BIOLOGIA PLANTARUM 62 (4): 732-740, 2018 DOI: 10.1007/s10535-018-0714-3

OsNOX3, encoding a NADPH oxidase, regulates root hair initiation and elongation in rice

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Abstract

Root hairs play important roles in plant nutrient and water acquisition. To better understand the genetic mechanism controlling root hair development in rice (*Oryza sativa* L.), a rice mutant with root hair defects was isolated and characterized. Cryo-scanning electron microscope showed that the density and length of root hairs in the mutant were significantly reduced compared to the wild type (WT). Map-based cloning and complementation test revealed that the mutation occurred in a NADPH oxidase gene *OsNOX3* (LOC_Os01g61880). The *OsNOX3* displays high sequence similarity with the previously characterized *NOX* genes *RTH5* in maize and *RHD2* in *Arabidopsis*, which play critical roles in root hair development. Expression pattern analysis indicated that *OsNOX3* was expressed in various tissues throughout the plant with high expression in roots and root hairs. Subcellular localization analysis confirmed that OsNOX3 was located on the plasma membrane. Staining assays showed that the content of superoxide and hydrogen peroxide were significantly reduced in root hair tips of *Osnox3* when compared to WT. Our results showed critical roles of OsNOX3 in regulating both root hair initiation and elongation in rice, which is similar to RTH5 but different from RHD2, confirming the difference of genetic mechanisms regulating root hair morphogenesis in monocot and dicot plants.

Additional key words: gene expression, map-based cloning, *Oryza sativa*, SEM, subcellular localization.

Introduction

Root hairs are tubular outgrowths of specialized epidermis cells and comprise up to 77 % of the surface area in crop roots (Parker *et al*. 2000). They are important for efficient absorption of water and mineral nutrients and serve as a site of interaction with the abiotic and biotic rhizosphere (Gilroy and Jones 2000). Root hair development is divided into three stages: cell fate specification, root hair initiation, and elongation (Schiefelbein 2000, Foreman and Dolan 2001).

Epidermal cells are classified into hair-forming cells (trichoblast) and non-hair-forming cells (atrichoblast), and root hairs are formed as specialized projections from modified trichoblasts in the elongation zone of a root (Row and Reeder 1957). Three types of epidermal patterning have been described in plants: *1*) all root epidermal cells appear to be capable of producing root hairs, *2*) the epidermis is composed of two types of cells of different lengths and only the shorter cells can produce root hairs, and *3*) a striped pattern, where the position of epidermal cells in a cleft between two underlying cortical cells determines the formation of root hairs (Dolan 1996, Marzec *et al*. 2014). The molecular mechanisms

Submitted 14 November 2017, *last revision* 18 April 2018, *accepted* 15 May 2018.
Abbreviations: EMS - ethylmethanesulfonate; GFP - green fluorescent protein; MS - Murashige and Skoog; ROS - reactive oxygen species; RT-PCR - reverse transcription PCR; SEM - scanning electron microscope; SSR - simple sequence repeat; STS - sequence tagged site; TM - transmembrane; WT - wild type.
Acknowledgments: This work was supported by the National Natural Science Foundation of China (grant No. 31371595), the Zhejiang Provincial Natural Science Foundation of China (LY17C020002), the Natural Science Foundation of Ningbo (2017A610291, 2017A610292), and the K.C. Wong Magna Fund at Ningbo University. The first two authors contributed equally to this work.
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underlying the third type of root hair patterning, which is position-dependent, have been extensively studied in the model species *Arabidopsis* (Grebe 2012, Salazar-Henao *et al.* 2016). There are variations in cell division patterns and cell expansion of root hairs among grasses (Marzec *et al.* 2015, Dolan 2017). In *Zea mays*, the last division of surface cells produces two equally sized daughter cells, both of which can produce root hairs (Clowes 2000). In *Brachypodium distachyon,* root hairs develop from smaller products of asymmetric cell division (Kim and Dolan 2011), whereas in *Oryza sativa* and *Hordeum vulgare*, a symmetrical shootward-last division was observed and after root hair initiation, atrichoblasts elongate more than trichoblasts (Kim and Dolan 2011, Marzec *et al*. 2013).

To date, thirteen genes related to root hair development have been reported in rice. Six of them are involved in cell wall modification: three cell wall loosening (expansin genes *OsEXPA17*, *OsEXPA30*, and *OsEXPA8)*) and one β-expansin gene (*OsEXPB5*) play crucial roles in mediating cell wall extension during root hair morphogenesis (Won *et al*. 2010, Yu *et al*. 2011, Ma *et al*. 2013). Further, *OsCSLD1*, a cellulose synthase-like D1 gene, is essential for the synthesis of polymers for the fast-growing primary cell wall at the root hair tip (Kim *et al*. 2007) and a xyloglucan 6-xylosytransferase (*OsXXT1*) is required for epidermal cell wall strength (Wang *et al*. 2014a). Moreover, *OsSNDP1* encoding a Sec14-nodulin domain-containing protein (Huang *et al*. 2013a), *OsFH1* encoding a rice formin homology (Huang *et al*. 2013b), *OsAPY1* encoding an apyrase protein (Yuo *et al*. 2009), and *OsRHL1* encoding a root hair-specific basic helix-loop-helix (bHLH) transcription factor (Ding *et al*. 2009) are required for root hair elongation. More recently, a putative mannosyl-oligosaccharide glucosidase (*OsMOGS*; Wang *et al*. 2014b) and two WUSCHEL-related homeobox genes *WOX11* and *OsWOX3A* (Yoo *et al*. 2013, Cheng *et al*. 2016) were reported to be involved in both initiation and elongation of root hairs in rice.

Compared with dicotyledonous species, however, our knowledge on molecular mechanisms controlling root hair development in monocots is still limited. With the aim to determine if rice NADPH oxidase 3 gene (*OsNOX3*) is important for root hair development we isolated a new short-root-hair mutant and identified the causal gene by map-based cloning and complementation assays.

Materials and methods

Plants, growth conditions, and mutant screening: The *Osnox3* mutant was isolated from an ethyl methanesulfonate (EMS)-mutagenized population of rice (*Oryza sativa* L. var. *indica* cv. Kasalath). For hydroponic experiments, seeds were directly grown in standard rice culture solution with a pH of 5.5 (Yoshida *et al*. 1976) after germination in water. Plants were grown in a greenhouse under a photosynthetic photon flux density of approxi-mately 200 μmol(photons) m^{-2} s^{-1}, a 12-h photo-period, an air humidity of about 70 %, and day/night temperatures of 32/22 °C. A mapping population was generated from the cross between *Osnox3* mutant and the wild type (WT) rice var. *japonica* cv. Nipponbare.

Morphological analysis: The germinated seeds were grown in rice culture solution. The root hairs on the seminal roots were examined under a stereomicroscope (*DC 300*, *Leica*, Nussloch, Germany) 7 d after germination. Plant height and seminal root length were measured. The number and length of all emerged lateral roots on seminal roots were analyzed with *WinRhizo* software (*Regent Instruments*, Quebec, Canada). For root hair measurement, seeds were surface-sterilized in 10 % (m/v) sodium hypochlorite for 15 min and thoroughly washed three times with sterile distilled water. The seeds were subsequently plated on Murashige and Skoog (MS) medium with 1 % (m/v) *Phytagel* (*Sigma*, St. Louis, USA) and grown for 3 d vertically. Root samples were placed on moist nitrocellulose paper mounted on a stub and immersed in liquid nitrogen slush, then transported under vacuum to a cryo preparation chamber. Ice was sublimed at -90 °C and the specimens were sputter-coated with gold and observed using a *Hitachi S-3000N* scanning electron microscope (SEM; *Hitachi*, Naka, Japan) with a *Gatan Alto 2100* Cryo preparation system (*Gatan*, Abingdon, UK) (for more detail see Ding *et al*. 2009).

Mapping and cloning of *OsNOX3*: *OsNOX3* was primarily mapped with simple sequence repeat (SSR) and sequence tagged site (STS) markers using 100 F_2 mutant plants. For fine mapping of *OsNOX3*, three new polymorphic markers were developed, including STS1, STS2, and STS3. The annotation of genes within the mapped region was obtained (http://rice.plantbiology.msu.edu/cgibin/gbrowse/rice/). The candidate gene *OsNOX3* was amplified from both *Osnox3* mutant and WT. The PCR products were cloned into T-vector and then sequenced. Primer sequences are listed in Table 1 Suppl.

Construction of vectors and plant transformation: The coding region of *OsNOX3* with stop codon was isolated by PCR amplification. The PCR product was ligated into the pUCM-T vector (*Promega*, Madison, USA) and sequenced. Then, the fragment was excised from the pUCM-T vector by *Sac*I and *Sal*I digestion and subcloned into the corresponding site of pCAMBIA1301(35S) vector. A 2 932 bp promoter was obtained by PCR. Primer sequences are listed in Table 1 Suppl. The resulting DNA fragment was inserted into

pCAMBIA1300NH-GUSplus *via* the *Hin*dIII/*Kpn*I sites to get a transcriptional fusion of *OsNOX3* promoter and the β-glucuronidase (GUS) coding sequence, *OsNOX3pro-GUS*. This was used for *Agrobacterium*-mediated rice transformation of WT or *Osnox3* as previously described (Chen *et al*. 2003).

Domains within OsNOX3 were predicted with several bioinformatic tools, including *CDD* (https://www.ncbi.nlm.nih.gov/cdd/), *InterProScan* (www.ebi.ac.uk/Tools/pfa/iprscan), *SMART* (smart.embl-heidelberg.de), and *TMHMM* (www.cbs.dtu.dk/services/TMHMM).

Histochemical analysis and subcellular localization of OsNOX3: Histochemical GUS analysis was performed as described (Ding *et al*. 2017). Transgenic plant samples were incubated with GUS staining solution (100 mM NaH_2PO_4 buffer pH 7.0, 0.5 % (v/v) *Triton X-100*, 0.5 mg cm^{-3} 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), and 20 % (v/v) methanol) overnight at 37 °C. Then, tissues were mounted on slides and photographed (*Leica MZ95*, Nussloch, Germany).

To make a vector *OsNOX3pro-OSNOX3-GFP*, a 2 931 bp fragment upstream of the start codon of *OsNOX3* was amplified from the genomic DNA of cv. Kasalath and a 2 530 bp coding region of *OsNOX3* without the stop codon was amplified from Kasalath cDNA, respectively. The two fragments were inserted in-frame before the coding sequence of a green fluorescent protein (GFP) of a modified pCAMBIA1300-sGFP plasmid. Primer sequences are listed in Table 1 Suppl. The resulting construct was sequenced to verify in-frame fusion and transformed into *Agrobacterium* strain EHA105. Transient infection of *Nicotiana benthamiana* was conducted as previously described (Van Loock *et al*. 2010). *CHL1-mCherry* located on the plasma membrane was co-transformed as a marker (Lv *et al*. 2014). The GFP and mCherry signals were visualized using a laser-scanning confocal microscope (*Leica SP5*). The excitation wavelength used for GFP and mCherry was 488 nm and 543 nm, respectively. The detection wavelength used was 493 to 542 nm for GFP and 578 to 625 nm for mCherry, respectively.

Reverse transcription (RT) semiquantitative PCR: Total RNA was isolated from roots with *RNeasy* plant mini kit with an additional treatment of an *RNase-free DNase I* (*Qiagen*, Hilden, Germany). The first-strand cDNA was synthesized using *SuperScript II* reverse transcriptase (*Invitrogen*, Carlsbad, USA) and used as RT-PCR templates. RT-PCR was performed using gene-specific primers designed by the *PRIMEREXPRESS* software (*Applied Biosystems*, Foster City, USA). Amplification of actin was performed as a control. The PCR products were analyzed on 1 % (m/v) agarose gels.

Detection of superoxide and hydrogen peroxide: The experiment was conducted as published previously (Nestler *et al.* 2014). For superoxide detection, root samples were incubated in 0.5 mM nitroblue tetrazolium chloride (NBT) dissolved in 0.1 M KCl/0.1 M NaCl. For hydrogen peroxide detection, two dyes were used: 3,3-diaminobenzidine (DAB) and 2,7-dichlorodihydro-fluorescein diacetate (H_2DCF-DA) (*Molecular Probes*, Irvine, USA). Four-day-old seedlings were incubated overnight in 1 mg cm^{-3} DAB dissolved in water. The H_2DCF-DA staining was conducted according to the manufacturer's instructions. Four-day-old seedlings were incubated for 45 min in the dark in the detection solution. For NBT and DAB staining, primary roots were mounted on slides and photographed using a stereomicroscope (*Leica MZ95*). For H_2DCF-DA staining, primary roots were mounted on slides and examined using a laser scanning confocal microscope (*Leica SP5*) with excitation and emission wavelength at 488 and 525 nm, respectively. More than 5 roots were examined for each genotype and the experiment was repeated twice.

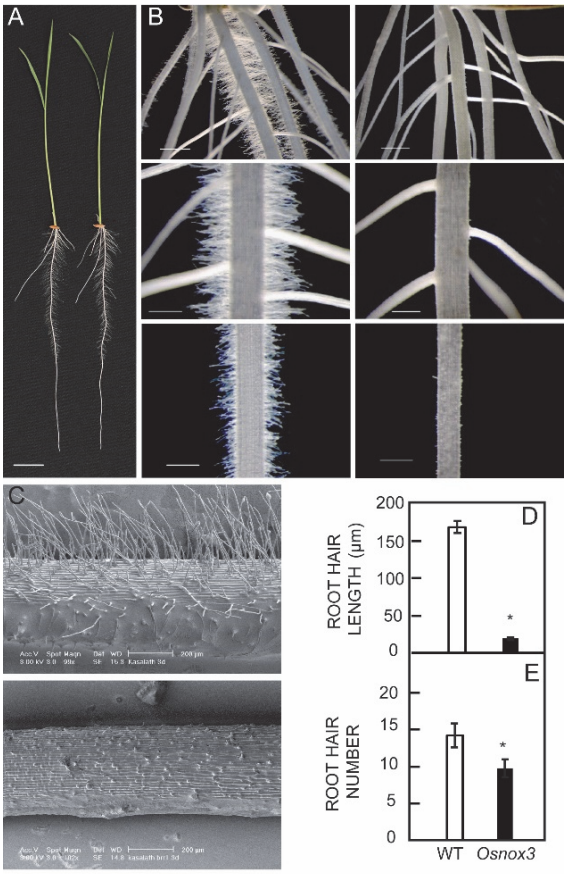

Fig. 1. Phenotypic characterization of the short root hair mutant *Osnox3*. *A* - 7-d-old seedlings of the WT (*left*) and *Osnox3* (*right*). *B* - Stereomicroscope images of roots of WT (*left*) and *Osnox3* (*right*). *C* - Cryo-SEM images of root hairs at 2 to 3 mm from the root apex of the WT (*top*) and *Osnox3* (*bottom)*. Seedlings were grown vertically for 3 d on MS media. *D,E* - Root hair length (*D*) and numbers (*E*) in the region 2 to 3 mm from the root apex. 200 root hairs (10 hairs/root) were counted for root hair length in a 200×200 μm^2 section. Means ± SDs. * indicate significant differences from the control at $P < 0.01$ (Student *t*-test). *Scale bars* = 2 cm in *A*, 0.5 mm in *B*, and 0.2 mm in *C*.

Results

To study the regulation mechanism of root hair growth, EMS-mutagenized population of rice was screened in the culture solution. A mutant *Osnox3* with significantly shorter root hairs than WT was selected (Fig. 1*B*). Except for root hair length, there was no significant difference in root and shoot parameters between WT and mutant seedlings (Fig. 1*A*).

To examine the morphology of epidermal cells in more detail, the WT and mutant seedlings were grown on MS media for 3 d after germination. Root hair length and number on primary roots at 2 to 3 mm from the root apex were investigated using cryo-SEM (Fig. 1*C*). The length of root hairs on primary roots of the mutant was decreased to 11.4 % of that in the WT (Fig. 1*D*). Moreover, root hair density of the mutant was reduced to 69 % of the WT (Fig. 1*E*).

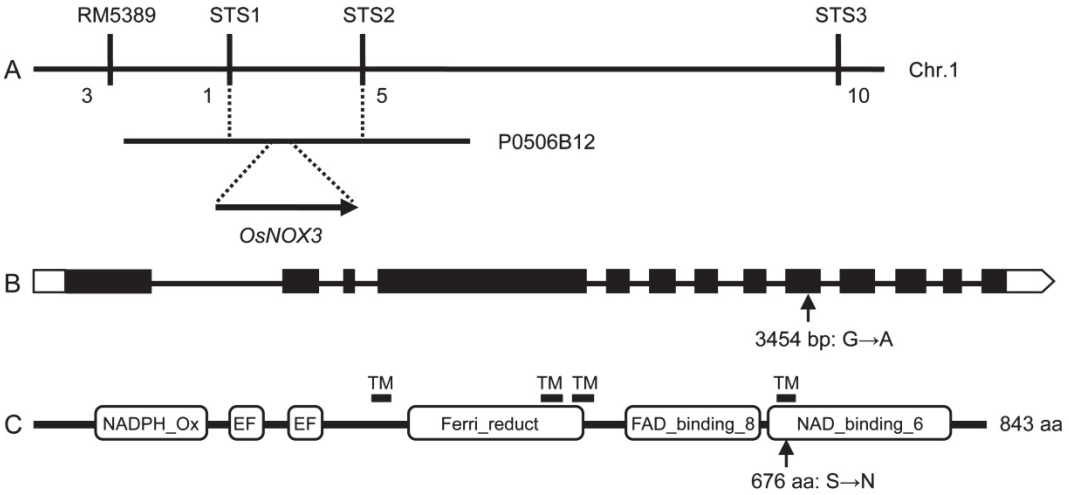

Fig. 2. Mapping of *OsNOX3*. *A* - Map-based cloning of *OsNOX3* on chromosome 1. The markers and numbers of recombinants in 1 335 F_2 mutants are listed. *B* - The gene structure of *OsNOX3*. *Black boxes* and *lines* represent exons and introns, respectively, *white boxes* indicate the 5' and 3' untranslated regions. The *arrow* indicates the G to A point mutation in *Osnox3*. *C* - OsNOX3 protein structure. The point mutation results in a substitution of serine (S) by asparagine (N) in the NAD_binding_6 domain.

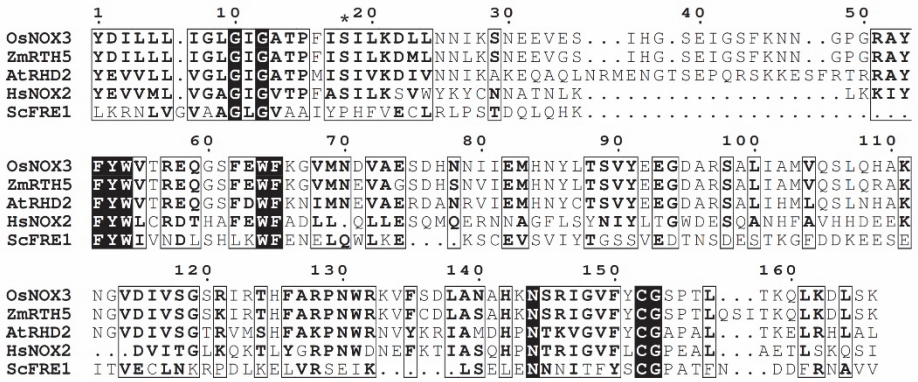

Fig. 3. Alignment of the NAD_binding_6 domain of OsNOX3 and its closest homologs from human (Hs), yeast (Sc), maize (Zm), and *Arabidopsis* (At). The mutated residue in *Osnox3* was marked by *asterisk*.

Genetic analysis of 200 F_2 progenies derived from the cross between the mutant and the *Oryza sativa* var. *japonica* cv. Nipponbare revealed that the mutant possessed a recessive mutation at a single nuclear locus. Then, a map-based cloning strategy was adopted to locate the mutant gene. The locus was first mapped to the long arm of chromosome 1 near the marker RM5389 (Fig. 2*A*). Afterwards, new sequence-tagged site (STS) markers (designated as STS1, STS2) were developed for fine mapping. The locus was then fine-mapped to a 67 kb region between STS1 and STS2 in a BAC clone P0506B12 using 1 335 mutant F_2 plants. Within the region, one gene (LOC_Os01g61880) was annotated to encode an NADPH oxidase OsNOX3 (also named as OsRbohE). The coding region of *OsNOX3* was then sequenced from both the WT and mutant plants. One mutant-specific G to A point mutation was found after 3 454 bp from the start codon on the ninth exon of *OsNOX3* (Fig. 2*B*), resulting in a change of the 676th amino acid residue from serine (S) to asparagine (N)

(Fig. 2*C*).

The *OsNOX3* gene containeds 12 introns and 13 exons, respectively. The protein coding region was 2 532 bp in length and encoded an 843 amino acid of protein with a predicted molecular mass of ~95 kDa (Fig. 2*B*). The OsNOX3 protein was predicted to contain four transmembrane (TM) domains, one NADPH oxidase domain characteristic for NADPH oxidases, two EF hand motifs, one ferric reductase domain, one FAD-binding domain, and one NAD-binding domain (Fig. 2*C*). The mutated serine residue resided in the NAD-binding domain and was found to be conserved among different species (Fig. 3), which might explain the severe root hair defects in *Osnox3* mutant.

To confirm that the single nucleotide substitution in *Osnox3* is responsible for the mutant phenotype, complementation analysis was performed using *Agrobacterium tumefaciens*-mediated transformation. The 2532 bp coding region of *OsNOX3* was cloned into the pCAMBIA1301 vector driven by the 35S promoter and used for transformation of *Osnox3*. More than thirty independent transgenic lines were obtained. All the positive transgenic lines showed normal root hairs on primary roots (Fig. 4*A*). Insertion and expression of the transgene were confirmed by RT-PCR (Fig. 4*B*). Those results confirm that the single-base mutation in *Osnox3* causes the root hair defects.

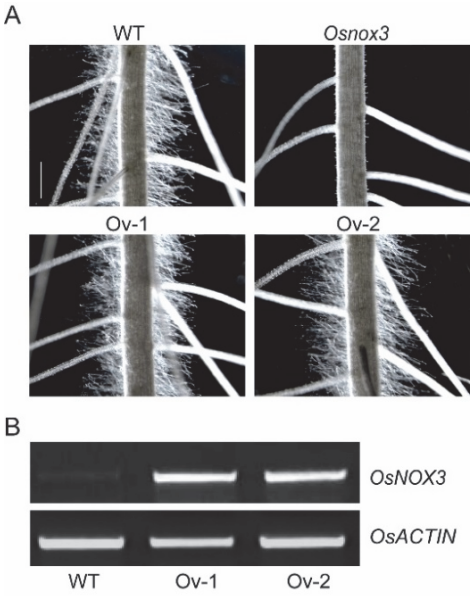

Fig. 4. Complementation of the *Osnox3* mutant. *A* - Complementation of the root hair phenotype in *Osnox3*. Ov-1 and Ov-2 represent two independent over-expression transgenic lines in the *Osnox3* background. *B* - RT-PCR analysis of *OsNOX3* expression in roots of WT and Ov-1 and Ov-2. *Scale bar* = 0.5 mm (*A*).

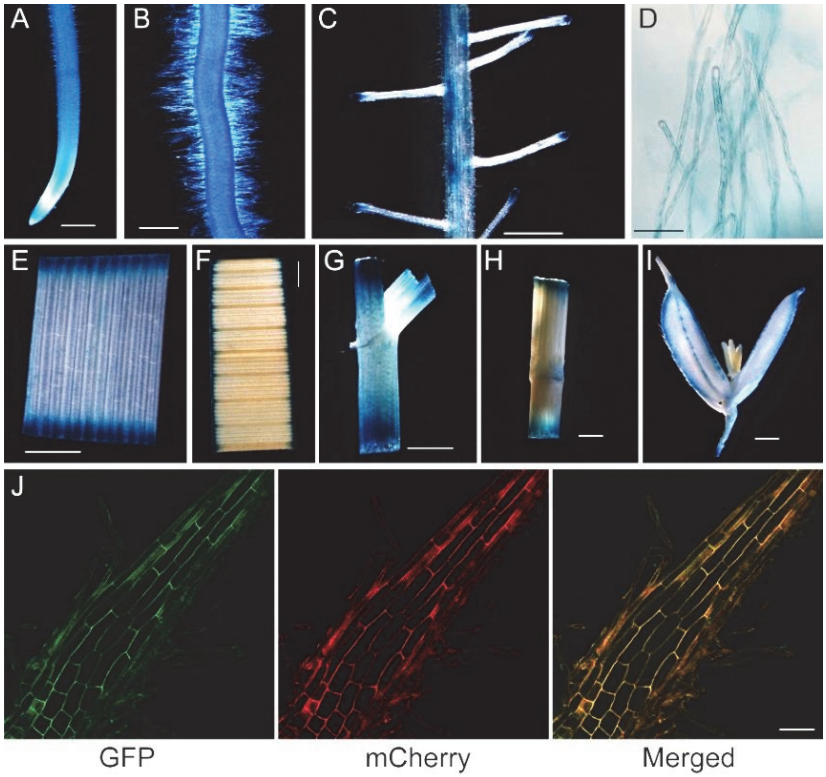

Fig. 5. Expression pattern of *OsNOX3* and subcellular localization of OsNOX3. *A-I* - Promoter-GUS fusion studies reveal the expression of *OsNOX3* in the root tip (*A*), root hair zone (*B*), lateral root (*C*), root hairs (*D*), young leaf (*E*), mature leaf (*F*), young node region of stem (*G*), mature node region of stem (*H*), and young spikelet (*I*). *J* - OsNOX3 targets GFP to plasma membrane in transiently transformed *Nicotiana benthamiana* root cells. The CHL1-mCherry is used as the plasma membrane marker. *Scale bars* = 0.5 mm (*A* - *C*), 50 μm (*D*), 1 mm (*E* - *I*), and 100 μm (*J*).

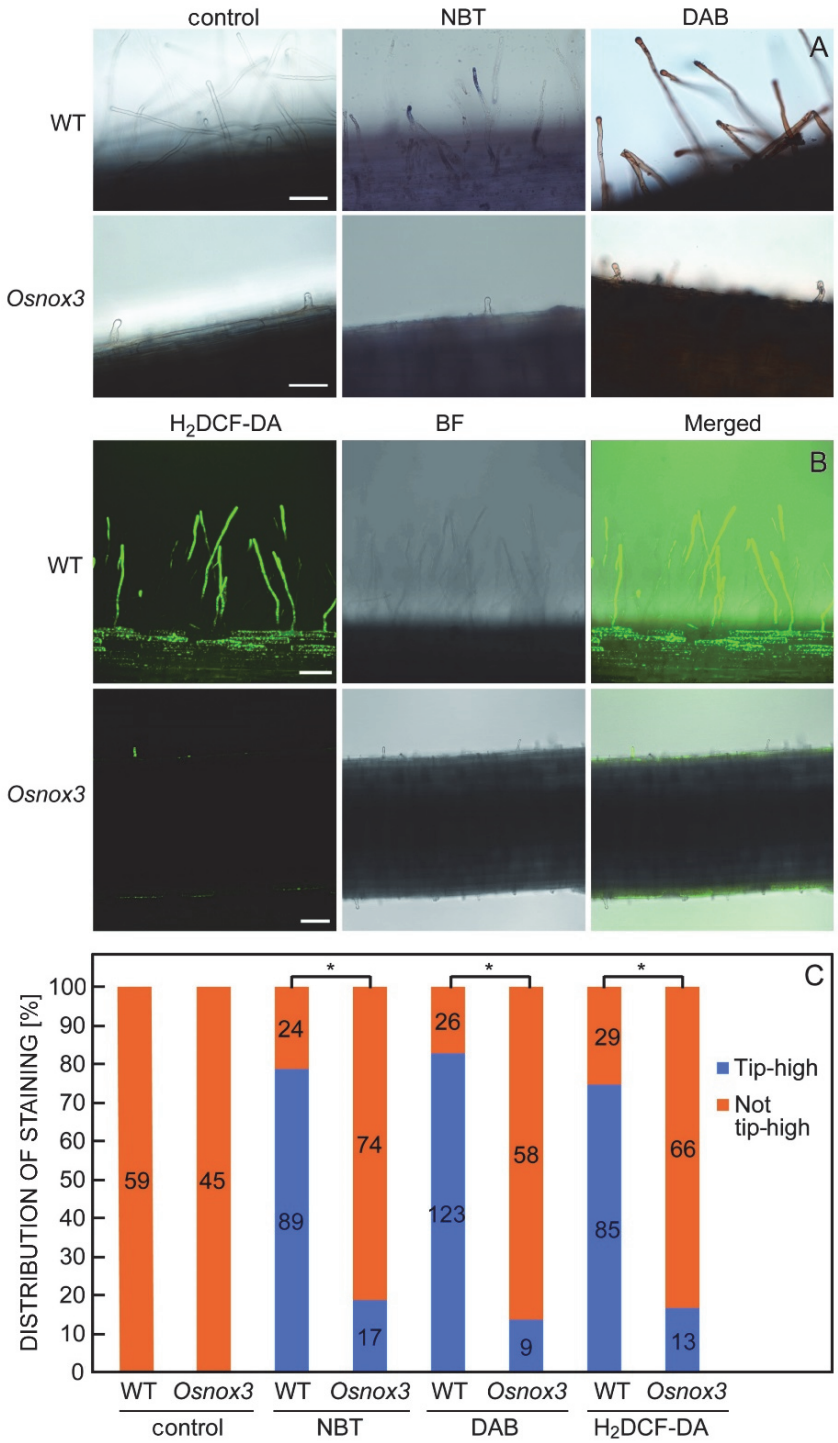

Fig. 6. Reactive oxygen species (ROS) content in *Osnox3*. *A* - ROS staining of WT (*upper lane*) and *Osnox3* (*lower lane*) root hairs using NBT and DAB. *B* - H_2DCF-DA staining of WT (*upper lane*) and *Osnox3* (*lower lane*). BF - bright field. *C*- Ratio distribution of tip-high staining signals in root hairs. Number of root hairs analyzed is indicated. * - differences significant at $P < 0.01$ (Fisher's exact test). *Scale bars* = 50 μm (*A*) and 100 μm (*B*). Root hairs with higher root hair tip staining compared with other parts of root hairs and/or epidermis cells were taken as tip-high, while others were taken as not tip-high.

To further determine the expression pattern of *OsNOX3*, a 2 931 bp promoter before its coding region was fused to the GUS reporter gene. This chimeric gene cassette was introduced into WT plants *via* *Agrobacterium*-mediated transformation. Histochemical staining for GUS activity in T_2 plants showed that *OsNOX3* was ubiquitously expressed in main root tips, lateral root tips, roots hair zone, root hairs, leaves, stems,

and young spikelets (Fig. 5*A-I*). Moreover, to examine the subcellular localization of OsNOX3, its promoter and coding sequence were fused in-frame to the N-terminus of *GFP*. An *Agrobacterium*-mediated infection assay was carried out to detect its transient expression in young tobacco roots. Fluorescence analysis showed that the fusion protein co-localized with a co-transformed plasma membrane marker (Fig. 5*J*), indicating that OsNOX3 was located on the plasma membrane.

In order to determine whether there were less reactive oxygen species (ROS) in root hairs of *Osnox3* mutant, NBT staining for superoxide and DAB and H_2DCF-DA staining for hydrogen peroxide was conducted. Compared with WT, staining signals for all three dyes were significantly reduced in root hairs of *Osnox3* (Fig. 6*A-B*). Furthermore, presence or absence of high ROS staining signals in root hair tips were quantified as previously reported (Nestler *et al*. 2014). While 79 % of WT root hairs exhibited a tip-high superoxide (NBT) signal, it was significantly reduced to 19 % in *Osnox3* root hairs. Similarly, 82 and 75 % of WT root hairs showed a high hydrogen peroxide signal in root hair tips by DAB or H2DCF-DA staining, respectively, while those for *Osnox3* were only 13 and 16 % (Fig. 6*C*).

Discussion

NADPH oxidases (NOXs), also known as respiratory burst oxidase homologs (RBOHs), are key enzymes that catalyze the generation of reactive oxygen species (ROS) in plants. Rice contains at least nine typical NOXs (OsNOX1-9) (Wong *et al*. 2007, Wang *et al*. 2013). Different rice NOX have diverse functions: OsNOX2 and OsNOX6 (Yoshie *et al*. 2005) as well as OsNOX1 and OsNOX9 (Nagano *et al*. 2016) are involved in immune responses. The plasma membrane OsNOX2 also plays a crucial role in developmental regulation and drought-stress response (Wang *et al*. 2016). Recent studies revealed that OsNOX9 is involved in aerenchyma formation in roots (Yamauchi *et al*. 2017). Here we report the cloning and characterization of *OsNOX3*, a gene which is required for root hair initiation and elongation in rice.

The role of plant NOXs in root hair growth has been reported in *Arabidopsis* and maize (Foreman *et al*. 2003, Nestler *et al*. 2014). There are ten NOX proteins named AtRbohA-J in *Arabidopsis thaliana* (Torres and Dangl 2005). The loss-of-function mutant of *AtRbohC* (*RHD2*) forms very short root hairs that do initiate bulges but do not elongate (Foreman *et al*. 2003). Moreover, a NOX from maize, RTH5, was shown to play a critical role for the transition from bulge formation to tip growth of root hairs (Nestler *et al*. 2014). The length and density of root hairs on roots of *rth5* were significantly decreased. Similar to *rth5*, *Osnox3* showed significantly shorter root hairs and reduced root hair density in all root types while other root parameters and aboveground development remained unaffected (Fig. 1). Hence, Os*NOX3* and *RTH5* specifically control root hair elongation and epidermis specification and/or root hair initiation.

Phylogenetic analysis showed that Os*NOX3* and *RTH5* are members of a monocot-specific sub-clade of group I and *OsNOX3* is also the closest homolog of *RTH5* (Nestler *et al*. 2014). *RHD2* was shown to control root hair elongation (Schiefelbein and Somerville 1990). Moreover, the *rhd2* mutant displayed stunted root growth (Foreman *et al*. 2003), an effect not observed in *Osnox3* and *rth5* mutants. It has been shown that increase of pH from 5 to 6 results in the restoration of root hair elongation in *Atrhd2* mutant (Monshausen *et al*. 2007). Thus, we also tested the response of root hair development of *Osnox3* mutant to different pH (Fig. 1 Suppl). It was found that the elongation of root hairs was partially inhibited in WT when the pH of culture solution was increased to 7.5 or decreased to 3.5. However, no change of root hair morphology was observed. On the contrary, there was no significant difference of root hairs in *Osnox3* under different pH. Our observations suggest that the functioning mechanism of the *NOX* gene is different between monocots and eudicots. Furthermore, in *Osnox3* and *rth5* the root hair density of mutants was reduced, while in *rhd2* the formation of root hair bulges was normal, indicating that the molecular mechanisms regulating the epidermal cells in different plant species are only partially conserved**.**

The asymmetric distribution of Rboh activity was shown to regulate ROS signalling in root hair growth and xylem differentiation (Foreman *et al*. 2003, Barcelo 2005, Carol *et al*. 2005). A large body of evidence from plants and animals indicates the distribution of NOX systems on the plasma membrane (Segal 2016). OsNOX1, OsNOX2, OsNOX8, and OsNOX9 are all located in the plasma membrane (Wong *et al*. 2007, Nagano *et al*. 2016, Wang *et al*. 2016). Our study showed that OsNOX3 was localized in the plasma membrane (Fig. 4*J*), similarly as its closest homolog RTH5 in maize. Furthermore, ROS staining assays showed that the content of superoxide and hydrogen peroxide in root hair tips of *Osnox3* was significantly lower than in WT (Fig. 6), which was similar to *rth5*. These results suggest the conservation of the regulation mechanism of root hair development among different monocot species.

In summary, we report herein the high expression of *OsNOX3* gene in rice roots and root hairs. We characterized this gene and confirmed its localization on the plasma membrane and its functions in the initiation and elongation of root hairs.

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