



E3 ubiquitin ligase SOR1 regulates ethylene response in rice root by modulating stability of Aux/IAA protein

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Edited by Mark Estelle, University of California, San Diego, La Jolla, CA, and approved March 19, 2018 (received for review November 8, 2017)

Plant hormones ethylene and auxin synergistically regulate plant root growth and development. Ubiquitin-mediated proteolysis of Aux/IAA transcriptional repressors by the E3 ubiquitin ligase SCF^{TIR1/AFB} triggers a transcription-based auxin signaling. Here we show that rice (*Oryza sativa* L.) soil-surface rooting 1 (SOR1), which is a RING finger E3 ubiquitin ligase identified from analysis of a rice ethylene-insensitive mutant *mhz2/sor1-2*, controls root-specific ethylene responses by modulating Aux/IAA protein stability. SOR1 physically interacts with OsIAA26 and OsIAA9, which are atypical and canonical Aux/IAA proteins, respectively. SOR1 targets OsIAA26 for ubiquitin/26S proteasome-mediated degradation, whereas OsIAA9 protects the OsIAA26 protein from degradation by inhibiting the E3 activity of SOR1. Auxin promotes SOR1-dependent degradation of OsIAA26 by facilitating SCF^{OsTIR1/AFB2}-mediated and SOR1-assisted destabilization of OsIAA9 protein. Our study provides a candidate mechanism by which the SOR1-OsIAA26 module acts downstream of the OsTIR1/AFB2-auxin-OsIAA9 signaling to modulate ethylene inhibition of root growth in rice seedlings.

rice | root elongation | ethylene response | Aux/IAA protein | ubiquitination

Both the plant hormones ethylene and auxin regulate root growth. Recent studies in *Arabidopsis* have portrayed an ethylene–auxin interaction in regulating root growth, in which the ethylene elevates auxin accumulation and then triggers TIR1/AFB2-mediated signal transduction (1–4). The molecular details that ethylene promotes auxin accumulation have been studied comprehensively, involving ethylene-induced WEI2/ASA-, WEI7/ASB1-, and TAA1-dependent auxin biosynthesis (5, 6) as well as PIN2- and AUX1-facilitated auxin transport (7, 8) in roots. However, the mechanism underlying auxin signaling-mediated ethylene regulation of root growth still requires elucidation.

Rice is a monocotyledonous and semiaquatic plant, and ethylene plays essential roles in the adaptive growth of rice in water-saturated environments (9–11). Previously, we have screened for mutants with altered ethylene sensitivity to identify modulators in the regulation of ethylene responses. A set of mutants with an abnormal growth of the roots in the presence of ethylene was identified and named as *mao hu zi* (*mhz*), a Chinese name with an English meaning of cat whiskers (12). Through map-based cloning, several corresponding genes have been identified, including *MHZ7/OsEIN2* (12), *MHZ6/OsEIL1* (13), *MHZ5* (14), and *MHZ4/OsABA4* (15). *MHZ7/OsEIN2* and *MHZ6/OsEIL1* encode proteins homologous to the EIN2 and EIN3 of the *Arabidopsis* ethylene signaling pathway, suggesting that the major components of ethylene signaling are conserved. Moreover, ethylene induces expressions of the carotenoid isomerase gene *MHZ5* and the ABA biosynthesis gene *MHZ4/OsABA4* to drive the metabolic flux into the ABA biosynthesis for regulation of rice root growth (14, 15). These analyses revealed that other plant hormones may modulate ethylene-regulated root growth in rice.

In this study, we report identification of *MHZ2/SOR1* from analysis of a rice root-specific ethylene-insensitive mutant *mhz2/sor1-2*. *MHZ2/SOR1* encodes an E3 ubiquitin ligase and affects root growth by modulating Aux/indole acetic acid (IAA) protein

stability. SOR1 interacts with and ubiquitinates OsIAA26, an atypical Aux/IAA protein, for degradation. OsIAA9, a canonical Aux/IAA protein, connects the SOR1-OsIAA26 signal module to the OsTIR1/AFB2-mediated auxin signaling pathway by interacting with SOR1.

Results

Identification of Rice *mhz2/sor1* Mutants. A set of rice ethylene-response mutants (*mhz*) have been identified in our previous study and the *mhz2* was analyzed in this study. The *mhz2* seedlings showed root-specific defects in ethylene-inhibited root growth at a wide range of hormone concentrations (Fig. 1*A* and *B*). The *mhz2* seedlings also display defects in gravity response (Fig. 1*A* and *SI Appendix, Fig. S1A*) and soil-surface rooting phenotypes in the field (*SI Appendix, Fig. S1B*).

We cloned the *MHZ2* gene using a map-based approach (*SI Appendix, Fig. S2*). As *MHZ2* (Os04g01160) is the same as the rice *SOR1* (16), we renamed the *MHZ2* as *SOR1*. Our mutant *mhz2* was also renamed as *sor1-2* (*SI Appendix, Fig. S2B*) following the original mutant *sor1-1* (16). Three additional mutant alleles were also identified (*SI Appendix, Fig. S2B*). *SOR1* encodes a plant-specific RING-type protein (17), containing a nuclear localization signal (NLS), a RING domain, and a von Willebrand factor type A (VWA) domain (Fig. 1*C* and *SI Appendix, Fig. S2C*). *SOR1* protein is conserved in other plant species (16) (*SI Appendix, Fig. S2D*) and is similar to *Arabidopsis* WAV3, which is an

Significance

Auxin signaling components participate in ethylene-mediated inhibition of root elongation. However, the interplay between TIR1/AFB2-auxin-Aux/indole acetic acid (IAA) signaling and ethylene response remains to be elucidated in detail. In this study, we report an E3 ubiquitin ligase soil-surface rooting 1 (SOR1), which targets a noncanonical Aux/IAA protein OsIAA26 for 26S proteasome-mediated degradation. The E3 ligase activity of SOR1 can be repressed by the canonical Aux/IAA protein OsIAA9, which is the target of OsTIR1/AFB2. Our study identifies a potential regulator that modulates auxin-mediated ethylene response at the auxin signaling level.

Author contributions: H.C. and J.-S.Z. designed research; H.C., B.M., Y.Z., S.-J.H., and X.L. performed research; S.-Y.T. and Q.X. contributed new reagents/analytic tools; H.C., S.-Y.C., and J.-S.Z. analyzed data; and H.C. and J.-S.Z. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <https://www.ncbi.nlm.nih.gov/geo> (accession no. [GSE51152](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51152) and [SRP041468](https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=SRP041468)) and BIG Data Center: CRA000688.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1719387115/-DCSupplemental.

Published online April 9, 2018.

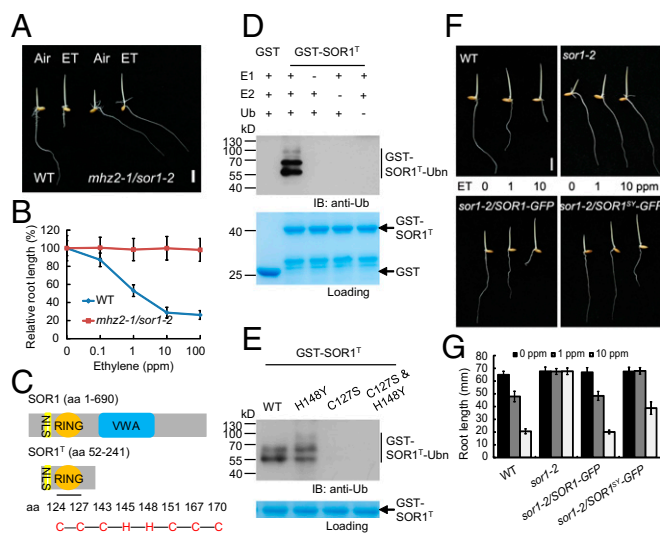


Fig. 1. Characterization of *mhz2/sor1* mutant and SOR1 protein. (A) Response of wild-type (WT) and *sor1-2* etiolated seedlings to 10 ppm ethylene (ET). (Scale bar, 10 mm.) (B) Quantification of the ethylene response of WT and *sor1-2* roots. The root lengths relative to the controls (0 ppm) are shown. Bars indicate SD ($n \geq 30$). (C) Diagrams of SOR1 and its truncated version SOR1^T used for E3 ligase activity studies. Conserved amino acids of RING domain and their positions in full-length protein are shown. (D) GST-SOR1^T protein (~45 kDa) displays E3 ubiquitin ligase activity in vitro. GST itself was used as a negative control. Coomassie Brilliant Blue (CBB) staining served as a loading control. (E) C127S mutation disrupts the E3 ligase activity of GST-SOR1^T. (F) Phenotypic analysis of *sor1-2* transformed with SOR1-GFP with or without mutations in the RING domain. SOR1^{SY} harbors C127S and H148Y double mutations. (Scale bar, 10 mm.) (G) Quantification of the root ethylene response in WT, mutant, and rescued lines shown in F. The data show mean \pm SD ($n \geq 30$).

E3 ubiquitin ligase and regulates root gravitropism by affecting auxin response in *Arabidopsis* (18). To further confirm that the SOR1 mutation was responsible for the ethylene-insensitive phenotype, the full-length genomic DNA fragment of SOR1 was transformed into the *sor1-2* mutant (SI Appendix, Fig. S2E), and the transgene-positive seedlings restored the ethylene-response phenotype and gravity response phenotype (SI Appendix, Fig. S2F). These results indicate that the Os04g01160 locus corresponds to the SOR1 gene.

We further examined the genetic relationship between SOR1 and *MHZ7/OsEIN2*, the central component of ethylene signaling in rice (12). The SOR1 mutation suppressed the short root phenotype of the *OsEIN2*-overexpressing seedlings under both normal and ethylene-treated conditions (SI Appendix, Fig. S3 A and B), suggesting that the SOR1 gene acts downstream of *OsEIN2* to regulate root growth.

SOR1 Functions as an E3 Ubiquitin Ligase in Modulating Ethylene-Inhibited Root Growth. Because proteins with a RING domain usually function as E3 ubiquitin ligases (18–20), we tested whether SOR1 also possesses E3 ligase activity. We expressed a truncated SOR1 version (amino acids 52–241) containing the RING domain, which is tagged with GST and referred to as GST-SOR1^T (Fig. 1C), since expression of the recombinant full-length SOR1 is not successful. In the presence of E1, E2, and ubiquitin, the GST-SOR1^T had the ability of self-ubiquitination (Fig. 1D). We further introduced mutations at the Cys-127 (C127S) and/or His-148 (H148Y) positions in the RING domain. The C127S mutation in the GST-SOR1^T version completely disrupted the E3 ligase activity; however, the H148Y mutation does not affect the activity (Fig. 1E). These results indicate that the conserved residue Cys-127 is required for SOR1 E3 ligase activity.

To address whether E3 ligase activity is required for the SOR1-mediated root responses to ethylene and gravity, the 35S promoter-driven SOR1-coding sequence or its mutant (SOR1^{SY}) harboring the C127S and H148Y double mutations was fused with *green fluorescent protein* (GFP) and transformed into the *sor1-2* mutant. SOR1-GFP rescued the root sensitivity to both ethylene and gravity; however, SOR1^{SY}-GFP did not rescue the ethylene-inhibited root growth at 1 ppm but partially rescued at 10 ppm ethylene, and SOR1^{SY}-GFP largely restored the gravity response (Fig. 1 F and G and SI Appendix, Fig. S3 C and D). These results indicate that the SOR1 E3 ligase activity is substantially required for the ethylene inhibition of root growth especially at lower ethylene concentration.

The Ethylene-Insensitivity of *sor1* Is Related to Alteration of Auxin Response. In *Arabidopsis*, mutations of several proteins required for auxin functions (4–8, 21) have been identified to cause defects of ethylene response phenotypes. The defects of the *sor1-2* roots in both ethylene and gravity responses suggest that SOR1 may mediate ethylene-inhibited root growth through auxin functions. We then tested whether auxin is required for ethylene response in rice roots and found that application of L-kynurenine (L-Kyn), an inhibitor of auxin biosynthesis (22), repressed the ethylene-induced inhibition of root growth (SI Appendix, Fig. S4 A and B). Furthermore, the effect of L-Kyn on ethylene-regulated root growth could be overcome by supplementation with the auxin analog naphthalene acetic acid (NAA, 20 nM) (SI Appendix, Fig. S4 C and D). These results indicate that auxin is required for ethylene inhibition of root growth.

To further examine whether TIR1-mediated nuclear auxin signaling is required for root response to ethylene, the *Mir393a*-overexpressed transgenic plants (23, 24), with the reduction of the auxin receptor gene *OsTIR1/AFB2* expression were analyzed (SI Appendix, Fig. S5A). We found that these transgenic seedling roots displayed insensitivity to ethylene at a concentration of 1 ppm (SI Appendix, Fig. S5 B and C). Furthermore, the application of inhibitors (4-Cl-PEO-IAA and auxinole) (25) blocking the interaction of the receptor and the Aux/IAA protein also reduced the ethylene inhibition of the rice roots (SI Appendix, Fig. S6). These results indicate that the auxin pathway is required for ethylene-inhibited root growth in rice, similar to previous findings in *Arabidopsis*.

We then explored whether the SOR1 mutation would change the auxin accumulation and/or sensitivity and found that *sor1-2* roots had similar auxin accumulation and similar transporter gene expression in comparison with the wild-type (WT) roots (SI Appendix, Fig. S7 A and B). Moreover, supplementation of exogenous NAA, which is able to override the effect of L-Kyn in rice roots (SI Appendix, Fig. S4 C and D) and restore the root ethylene sensitivity in mutants defective in auxin production and transport in *Arabidopsis* (6, 26), did not restore the root ethylene response and gravity response in the *sor1-2* mutant (SI Appendix, Fig. S7 C and D). The *sor1-2* roots showed resistance to lower concentrations of NAA in comparison with the wild-type roots (Fig. 2A), suggesting that SOR1 mutation may alter auxin response in rice roots.

From our microarray and transcriptome data of the root tips treated with ethylene (National Center for Biotechnology Information: GSE51152 and SRP041468), three *Aux/IAA* genes, *OsIAA26*, *OsIAA9*, and *OsIAA20*, relevant to the auxin response were identified as ethylene-responsive genes, and the ethylene-induced expression of these genes fully depended on SOR1 functions (Fig. 2B). However, only around half of auxin-induced expression of these three genes depended on the SOR1 function (Fig. 2C). These results imply that SOR1 is fully required for the ethylene response but only partially required for the auxin response at the molecular level in roots. Moreover, inhibition of auxin synthesis by L-Kyn application or repression of auxin signaling by knockdown of auxin receptor genes *OsTIR1/AFB2* also blocked ethylene-induced expression of *OsIAA26*, *OsIAA9*, and *OsIAA20* (Fig. 2D and SI Appendix, Fig. S5D). Collectively, these results suggest that

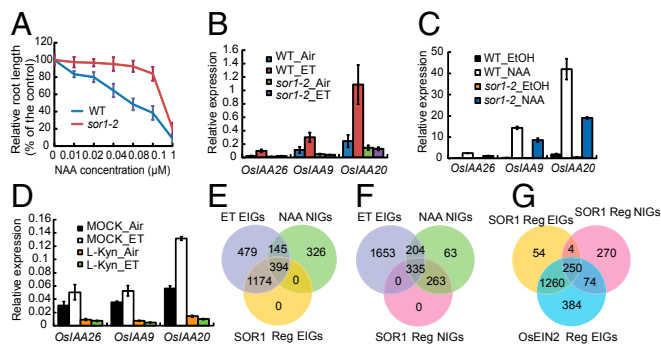


Fig. 2. *SOR1* mutation alters auxin response in rice root. (A) Quantification of the auxin response of WT and *sor1-2* roots. The rates of root growth inhibition relative to the controls (0 μ M) are shown. Bars indicate SD ($n \geq 30$). (B) Ethylene inductions of *OsIAA26*, *OsIAA9*, and *OsIAA20* fully require *SOR1* function. Relative expression of *Aux/IAA* genes in WT and *sor1-2* root tips was evaluated in response to 100 ppm ethylene. (C) Auxin inductions of these genes partially require *SOR1* function. NAA (10 μ M) was used for treatment. (D) Ethylene inductions of *OsIAA26*, *OsIAA9*, and *OsIAA20* require auxin function. The auxin biosynthesis inhibitor L-Kyn (10 μ M) was used for the treatment. The data show mean \pm SE. Four independent biological repeats were performed and analyzed. All of the mRNA levels were normalized to *OsACT1N2*. (E–G) Venn diagram shows overlaps between genes induced by NAA or by ethylene (ET) treatment and the genes related by *SOR1* or *OsEIN2* in rice root tips. The genes regulated more than twofold were used for analysis. ET EIGs, ET ethylene-induced genes; NAA NIGs, NAA-induced genes; *SOR1* Reg EIGs, *SOR1*-regulated ethylene-induced genes; *SOR1* Reg NIGs, *SOR1*-regulated NAA-induced genes; and *OsEIN2* Reg EIGs, *OsEIN2*-regulated ethylene-induced genes.

SOR1 may modulate root responses to ethylene through affecting a portion of the auxin response.

Transcriptome analysis (BIG Data Center: CRA000688) was further performed to dissect *SOR1*-modulated gene expression in root tips. We found that *SOR1* controlled $\sim 77\%$ of the *OsEIN2*-mediated ethylene-induced genes. It also mediated $\sim 70\%$ of auxin-induced genes, among which $\sim 56\%$ were ethylene-induced genes (Fig. 2 E–G). These analyses imply that *SOR1* affects ethylene response genes, among which a subset belongs to auxin response genes.

***SOR1* Interacts with and Ubiquitinates *OsIAA26*.** Since mutation of *SOR1* leads to deficiency of auxin-dependent ethylene response in roots (Fig. 2 A–D), and the reduced root responses of the *MiR393aOE* (*SI Appendix*, Fig. S5B) and *sor1-2/SOR1^{SY}-GFP* (Fig. 1F) seedlings to 1 ppm ethylene were very similar, we hypothesized that *SOR1* may be involved in the *OsTIR1/AFB2*-mediated auxin signaling pathway. There are 31 members in the rice *Aux/IAA* family, and most of these have four conserved domains, i.e., domains I–IV. However, a few of these do not have conserved domain II (27, 28). Among *SOR1*-dependent ethylene-induced *Aux/IAA* genes, the *OsIAA9* and *OsIAA20* encode *Aux/IAA* proteins with conserved domains, whereas the *OsIAA26* encodes an atypical *Aux/IAA* protein with substitutions in domain II (Fig. 3A). *OsIAA26* has canonical domain I contributing to transcriptional regulation and shows transcriptional repression activity (*SI Appendix*, Fig. S8). In the presence or absence of IAA, *SOR1* could interact with both *OsIAA26* and *OsIAA9*, but not *OsIAA20*, in a yeast two-hybrid assay. In contrast, in the presence of IAA, the auxin receptor *OsTIR1* interacted with *OsIAA20*, and the auxin receptor *OsAFB2* interacted with both *OsIAA9* and *OsIAA20* (Fig. 3A).

We further investigated whether *SOR1* can ubiquitinate *OsIAA26* and *OsIAA9*. We performed *in vitro* ubiquitination experiments using the GST-*SOR1^T* and the *OsIAA26* and *OsIAA9* proteins tagged with maltose-binding protein (MBP). While MBP-*OsIAA9* was not ubiquitinated, MBP-*OsIAA26* can be ubiquitinated by

SOR1^T, and the C127S mutation in the RING domain of *SOR1^T* disrupted *OsIAA26* ubiquitination (Fig. 3 B and C).

The association of *SOR1* with *OsIAA26* is further supported by a pull-down assay. Full-length *SOR1* proteins expressed in tobacco leaves can be pulled down by incubation with MBP-*OsIAA26* proteins (Fig. 3D). Furthermore, we employed bimolecular fluorescence complementation (BiFC) assays in tobacco leaf cells to check the interaction between *SOR1* and *OsIAA26*. The yellow fluorescence that represents a positive interaction can be detected in the combination of *OsIAA26* and *SOR1^{C127S}*, a version without E3 ligase activity (Fig. 3E). However, the combination between normal *SOR1* and *OsIAA26* did not generate a positive interaction signal, probably due to rapid degradation of the ubiquitinated proteins.

***SOR1* Targets *OsIAA26* for Degradation.** To reveal whether *OsIAA26* can be targeted directly for degradation by *SOR1*, the *OsIAA26*-GFP and *SOR1* were coexpressed in tobacco leaves to perform *in vitro* degradation assays. Results showed that *SOR1* promoted degradation of the *OsIAA26*-GFP (Fig. 4A). Moreover, the atypical *Aux/IAA* protein *OsIAA26* can be destabilized by auxin with different incubation times or with different concentrations (Fig. 4 B and C), supporting that *Aux/IAA* proteins with substitutions in domain II could also be degraded slowly by auxin treatment (29). These results indicate that *SOR1* facilitates *OsIAA26* degradation and auxin may play a positive role in this process.

We further employed a cell-free degradation assay system (30–32) to explore whether *SOR1* regulates *OsIAA26* degradation in rice. In these assays, *Escherichia coli*-expressed MBP-*OsIAA26* proteins were incubated with protein extracts from various rice materials for different times and the remaining MBP-*OsIAA26* levels were examined. We found that the loss of *SOR1* in the *sor1-2* mutant or the disruption of the *SOR1* E3 ligase activity in the *sor1-2/SOR1^{SY}-GFP* transgenic plants prevented the degradation of the MBP-*OsIAA26* protein (Fig. 4D). The reduction of auxin receptors in the *MiR393OE* transgenic plants also slightly prevented

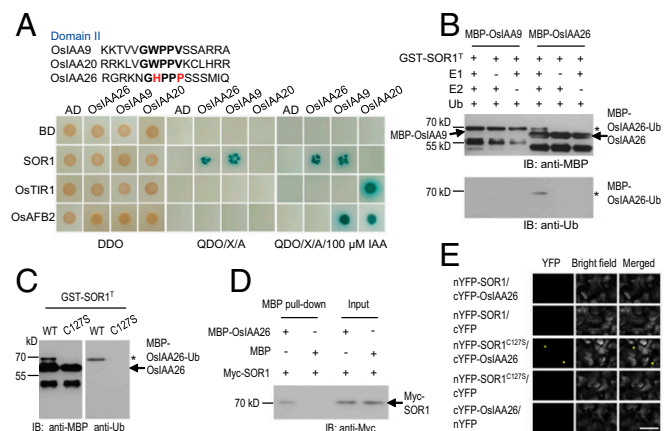


Fig. 3. *SOR1* interacts with and ubiquitinates *OsIAA26* protein. (A) *SOR1* interacts with *OsIAA26* and *OsIAA9* in a yeast two-hybrid assay. Yeast transformants were grown on the DDO media [synthetic defined (SD)–Trp–Leu] and on the DQO/X/A media (SD–Trp–Leu–His–Ade plus X- α -gal and aureobasidin A) with or without 100 μ M IAA. Greenish blue indicates positive interactions. *OsIAA9* and *OsIAA20* are canonical *Aux/IAA* proteins with a conserved domain II. *OsIAA26* is a noncanonical *Aux/IAA* protein. Interactions of the three *Aux/IAA* proteins with auxin receptors *OsTIR1* and *OsAFB2* were also tested. (B) The truncated *SOR1* version containing the RING domain (GST-*SOR1^T*) ubiquitinates *OsIAA26*, but not *OsIAA9*. Asterisks indicate ubiquitinated MBP-*OsIAA26* proteins. (C) Ubiquitination of *OsIAA26* depends on the intact RING finger of *SOR1*. (D) *SOR1* associates with *OsIAA26* in a pull-down assay. The assay was performed using MBP-*OsIAA26* recombinant protein and extracts were prepared from tobacco leaves expressing Myc-*SOR1* protein. (E) *SOR1^{C127S}*, but not *SOR1*, can be detected to interact with *OsIAA26* in the BiFC assay. Yellow fluorescence indicates positive interactions. (Scale bar, 50 μ m.)

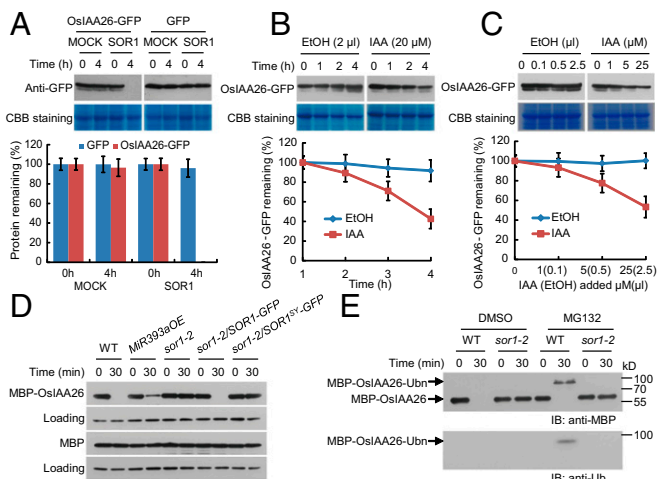


Fig. 4. OsIAA26 is a substrate of SOR1. (A) OsIAA26-GFP protein levels in the absence or presence of SOR1. Crude plant protein extracts from tobacco leaves coexpressing SOR1 and OsIAA26-GFP or GFP were incubated at 30 °C for degradation assays. Coomassie Brilliant Blue (CBB) staining served as a loading control. Three independent biological repeats were performed and analyzed, and the relative protein levels were measured using ImageJ software. (B) OsIAA26-GFP protein levels in response to IAA at different time points. Ethanol solvent (EtOH) was used as a control. (C) OsIAA26-GFP protein levels in response to different concentrations of IAA. Crude plant protein extracts from tobacco leaves expressing OsIAA26-GFP were incubated with IAA for 4 h. (D) Stability of MBP-OsIAA26 in protein extracts from various indicated rice seedling roots. *E. coli*-expressed MBP-OsIAA26 proteins were incubated with different plant protein extracts prepared from indicated materials. MBP itself was used as a control. (E) Analysis of MBP-OsIAA26 ubiquitination in protein extracts prepared from WT and *sor1-2* with or without MG132 (100 μ M) treatment. DMSO was used as a control. MBP-OsIAA26-Ubn indicates ubiquitinated MBP-OsIAA26.

MBP-OsIAA26 degradation (Fig. 4D). We further used the MG132 to inhibit the 26S proteasome-mediated protein degradation and checked the MBP-OsIAA26 ubiquitination status. We found that, in the presence of MG132, the MBP-OsIAA26 is polyubiquitinated in the WT protein extracts but not ubiquitinated in the *sor1-2* extracts (Fig. 4E). These results indicate that SOR1 is responsible for the MBP-OsIAA26 ubiquitination and facilitates its degradation by the 26S proteasome pathway, and auxin receptors OsTIR1/AFB2 also play some roles in the OsIAA26 degradation process.

OsIAA9 Interacts with SOR1 and Inhibits Its E3 Ligase Activity. From the above experiments, we found that SOR1 could interact with both OsIAA26 and OsIAA9 in the yeast two-hybrid assays (Fig. 3A); however, the SOR1 could not ubiquitinate OsIAA9 (Fig. 3B). We further studied the interaction of SOR1 and OsIAA9 by BiFC assay, and found that they could also associate (Fig. 5A). However, unlike the case for SOR1 and OsIAA26, the interaction signal between SOR1 and OsIAA9 was not altered when SOR1 had the E3 ubiquitin ligase activity. Using the pull-down assays, we further found that OsIAA9, different from OsIAA26, interacted with truncated SOR1 version SOR1^T (Fig. 5B and *SI Appendix*, Fig. S9), and this interaction required an intact RING finger (*SI Appendix*, Fig. S9). These results indicated that OsIAA9 associates with SOR1 at the RING domain, possibly affecting the E3 ubiquitin ligase activity of SOR1.

To test whether OsIAA9 would affect SOR1 E3 ligase activity, we performed *in vitro* ubiquitination assays, and found that the OsIAA9 can inhibit the E3 ubiquitin ligase activity of SOR1 and thus prevent SOR1-mediated OsIAA26 ubiquitination (Fig. 5C). We further tested the OsIAA9 effect on SOR1-mediated OsIAA26 degradation in *in vitro* degradation assays using tobacco leaves transiently coexpressing SOR1, OsIAA26, and OsIAA9, and found that OsIAA9 reduced the SOR1-mediated degradation of OsIAA26

(Fig. 5D). When the auxin IAA was included in the assay, the OsIAA9-dependent stabilization of OsIAA26-GFP was affected (Fig. 5E). Moreover, we found that OsIAA26 protein was more stable in OsIAA9-overexpressed rice than in WT or OsIAA20-overexpressed rice (Fig. 5F–H). These results indicate that the OsIAA9 can protect OsIAA26 from SOR1-mediated degradation by inhibiting the E3 ligase activity of SOR1.

OsIAA9 Degradation Is Mediated by OsTIR1/AFB2 and Facilitated by SOR1. Because OsIAA9 is a canonical Aux/IAA protein (Fig. 3A), and OsIAA9 regulates OsIAA26 stability (Fig. 5D, E, and H), we propose that auxin may regulate the SOR1-dependent degradation of OsIAA26 through possible recruitment of the OsIAA9 protein from SOR1 to OsTIR1/AFB2 for ubiquitination and degradation. Pull-down assays were performed and the results showed that the interaction between OsIAA9 and auxin receptor OsAFB2 is enhanced in the presence of NAA (Fig. 6A).

We also examined the stability of OsIAA9 when incubated with protein extracts from various rice materials. The MBP-OsIAA9 can be degraded in the WT protein extracts. However, when the auxin receptor gene *OsTIR1/AFB2* expression was reduced in the *Mir393aOE* extracts, the MBP-OsIAA9 protein showed increased stability (Fig. 6B). Similarly, the MBP-OsIAA9 in the *sor1-2* extracts also showed increased longevity (Fig. 6B). In the *sor1-2/SOR1-GFP* plant extracts, the MBP-OsIAA9 degradation was recovered. It is interesting to note that when the SOR1 E3 ligase activity was disrupted in the *sor1-2/SOR1^{SY}-GFP*, the MBP-OsIAA9 degradation remained (Fig. 6B). Together, these results indicate that degradation of OsIAA9

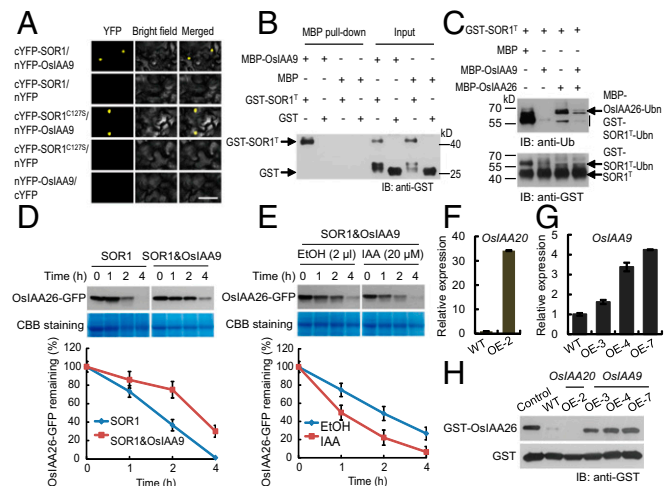


Fig. 5. OsIAA9 protects OsIAA26 from SOR1-mediated degradation. (A) SOR1 interacts with OsIAA9 in a BiFC assay. Yellow fluorescence indicates positive interactions. (Scale bar, 50 μ m.) (B) The truncated SOR1 version containing the RING domain (GST-SOR1^T) interacts with MBP-OsIAA9 in a pull-down assay. (C) OsIAA9 inhibits the E3 ligase activity of GST-SOR1^T *in vitro*. MBP was used as a control. (D) OsIAA9 reduces SOR1-mediated degradation of OsIAA26. Assays were performed using crude plant protein extracts from tobacco leaves coexpressing SOR1 and OsIAA26-GFP, with or without OsIAA9. Coomassie Brilliant Blue (CBB) staining served as a loading control. Three independent biological repeats were performed and analyzed, and the relative protein levels were measured using ImageJ software. (E) Degradation of OsIAA26 in the presence of both SOR1 and OsIAA9 in response to auxin. Assays were performed using protein extracts from tobacco leaves coexpressing SOR1, OsIAA26-GFP, and OsIAA9. Ethanol solvent (EtOH) was used as a control. (F–G) Real-time qPCR analysis for expression of *OsIAA20* and *OsIAA9* in their corresponding overexpressed transgenic lines. The mRNA levels were normalized to *OsACTIN2*. (H) Stability of GST-OsIAA26 in protein extracts from indicated transgenic rice seedling roots. *E. coli*-expressed GST-OsIAA26 proteins were incubated with plant protein extracts prepared from indicated materials. GST itself was used as a control.

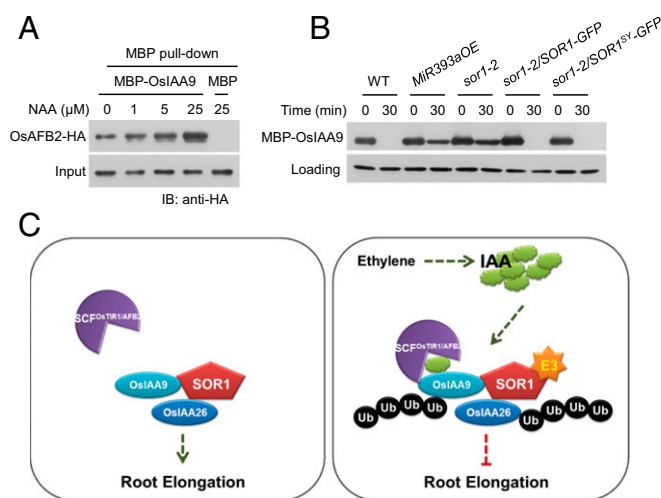


Fig. 6. Regulation of OsIAA9 stability and a working model for SOR1. (A) OsIAA9 interacts with OsAFB2 in the presence of auxin. Crude protein extracts prepared from tobacco leaves expressing OsAFB2 were used to do MBP pull-down assays. (B) Stability of MBP-OsIAA9 in protein extracts from various rice materials. *E. coli*-expressed MBP-OsIAA9 proteins were incubated with different plant protein extracts prepared from indicated materials. MBP itself was used as a control. (C) A working model for SOR1 functions in auxin-mediated ethylene response. In the absence of ethylene and auxin (Left), OsIAA9 inhibits the E3 ligase activity of SOR1 and thus stabilizes OsIAA26, facilitating normal root elongation. In the presence of ethylene and auxin (Right), ethylene-triggered IAA accumulation enhanced OsAFB2-mediated degradation of OsIAA9, with the help of SOR1. OsIAA9 removal released the E3 ligase activity of SOR1 and then promoted SOR1-mediated degradation of OsIAA26. The normal root elongation is thus repressed.

protein is mediated by auxin receptor OsTIR1/AFB2 in rice, and OsIAA9 degradation is also facilitated by the presence of SOR1; however, the E3 ligase activity of SOR1 is not required for OsIAA9 degradation.

Alteration of *OsIAA9* and *OsIAA26* Expression Affects Ethylene-Regulated Root Growth. Because OsIAA26 is ubiquitinated by SOR1 for degradation and OsIAA9 affects this process (Figs. 3–5), we further tested whether OsIAA26 and OsIAA9 have any roles in ethylene inhibition of root growth. The *OsIAA26*- and *OsIAA9*-overexpressed transgenic plants were generated and three higher expressers for each transgene were analyzed (SI Appendix, Fig. S10A and B). Both the *OsIAA26*- and *OsIAA9*-overexpressed seedlings showed reduced ethylene sensitivity in roots than the wild-type seedlings did (SI Appendix, Fig. S10C and D). Furthermore, mutations of *OsIAA9* and *OsIAA26* were generated by genome editing using the CRISPR/Cas9 system to further study their roles in root ethylene response (SI Appendix, Fig. S11A). Both *Osiaa9* and *Osiaa26* mutants showed similar ethylene response to WT (SI Appendix, Fig. S11B and C). However, mutation of the *OsIAA26* protein caused root wave growth in the presence of 10 ppm ethylene (SI Appendix, Fig. S11D). These results suggest that both OsIAA26 and OsIAA9 may function in the reduction of ethylene effects on root growth, and other Aux/IAA proteins may also be involved in ethylene-regulated root growth.

Discussion

E3 ubiquitin ligase-mediated protein degradation plays a central role in ethylene and auxin signaling. The stability of ethylene signaling protein EIN2 is modulated by the two F-box proteins ETP1/2 (33), while the EIN3 is regulated by another two F-box proteins EBF1/2 (34–36). The auxin receptor is a small family of related F-box proteins (37), which triggers nuclear auxin signaling by targeting transcriptional repressor Aux/IAA proteins for degradation (38). Here we find that SOR1, a

RING finger E3 ubiquitin ligase (Fig. 1C–E), ubiquitinates a non-canonical Aux/IAA protein OsIAA26 for degradation (Figs. 3 and 4), and the OsTIR1/AFB2-auxin-OsIAA9 signaling affects this process (Figs. 5 and 6A and B). Our study identifies a potentially unique component of auxin signaling to regulate ethylene-inhibited root growth.

Noncanonical Aux/IAA proteins can regulate auxin-related phenotypes in *Arabidopsis* (39); however, their protein stability cannot be directly modulated by SCF^{TIR1} E3 ubiquitin ligase (28, 40). In the present study, we discovered that SOR1 ubiquitinates a noncanonical Aux/IAA protein OsIAA26, for degradation (Figs. 3 and 4). In the *in vitro* assay, the OsIAA26 appeared to be monoubiquitinated by the truncated SOR1 version SOR1^T (Fig. 3B and C). However, in the rice protein extracts, the OsIAA26 can be polyubiquitinated by full-length SOR1 as judged from the protein size (Fig. 4E). This difference may be due to the different assay systems used or different SOR1 versions involved. Interaction assays showed that OsIAA26 interacts with full-length SOR1 but not with the truncated SOR1^T (Fig. 3D and SI Appendix, Fig. S9); however, the SOR1^T still ubiquitinates OsIAA26. This fact may suggest that the extra sequences outside the RING finger likely facilitate the ubiquitination process. Other possibilities could not be excluded.

Moreover, auxin also regulates the OsIAA26 protein degradation likely by OsTIR1/AFB2 receptors through SOR1, because in the *MIR393aOE* plants with reduced *OsTIR1/AFB2* expression, the OsIAA26 can be partially stabilized (Fig. 4D). This fact may be explained by the stabilization of OsIAA9 in *MIR393aOE* plants, which leads to SOR1 inactivation and OsIAA26 stabilization (Figs. 5C, D, and H and 6A and B). It is worth noting that among six noncanonical Aux/IAA proteins in rice, we only studied the OsIAA26, whose gene expression is induced by ethylene and the induction fully depends on SOR1 (Fig. 2B). Other noncanonical and/or canonical Aux/IAA proteins should be examined to test whether they are also the substrates of SOR1. It is also interesting to study whether SOR1 modulates other components of ethylene/auxin signaling pathways.

Aux/IAA family proteins usually function as transcriptional repressors (41, 42) and bind to ARF transcription factors to repress their transcriptional activity (43). Previous study has shown that HaIAA27 (44), one of the Aux/IAA proteins in sunflower, can interact with HaHSFA9, a non-ARF-type transcription factor, and represses its transcriptional activity. In this study, we show that OsIAA9, a canonical Aux/IAA protein (Figs. 3A and 6A and B), can function as a repressor for inhibition of the E3 ligase activity of SOR1 (Fig. 5C). Considering that SOR1 can interact with both OsIAA26 and OsIAA9 but only ubiquitinate the former, the OsIAA9 may play some inhibitory roles in the SOR1 E3 ubiquitin ligase activity toward its substrate OsIAA26. This conclusion is further supported by the fact that OsIAA9 protects OsIAA26 from degradation (Fig. 5D and H). This finding widens our understanding of the specific roles of each Aux/IAA protein. The mechanism involving the interplay of the three proteins is still not clear and requires further investigation.

It should be noted that although SOR1 cannot ubiquitinate OsIAA9 (Fig. 3B), it assists OsIAA9 degradation, and this assistance does not require E3 ubiquitin ligase activity (Fig. 6B). Considering that OsIAA9 can also interact with OsAFB2 in the presence of auxin and be targeted by these receptors for degradation (Figs. 3A and 6A and B), SOR1 may act as a facilitator for OsIAA9 degradation in this process. The VWA domain of SOR1 may function to recognize and promote ubiquitinated OsIAA9 for 26S proteasome-mediated degradation. This speculation may be supported by a previous study. Verma et al. (45) reported that, during delivery of ubiquitinated substrates to the 26S proteasome, deubiquitination and degradation of substrates delivered by Rad23 require a facilitator activity within the VWA domain of Rpn10. Further studies should clarify the possible functions of the SOR1 VWA domain in the process of Aux/IAA degradation.

While our study finds that ethylene affects root growth through SOR1-mediated auxin signaling, ethylene may also inhibit the second rate-limiting step at the *YUCCA* gene of auxin biosynthesis

to confer auxin levels suboptimal for root cell elongation as in the cases of *Brachypodium* (46) and rice (47), providing an alternative mechanism of ethylene–auxin interaction.

From our current studies, we propose a model in which SOR1 acts as an E3 ligase in regulating the rice root response to ethylene by modulating Aux/IAA protein stability (Fig. 6C). In the absence of ethylene, the canonical Aux/IAA protein OsIAA9 associates with SOR1 and blocks the SOR1-mediated ubiquitin-related degradation of OsIAA26. In the presence of ethylene, ethylene-triggered auxin accumulation facilitates the interaction of the auxin receptors OsTIR1/AFB2 with OsIAA9, leading to OsIAA9 degradation. A decrease of the OsIAA9 protein releases the E3 ligase activity of SOR1, thereby targeting OsIAA26 for degradation via the ubiquitin/26S proteasome pathway.

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Materials and Methods

Details of the experimental programs for ethylene response assay, quantitative real-time PCR analysis, recombinant protein purification, yeast two-hybrid assay, pull-down assay, BiFC assay, in vitro ubiquitination assay, and cell-free degradation assay are provided in *SI Appendix, SI Materials and Methods*. The primers used in this study are listed in *SI Appendix, Tables S1 and S2*.

ACKNOWLEDGMENTS. We thank M. Y. Zhu (Zhejiang University) and M. Y. Zhang [South China Botanical Garden, Chinese Academy of Sciences (CAS)] for providing the seeds of *MIR393OE*; K. Hayashi (Okayama University of Science) for providing the chemicals PEO-IAA, 4-Cl-PEO-IAA, and auxinole; and J. F. Chu (Center for Plant Hormone Determination, Institute of Genetics and Developmental Biology, CAS) for IAA measurement. This work is supported by the National Natural Science Foundation of China (Grant 31530004), the 973 Project (Grant 2015CB755702), and the State Key Laboratory of Plant Genomics.

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