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SHR overexpression induces the formation of supernumerary cell layers with cortex cell identity in rice

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ABSTRACT

The number of root cortex cell layers varies among plants, and many species have several cortical cell layers. We recently demonstrated that the two rice orthologs of the Arabidopsis *SHR* gene, *OsSHR1* and *OsSHR2,* could complement the *A. thaliana shr* mutant. Moreover, *OsSHR1* and *OsSHR2* expression in *A. thaliana* roots induced the formation of extra root cortical cell layers. In this article, we demonstrate that the overexpression of *AtSHR* and *OsSHR2* in rice roots leads to plants with wide and short roots that contain a high number of extra cortical cell layers. We hypothesize that SHR genes share a conserved function in the control of cortical cell layer division and the number of ground tissue cell layers in land plants.

1. Introduction

A vast majority of plant species, with the exception of some halophyte species, presents only one layer of endodermis (Inan et al., 2004), and the number of root cortex cell layers varies greatly among species as well as between root types in the same plant. For instance, *A. thaliana* roots have two cortex cell layers, whereas rice contains one to more than 10 cell layers, depending on the root type and its development (Coudert et al., 2010; Pauluzzi et al., 2012). In the Arabidopsis root, endodermis and cortex tissues arise from an asymmetric periclinal division of a single initial cell that is called the cortical endodermal initial daughter (CEID) (Dolan et al., 1993). In rice and several species with multiple cortex cell layers, the formation of the cortex follows a process fairly similar to that described for *A. thaliana*, except for the occurrence of multiple cell divisions of a single or several CEIDs. For example, in rice, cortex cell layers may be produced following the repetitive periclinal cell divisions of a single CEID or by the divisions of several independent CEIDs (Coudert et al., 2010; Pauluzzi et al., 2012).

At the molecular level, CEID division is controlled by *SHR* and *SCR* genes, which encode two transcription factors of the GRAS family (Cui et al., 2007; Helariutta et al., 2000; Wysocka-Diller et al., 2000). *SHR* is expressed in the stele, and its protein moves outward to the CEID where it activates *SCR*. Both proteins induce CEID division through the activation of CYCLIND6;1 to separate the clonally related cortex and endodermis cell layers (Sozzani et al., 2010). Whereas SCR and SHR are both needed to induce CEID division, SHR is also needed to specify endodermis identity (Helariutta et al., 2000). SHR is believed to be both necessary and sufficient for endodermal cell specification, and its interaction with SCR prevents the extra-movement of SHR, thereby limiting the number of endodermal cell layers to one (Cui et al., 2007). *SHR* overexpression experiments in *A. thaliana* and the observation of increased SHR movement resulting from the reduction of *SCR* expression demonstrate that SHR controls the frequency of CEID division in *A. thaliana* (Helariutta et al., 2000; Nakajima et al., 2001). Moreover, based on expression of SCR, the presence of suberin and the immunolocalization of cell wall antibodies, these experiments concluded that these supernumerary cell layers have an endodermal cell identity (Helariutta et al., 2000; Nakajima et al., 2001), suggesting that SHR may control the number of endodermal cell layers. In our work, root expression of *OsSHR1* or *OsSHR2,* which are the two rice *SHR* orthologs, increased the root ground tissue cell layer number; this phenomenon was correlated with SHR movement (Wu et al., 2014). We demonstrated that, in all cases, the overexpression of *AtSHR* or expression of *OsSHR1* and *OsSHR2* in *A. thaliana* induced the formation of supernumerary cortex cell layers but not, as previously suggested, the formation of extra endodermal cell layers. These results confirmed the hypothesis that the movement/ectopic expression of

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SHR controls cortex formation (Wu et al., 2014). Therefore, we proposed a model in which the formation of multiple layers of cortex in plants that relies on SHR movement.

In this paper, we tested the predictions of our model by increasing the expression of *AtSHR* and *OsSHR2* in rice to confirm the likelihood that *SHR* regulates the cortex cell layer number in plants.

2. Materials and methods

2.1. Plant material and growth conditions

The growth conditions of *Nipponbare* (*Oryza sativa* L.) plants are described in Sallaud et al. (2003). The transgenic lines used in this study (OE *AtSHR* (n=265), OE *OsSHR2* (n=290) and the control lines (n=90)) were made in the laboratory and analyzed at T0 generation.

2.2. Constructs and plant transformation

The *Agrobacterium tumefaciens* EHA105 strain, which harbors the pCAMBIA 5300 vector (CAMBIA, Canberra, Australia) as a binary vector, was used for the transformations. The binary vectors comprised a T-DNA cassette containing of the SHR CDS sequence driven by the ubiquitin promoter of maize. The CDS sequence (1596 bp) of the *SHR* gene (At4g37650) was cloned in the pCAMBIA 5300 vector (CAMBIA, Canberra, Australia) by the Genscript company (Piscataway, NY state, US) using *Xma*I/*Kpn*I restriction sites, and the of the OsSHR2 CDS sequence (OsO3g31880) (1812 pb) was cloned in the lab using *Xma*I restriction sites. Empty vector and both *AtSHR*- and *OsSHR2*-containing vectors were then transferred into *Agrobacterium tumefaciens* EHA 105 strain (Hood et al.) by the freeze–thaw transformation method (Chen et al., 1994). The three constructs were used to transform embryogenic calli prepared from rice mature seed embryos (Sallaud et al., 2003). The regenerated hygromycin-resistant plants, overexpressing *AtSHR*, *OsSHR2* and control lines were cultivated on Murashige and Skoog agar medium containing 50-mg/l hygromycin. Transgenic T0 seedlings were phenotyped directly in tubes. Samples of roots and leaves were then fixed, and the plants were transplanted in soil in a greenhouse.

2.3. Fresh root sectioning

Root tips were cut with a sharp blade and placed parallel, as described in Henry et al. (2016); then, they were embedded in a drop of 3% melted agarose (50 °C). Patches containing root tips were inserted into 3×1×1-cm wells filled with 3% melted agarose. After solidification, the blocks were resized and glued onto a vibratome plate to be sliced. The parameters of the vibratome (Hm650v (Thermo Scientific Microm)) were as follows: speed: 30; frequency: 70; amplitude: 0.8; and thickness: 60 µm. Sections were transferred into chamber slides (Lab-teak 177402) for immunostaining or onto slides humidified with 1X phosphate-buffered saline (PBS, Sigma-Aldrich P3813) for direct observation.

2.4. Immunolocalization with cell wall antibodies

Sections placed into chamber slides (3 per chamber) were first rinsed with 0.1-M glycine supplemented with 1X PBS and then twice with 1X PBS for 10 min each. A PBS solution containing 5% bovine serum albumin (BSA, Thermo Fisher 37520) was added overnight at 4 °C under agitation. Primary antibodies diluted 1/10 in blocking solution were applied overnight at 4 °C under agitation. Before the application of the secondary antibody, the sections were rinsed 3 times with PBS 1X for 10 min each. The secondary antibody was coupled to a fluorophore (Alexa 546 anti-mouse antibody (Invitrogen A11060)) and diluted 1/500 in blocking buffer. Root sections were incubated for 2 h under agitation and then rinsed 3 times with 1X PBS under agitation for 10 min each. The chambers were then removed from the glass substrate, and a few drops of Mowiol mounting media (Sigma-Aldrich 81381) were added. Finally, a coverslip was added to each glass slide, and the slides were allowed to dry for 36 h at 4 °C in the dark; numbers of lines analyzed for OE *AtSHR* (n=12), OE *OsSHR2* (n=9) and control (n=10).

2.5. Immunolocalization with AtSHR- and OsSHR2-specific antibodies

Radicles were grown on petri dishes, as described above. Roots were sampled at the 6-day-old stage, fixed in 4% paraformaldehyde in 1X PBS (pH=7.4) overnight at 4 °C, and rinsed twice with 1X PBS. The fixed tissues were dehydrated in ethanol, cleared in Histochoice Clearing Agent (HistoClear, Sigma Aldrich), and embedded in Paraplast (Fisher). Tissue sections (6 µm thick) were obtained using a Leica RM2255 microtome and mounted on Superfrost Plus slides (Fisher). Immunolocalizations were performed at Histalim Company (Montpellier, France) using a Ventana Medical Systems Discovery XT automated immunostainer. After deparaffinization, the samples were treated with trypsin-based antigen retrieval solution. Then, primary antibodies were applied at 1:100 dilution. Antibodies for actin (AS13 2640, AGRISERA) and the Golgi apparatus (AS08 325, AGRISERA) antibodies were used to control the sample quality. AtSHR and OsSHR2 were detected using specific antibodies produced in sheep and rabbit, respectively. The AtSHR antibody was kindly provided by R. Swarup (Nottingham University UK), whereas the OsSHR2 antibody was obtained from rabbit by Eurogentec Company using the synthetic peptide CSPPDQPHKSYPSSRG. The pre-diluted universal secondary antibody (n. 760–4205; Ventana Medical Systems) was then added. Hematoxylin was used as a counterstain. Immunoreactive sites were visualized using a diaminobenzidine tetrahydrochloride preparation (DAB, Sigma). Control experiments were performed similarly, but without the first antibody step and diluted, as described earlier. Immunolocalization was performed in two independent lines for OE OsSHR2 and AtSHR lines and 3 for Nipponbarre control lines.

2.6. Quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from 200 mg of 5-week-old roots and leaves of transgenic plants (RNA Isolation Kit, Macheray and Nagel, Germany). cDNA synthesis was performed with M-MLV RT (Pomega, US) with oligo dT, according to the manufacturer's recommendations. Primer pairs were designed to amplify fragments of *AtSHR* and the *OsEXP* housekeeping gene (Os06g11070). The following primers were used for real-time PCR: AtSHR_Fwd GTTTTCCAAGGACGAGCAAC; AtSHR_Rev: CACAAGCCACAAGATCAACG; xExp_Fwd: CGGTTAG CTAGAGTTCATGTGAGA; Exp_Rev: ATTGGAGTAGTGGAGTGCCA AA. Quantitative RT-PCR was performed using SYBR green master mix (Applied Biosystems), and the results were normalized against EXP expression. The experiment was repeated twice using two biological replicates.

2.7. Confocal microscopy

Immunolocalized sections were observed with a confocal microscope. Confocal images were obtained using a Zeiss LSM 510 multiphoton microscope or confocal Leica SP8 at a magnification of 40× for radicles. The cell walls were first visualized via autofluorescence using a 720-nm (Chameleon) or 405-nm laser. The secondary antibody—Alexa 546 anti-mouse—was then visualized using a He/Ne laser with a wavelength of 543 nm or a laser with a wavelength of 561 nm.