogen, Carlsbad, CA, USA). The resulting plasmid (*GOS2:cMYC-OsIAA6*; Fig. S1) was introduced into rice protoplasts as previously reported [53] but with slight modifications. Briefly, leaf sheaths from 100 rice seedlings were cut into 1–2 mm wide strips on a glass plate using a fresh sharp razor blade. The leaf sheath pieces were immediately transferred to a 15 ml digestion solution (0.6 M mannitol, 1.5% cellulase RS (Yakult, Japan), 0.75% macerozyme (Yakult, Japan), 1 mM $CaC1_2$, 0.1% bovine serum albumin, 5 mM beta-mercaptoethanol and 0.6 mM 4-morpholineethanesulfonic acid, pH 5.7) and were incubated for 4–5 h at 28 °C in the dark with gentle shaking. Filtration of the digestion solution with 70 μm and 40 μm nylon mesh (BD Bioscience, USA) to collect the protoplasts, which were then resuspended in 20 ml of a washing buffer, W5 solution (154 mM NaCl, 125 mM $CaC1_2$, 5 mM KC1, 5 mM D-glucose and 2 mM MES, pH 5.7). The pooled protoplast suspensions were centrifuged at 200 × *g* for 5 min to pellet the protoplasts, which were then resuspended in 1 ml of MMg solution (0.6 M mannitol, 15 mM $MgCl_2$ and 4 mM MES, pH 5.7). The protoplasts were quantified using a microscope (CX21; Olympus, Tokyo, Japan) and a hemocytometer. The *GOS2:cMYC-OsIAA6* and *Ubi1:GFP* constructs were then co-transfected into the isolated protoplasts using polyethylene glycol-mediated transformation [53]. After 12 h incubation at 28 °C, centrifugation was performed at 300 × *g* for 2 min for the protoplast harvest. Proteins were extracted with a protein lysis buffer (50 mM Tris–HCl pH 8.0, 100 mM NaCl, 1 mM dithiothreitol and 1% Triton X-100) and 40 μg of total protein was fractionated on a 10% SDS-PAGE gel and electrophoretically transferred to a Immobilin-P transfer membrane (Millipore, MA, USA) using a wet transfer apparatus (Hoefer, San Francisco, CA, USA). An anti-cMYC antiserum from rabbit (Santacruz Biotechnology, CA, USA) and anti-GFP antiserum from mouse (Nacalai tesque, Kyoto, Japan) were used at a dilution of 1:1000 and a 1:10,000 dilution of secondary antibodies were used (Promega, Madison, WI, USA). Signals were visualized