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Original Article

Tissue-specific enhancement of *OsRNS1* with root-preferred expression is required for the increase of crop yield

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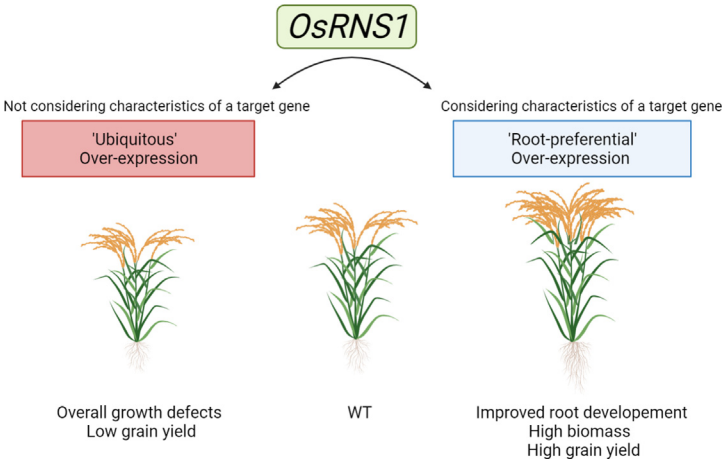
HIGHLIGHTS

- Root preferential promoters in rice was used for enhancing function of root preferential genes.
- Root preferential promoters in rice was more effective to increase grain yield without side effect frequently observed in those by ubiquitous ones.
- Enhanced degradation of RNAs in root is beneficial for crop biomass.
- Enhanced ability of ROS scavenge in root is beneficial for crop biomass.

GRAPHICAL ABSTRACT



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ABSTRACT

Introduction: Root development is a fundamental process that supports plant survival and crop productivity. One of the essential factors to consider when developing biotechnology crops is the selection of a promoter that can optimize the spatial–temporal expression of introduced genes. However, there are insufficient cases of suitable promoters in crop plants, including rice.
Objectives: This study aimed to verify the usefulness of a new rice root-preferred promoter to optimize the function of a target gene with root-preferred expression in rice.
Methods: osrns1 mutant had defects in root development based on T-DNA insertional mutant screening and *CRISPR* technology. To optimize the function of *OsRNS1*, we generated *OsRNS1*-overexpression plants under two different promoters: a whole-plant expression promoter and a novel root-preferred expression promoter. Root growth, yield-related agronomic traits, RNA-seq, and reactive oxygen species (ROS) accumulation were analyzed for comparison.
Results: OsRNS1 was found to be involved in root development through T-DNA insertional mutant analysis and gene editing mutant analysis. To understand the gain of function of *OsRNS1*, *pUbi1::OsRNS1* was generated for the whole-plant expression, and both root growth defects and overall growth defects were found. To overcome this problem, a root-preferential overexpression line using *Os1-CysPrxB* promoter (*Per*) was generated and showed an increase in root length, plant height, and grain yield compared to wild-type (WT). RNA-seq analysis revealed that the response to oxidative stress-related genes was

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significantly up-regulated in both overexpression lines but was more obvious in *pPer::OsRNS1*. Furthermore, ROS levels in the roots were drastically decreased in *pPer::OsRNS1* but were increased in the *osrns1* mutants compared to WT.

Conclusion: The results demonstrated that the use of a root-preferred promoter effectively optimizes the function of *OsRNS1* and is a useful strategy for improving root-related agronomic traits as well as ROS regulation.

Introduction

For improving agronomic traits via introducing ectopic genes, diverse types of promoters have been used, including constitutive, spatiotemporal and stress/chemical inducible promoters. The most commonly used constitutive promoters are the cauliflower mosaic virus (CaMV) 35S, rice *Actin1* (*Act1*), rice *GOS2*/rice eukaryotic translation initiation factor1-like gene (*eIF1*), rice *Phosphogluconate dehydrogenase (PGD1)* and maize *ubiquitin1* (*Ubi1*) promoters [1–4]. Many studies have succeeded in improving the function of the target gene by using constitutive promoters regardless of features of the target gene, such as being tissue-specific or stress inducible, but have frequently shown side effects such as impaired growth, reduced biomass, or early flowering [5–12]. For example, *AtFD*, *AtFDP, OsMADS26*, and *OsbHLH142* genes in rice, which were reported to have growth defects or pollen impairment through dysfunctional mutations, and overexpression lines of the genes driven by the *Ubi1* promoter have triggered several abnormal phenotypes, including semi-dwarf and retarded root/shoot growth or sterility [5–7,10]. In addition, over-expressing *AtMS1* under the control of the CaMV35S promoter and *HvMS1* under the control of the maize ubiquitin promoter caused stunted plants with sterile pollen in *Arabidopsis* and wheat, respectively [8,9]. Moreover, studies on the discovery and application of promoters that are optimal for target species are essential, as there are many cases where they do not work properly or show side effects according to applied species or tissues. For example, the *CaMV 35S* promoter operates efficiently in dicotyledon plants, but it has relatively poor activity in monocotyledon plants, such as rice and maize. It is also inactive in other cell types like pollen [13,14]. More interestingly, although the rice and tomato *rbcS* promoters reveal mesophyll-preferred expression, in rice, the expression of the β-glucuronidase (GUS) gene linked to the tomato *rbcS* promoter is significantly lower than that of the GUS gene linked to the rice *rbcS* promoter. [15]. The use of constitutive promoters can produce an abnormal phenotype regardless of enhancement of the target gene function. However, there are insufficient examples of promoters in rice for effective application after considering the characteristics of target genes or traits in rice.

The root system is important for the absorption of water and nutrients, support of plants, storage of nutrients, and the formation of symbioses with other microorganisms in the rhizosphere [16–18]. In fact, when a problem occurs in the roots, it directly affects the leaves and stems of the above-ground part of the plant. Therefore, the productivity of a crop is absolutely dependent on a healthy root system. A few studies have been conducted on the isolation, characterization, and application of root-specific promoters. *sa/T* gene was isolated from the roots of a rice cultivar sensitive to salt [19], and two genes preferentially expressed in the roots of rice, *RCc2* and *RCc3*, were identified from rice [20]. More recently, *Os1-CysPrxB (OsPer, LOC_Os07g44440)* was identified as a gene preferentially expressed in the whole root and embryos where the root was initiated [21], and the *Os03g01700* promoter was a newly discovered promoter expressed in the root epidermis of rice [22]. Among previously discovered root-specific promoters, *RCc3* and *Os03g01700* were used for practical applications in rice [22–27]. However, most studies over-expressing a target gene by using a root-specific promoter did not show a clear effect on the increasing yield under normal growth field conditions [22–25]. The discovery of new tissue-specific promoters is expected to be a viable option for successful biotech crop development.

RNase T2 enzymes are transferase-type endoribonucleases and retain acid RNase activity that produce oligonucleotides and/or mononucleotides with a terminal 3′ phosphate via a 2′, 3′ cyclic phosphate intermediate. *RNase T2* family genes exist in all eukaryotes and play important roles in a variety of biological processes. These RNases in plants can be divided into three subclasses [28–30]: Plant class I *RNase T2* genes are associated with responses to abiotic and biotic stresses [29,31,32]; plant class II *RNases T2* genes participate in senescence and play a housekeeping role through ribosomal RNA (rRNA) recycling [31,33–36]; and class III *RNases T2* genes are generally involved in self-incompatibility, although some class III *RNases T2* are involved in stress responses such as phosphate starvation without being involved in self-incompatibility [37–39]. Thus, plant *RNase T2* family genes perform various functions.

In a previous study, we conducted a genome-wide analysis for root-preferred genes in rice and identified 684 loci with root-preferred expression, which were validated with *in silico* analysis using meta-expression profiles of both *indica* and *japonica* varieties [40]. In this study, loss-of-function mutations of *OsRNS1* were confirmed, and one of the root-preferred genes mentioned above was identified, showing defects in root development. Furthermore, histochemical GUS assay and real-time quantitative polymerase chain reaction (qRT-PCR) analyses confirmed that *OsRNS1* has entire root-preferred expression but is more strongly expressed near the root elongation zone and the mature zone. To optimize the function of *OsRNS1* with root-preferred expression, we designed a novel comprehensive strategy by utilizing the *Os1-CysPrxB* (*Per*) promoter system with root-preferred expression, and the effect was compared with that of the *ubiquitin 1* (*Ubi1)* promoter as the most commonly utilized constitutive promoter. Detailed data using the wild-type (WT), overexpression lines of *OsRNS1* using the *Per* and *Ubi1* promoters as well as *osrns1* mutants have been presented and discussed.

Experimental procedures

Plant material, growth conditions

osrns1-1 (T-DNA insertional line of *OsRNS1*, 1A-06714) was isolated from our rice T-DNA insertion collection (Rice Functional Genomic Express Database, https://signal.salk.edu/cgi-bin/RiceGE) and was provided by Prof. G An, Kyung Hee University, Korea [41,42]. For the comparison of the phenotypes in young seedling stage, seeds of all transgenic plants [two *osrns1* mutants (*osrns1-1* by T-DNA insertion and *osrns1-2* by gene editing system), two types of *OsRNS1* over-expressing transgenic lines (*Ubi1::RNS1-1*, *Ubi1::RNS1-2*, *Per::RNS1-1*, and *Per::RNS1-3*)] and WT (*Oryza sativa L.* cv. *Dongjin*) were germinated on Murashige Skoog (MS) medium

under the controlled conditions of 28 °C day/25 °C night temperatures for 10 days, 8 h light/16 h dark cycle, and 78% relative humidity after sterilization with 50% (w/v) commercial bleach for 30 min with gentle shaking.

All plants were then transferred to soil for agricultural trait measurements [height, number of tillers, number of panicles, total grain weight (g), and seed fertility rate (%)] in paddy field conditions. In anatomical expression analysis, roots, leaf, panicle, mature flower, and seeds were collected at 10 and 15 days after pollination, and total RNA was extracted. Four biological replicates were prepared and analyzed separately.

Cloning and generation of OsRNS1-related transgenic rice plants

Rice seedlings at 10 days after germination were used for cloning experiments for CRISPR/Cas9, histochemical GUS assay, subcellular localization, and two types of over-expressing transgenic plants. To generate guide RNA by CRISPR/Cas9 system for *osrns1-2*, a 20-bp target site was selected in the second exon of *OsRNS1* by CRISPRdirect (https://crispr.dbcls.jp/) (Figure S8). An oligomer, which contains GGCA/AAAC overhangs for ligation, was annealed to at 37 ℃ for 60 min, denatured at 95 ℃ for 10 min and inserted into the Bsa I site of a pRGEB32 binary vector (Addgene plasmid ID: 63142; Supplemental Table S3). For cloning the *OsRNS1* promoter::*GUS* expression vector, a 2,188 upstream sequence from the start codon with 369 bp of the *OsRNS1* coding sequence was amplified by PCR, and the PCR product was cloned into the binary vector pGA3519 for GUS expression in plants. To clone *OsRNS1* native promoter::*OsRNS1* full coding sequence linked to the green fluorescent protein (GFP) reporter system, the 2,188 upstream sequence from the start codon with *OsRNS1* coding sequence without stop codon was amplified by PCR, and the PCR product was cloned to the modified pGA3427 by In-Fusion Cloning (In-Fusion HD Cloning Kit, Clontech, 639644, California, USA) [43]. To clone the two types of *OsRNS1* over-expressing transgenic lines (*pUbi1::OsRNS1*, and *pPer::OsRNS1*), the pGA3426 vector with *Ubi1* promoter was used for constitutive expression [43] and *Per* promoter was used for root-preferred overexpression system (Figure S6). A root-preferred overexpression system (Figure S6) was constructed by cutting the *Ubi1* promoter of the pGA3426 vector [43] using two restriction enzymes (BamH I and Spe I) and then adding 1,752 bp of the *Per* promoter with root-preferred expression [21]. The full sequence of the *Per* promoter is shown in Figure S7. The two types of *OsRNS1* overexpression lines were created by inserting 771 bp of *OsRNS1* coding region, including the Kozak sequence, into pGA3426 and the root overexpression system vector via the In-Fusion Cloning system (In-fusion HD Cloning Kit, Clontech, 639644, California, United States).

Then, all ligation products were transformed into *Escherichia coli* and Agrobacterium tumefaciens strain LBA4404 using the freeze–thaw method [44]. This is followed by co-cultivation into embryogenic callus from mature seeds (*O. sativa* L. cv. *Dongjin*). Regenerated plants were obtained as described previously [45–46]. The primers used in these analyses are summarized in Supplemental Table S3.

Histochemical GUS assay and microscopic analyses

Histochemical GUS staining was performed as described by [21]. The roots of transgenic plants were incubated in a GUS solution for two hours, and other samples were incubated in a GUS solution overnight at 37 °C after being vacuumed for 15 min, and then a 96% ethanol solution was exchanged at 65 °C to remove chlorophyll. The assayed samples were photographed with an Olympus BX61 microscope (Olympus, Tokyo, Japan).

Sub-cellular localization

To observe the sub-cellular localization of the OsRNS1 protein, we used a stable transgenic plant with GFP fusion protein in rice and agro-infiltration in tobacco. To detect sub-cellular localization in the cell wall, the pOsRNS1::OsRNS1::GFP fusion stable transgenic plants were grown on MS medium for 10 days and then stained with calcofluor white (Sigma, 18909-100ML-F) in the root according to [47]. The roots were immersed in 0.1% calcofluor white in ClearSee solution for more than 30 min and were washed two times in ClearSee solution. The images were analyzed with a fluorescence microscope and Zeiss LSM800 microscope with the following excitation/emission parameters for generating composite images: (i) GFP—AF488 493/517 nm; (ii) calcofluor white—CW2MR 425–475 nm.

RNA extraction and real-time PCR

All samples in this study were frozen in liquid nitrogen and ground with a Tissue Lyser II (Qiagen; Hilden, Germany). RNAs were extracted with the RNAiso Plus Kit according to the manufacturer's protocol (Takara Bio, Kyoto, Japan). Complementary DNA (cDNA) was synthesized [48]. For real-time PCR analysis, we used *OsUbi5* as the reference gene [49]. We used cycling conditions of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s. This experiment was repeated three times by using four independent biological replicates. Relative transcript levels and fold changes were calculated by the $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods [50], respectively. The primers used in these analyses are summarized in Supplemental Table S3.

Agricultural trait measurements of OsRNS1 over-expressing transgenic plants in a paddy field for three cultivation seasons (2018–2019 and 2021)

To evaluate the agricultural components of the *OsRNS1* over-expressing transgenic plants (*pUbi1::OsRNS1* and *pPer::OsRNS1*) under normal field conditions, two independent T2 (2018), T3 (2019), and T4 (2021) plants, together with the WT controls, were transplanted into a paddy field in certified genetically modified organism fields in Yongin, Korea (37° 14′ N) (Table 1; Supplemental Table x). For the field tests, 10 seedlings were planted per line. The following agronomic traits were scored: height, number of tillers, number of panicles, total grain weight (g), and seed fertility rate (%). Agronomic trait measurements between the WT and two types of *OsRNS1* over-expressing transgenic plants (*Ubi1::RNS1-1*, *Ubi1::RNS1-2, Per::RNS1-1,* and *Per::RNS1-3*) was performed with ten independent biological replicates.

RNA-sequencing analysis

Utilizing the Illumina platform, three independent total RNA samples from the roots of each of the *Per::RNS1-2*, *Ubi1::RNS1-1* and control seedlings were paired-end sequenced on a NovaSeq 6000 sequencing system. In each transcriptome sample, 100-bp paired-end sequences were assessed with a FastQC. Any adapter contaminations or low-quality sequences (-q 30) were removed using both fastp and its wrapper tool, Trim Galore*!*. Read pairs were aligned to the rice genome (International Rice Genome Sequencing Project [IRGSP] 1.0 reference genome) using Top Hat, and read counts for each gene were determined with a cufflink. DEGs were evaluated using cuffdiff for comparing two promoter transgenic plants (*Ubi1::RNS1-2* and *Per::RNS1-1*) with the WT. Genes with p-values <0.05 and $\log_2$fold-changes >1.5 were considered to be differentially expressed. Further screening among the initial DEGs was based on fragments per kilobase per million frag-

Table 1
Test of agronomic traits of *OsRNS1*-overexpression plants for two cultivation seasons. **(2018–2019).** Plants were grown in a genetically modified organism field at Yongin, Korea (37°14′N) in 2018–2019. In addition, data in the third cultivation season is prepared in Supplemental Table S4. Values are represented as the means with standard deviations of 10 replications.

	First cultivation season (2018)				
	WT	*Ubi1::RNS1-1*	*Ubi1::RNS1-2*	*Per::RNS1-1*	*Per::RNS1-3*
Height (cm)	110.2 ± 3.08	113.2 ± 3.11	110.4 ± 2.61	135 ± 11.18	137.6 ± 10.06
Panicle number	15.7 ± 4.70	14.6 ± 1.81	13.2 ± 1.78	23.4 ± 5.59	22.6 ± 8.28
Seed weight (g)	22.9 ± 8.24	15.75 ± 4.79	12.6 ± 3.52	38.28 ± 9.34	34.12 ± 6.38
	Second cultivation season (2019)				
	WT	*Ubi1::RNS1-1*	*Ubi1::RNS1-2*	*Per::RNS1-1*	*Per::RNS1-3*
Height (cm)	111.8 ± 5.58	105.8 ± 6.43	105.2 ± 3.91	133.7 ± 5.96	132 ± 5.62
Panicle number	17.5 ± 3.77	16.2 ± 5.13	15.4 ± 1.8	26.2 ± 6.17	23.5 ± 7.03
Seed weight (g)	26.1 ± 7.47	17.45 ± 3.79	12.4 ± 2.05	37.22 ± 9.34	32.62 ± 7.0
Fertility percent (%)	79.2 ± 9.67	58.4 ± 7.97	29.1 ± 8.04	76.1 ± 8.42	74.7 ± 12.07

ments mapped values with quartile normalization (Fig. 5 and Supplemental Table S2). Gene ontology (GO) enrichment was analyzed using the Rice Oligo Array Database (https://ricephylogenomics-khu.org/ROAD_old/analysis/go_enrichment.shtml) with $P \leq 0.05$ and gene numbers ≤ 4. The enrichment result was visualized using R. A heatmap of the selected DEGs was created using the Multi Experiment Viewer (MeV_4-9–0) software tool.

Quantification of cellular RNA contents

Whole seedlings after 10-day germination were stained for 1 h with 2 μM SYTO® RNASelect™ Green Fluorescent Cell Stain (SYTO® RNASelect™) in 1 × phosphate buffered saline (PBS) at room temperature, washed three times with 1 × PBS, and observed under a fluorescence microscope to produce green fluorescent images in root as described above. Five biological replicates were prepared separately and analyzed three times.

Quantification at the reactive oxygen species (ROS) level

The presence of ROS was examined in roots from 10-days old rice seedlings were incubated in 10 μM CM-H2DCFDA for 30 min in dimethyl sulfoxide and in 1 mg/ml of 3, 3′-diaminobenzidine (DAB) solution for 30 min. After being washed three times with PBS, the tissues were observed with Zeiss LSM800 and Olympus BX61 microscopes. Four biological replicates were prepared separately and analyzed three times.

Results

OsRNS1 is required for root development

Recently, we carried out integrated omics analysis of root-preferred genes and identified a *OsRNS1* gene showing root-preferred expression patterns in the *OsRNS* family [40]. To explore the functional roles of *OsRNS1*, we first used a gene indexed mutant having T-DNA insertion in the first intron of the gene and analyzed the phenotypes in the homozygous mutant (*osrns1-1; 1A-06714*). We then found that *osrns1-1* showed approximately 20% less root length and 18% less stem length than those of the WT (Fig. 1). To confirm the knockout phenotype, we generated off-frame mutants of *OsRNS1* by introducing one gRNA. Mutations of the target region were detected via sequencing analysis of independent T2 transgenic plants. As expected, the mutant (*osrns1-2*) carrying *OsRNS1*-sgRNA showed similar phenotypes as *osrns1-1* (Fig. 1). Furthermore, both mutants showed dwarfism during the vegetative stage in the field condition (Figure S1). Our results suggest that *OsRNS1* is not only involved in root development but also affects growth.

OsRNS1 is preferentially expressed in rice root elongation

To know the more precise expression patterns of *OsRNS1* according to different tissue/organ types, we generated and analyzed transgenic plants harboring the GUS reporter system and the GFP reporter system under the control of its own promoter. Histochemical examination of roots from transgenic plants sampled at 10 days after germination (DAG) revealed that *OsRNS1* is strongly expressed in the root elongation zone and maturation zone (Fig. 2). Examination of other organs from transgenic plants sampled at 90 DAG in the field has been carried out, and we found that *OsRNS1* is not expressed in other organs except anther (Figure S2). In addition, observation for roots from transgenic plants expressing GFP under the control of the *OsRNS1* promoter at 7 DAG confirmed the root-preferred expression of *OsRNS1* (Fig. 2). These results indicate that *OsRNS1* mainly functions in root elongation and maturation zones.

OsRNS1 protein localized at the cell wall and in the intracellular region

Using the ProtComp Version 9.0 tool from softberry (http://www.softberry.com/) to estimate the sub-cellular location of the OsRNS1 protein, we found that the OsRNS1 protein is targeted to the extracellular region (Table S1). To determine the estimation, we generated transgenic rice plants expressing *pOsRNS1::OsRNS1::GFP* and used *p35S::OsRNS1::GFP* vector for the transient analysis system by agro-infiltration in *Nicotiana benthamiana* (tobacco). As a result, the GFP signal producing yellow fluorescence on the cell wall in rice root overlapped the cyan fluorescence signal when calcofluor white was used as a cell wall specific dye. Images showing the merger of green and cyan channels in the cell wall indicates that OsRNS1 is localized at the cell wall. Green fluorescence was also detected in the intracellular region (Fig. 2). Through agro-infiltration, we observed similar results in tobacco through a transient assay, thus indicating that the OsRNS1 protein localizes at the cell wall and in the intracellular region (Fig. 2 and Figure S3).

Root-preferred Os1-CysPrxB (Per) promoter effectively enhanced the expression of OsRNS1 in the root and enhanced root-related agronomic traits

To enhance the function of the *OsRNS1* gene, we generated *OsRNS1*-overexpression plants by expressing its coding sequence with the *ubiquitin 1* promoter (*Ubi1*) for ubiquitous expression [3,14]. However, contrary to expectations, it showed not only root growth defects but also overall growth defects (Fig. 3). The reason is that the use of constructive overexpression of the target gene is known to sometimes lead to undesirable phenotypes such as low

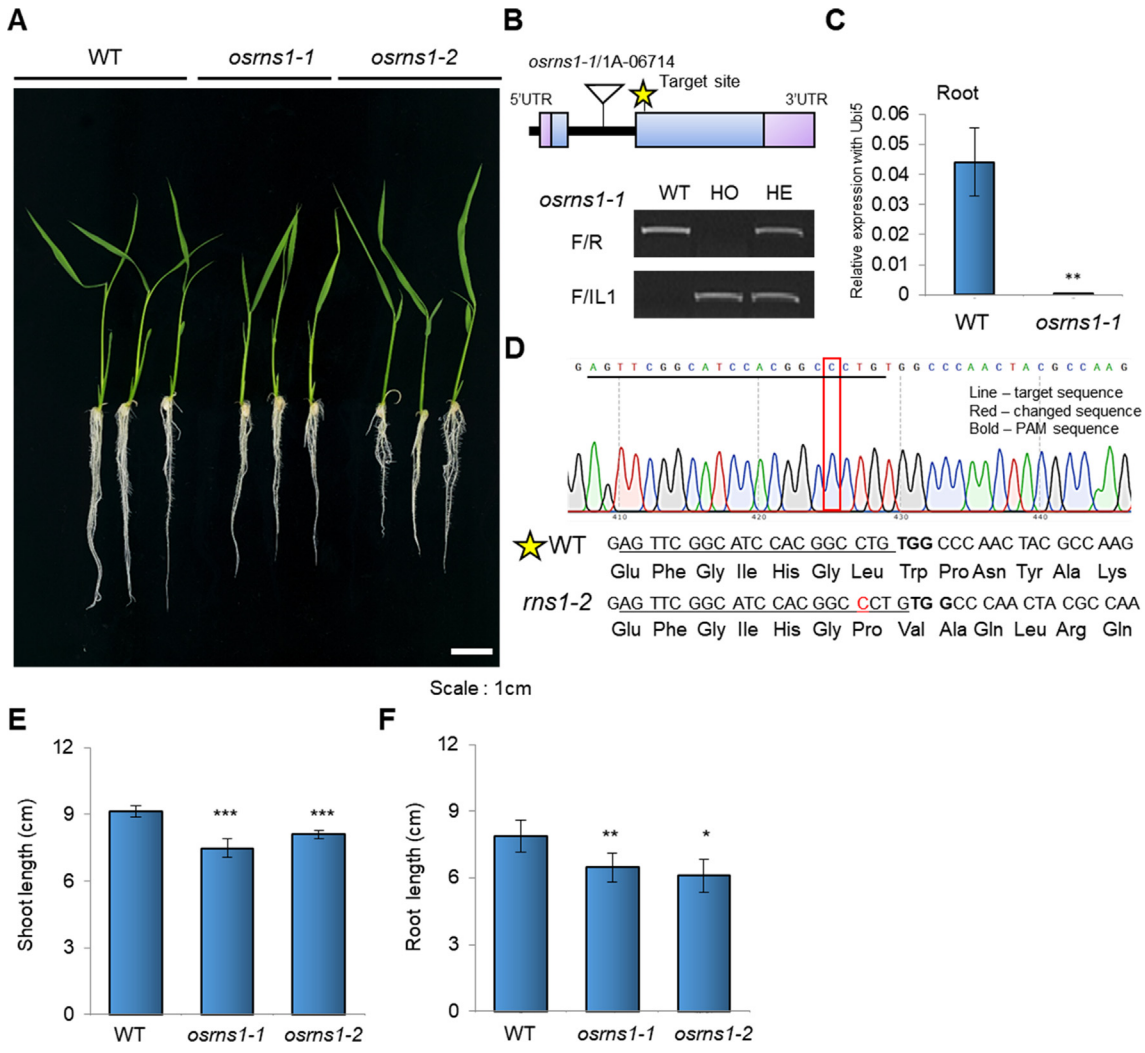

Fig. 1. *OsRNS1* is required for root development and normal plant growth. Comparison between the phenotypes of wild-type (WT) and two *rns1* mutant allele plants (*osrns1-1* (T-DNA insertional mutant) and *osrns1-2* (*OsRNS1*_sgRNA)) using 10-day seedlings on MS media (A). (B) Diagram of T-DNA insertions in the first intron of *OsRNS1* (triangle; *osrns1-1*) and CRISPR target site in the second exon of *OsRNS1* (star; *osrns1-2*). (C) Confirmation of *OsRNS1* expression in *osrns1-1* with the *OsUbi5* gene as an internal control and (D) confirmation of the target sequence in *osrns1-2*. Comparison of the measurements of shoot (E) and root (F) lengths among the WT and two knockout mutants was performed with 10 independent biological replicates (scale bar: 1 cm). * *P*-value < 0.05, ** *P*-value < 0.01, and *** p-value < 0.001 were determined by the Student's *t*-test.

modification rates, growth defects, and reduced crop yields, so it can be assumed that such results have been obtained [5–9]. On the basis of the expression analysis of *OsRNS1* and the phenotypes of the *osrns1* mutants, we estimated that *OsRNS1* plays major roles in root development. We predicted that the use of root-dominant promoters would be a good strategy to optimize the function of *OsRNS1* with strong root expression to overcome these problems. We generated *OsRNS1*-overexpression plants by expressing its coding sequence under the *Os1-CysPrxB (Os1-CysPrxB;OsPer, LOC_Os07g44440)* promoter (*Per)* for entire root-preferred expression in rice [21] (Fig. 3a–d) and identified 3 and 4 T1 plants for *pPer*::*OsRNS1* and *pUbi1*::O*sRNS1* after checking the correlation between expression data and phenotype, respectively (Figure S9). We then selected two independent T4 homozygous lines of both *pPer*::*OsRNS1* and *pUbi1*::*OsRNS1* plants for further analysis and collected the transgenic T1 to T4 seeds. The transcript levels of *OsRNS1* in the *pPer*::*OsRNS1* and *pUbi1*::*OsRNS1* plants were confirmed by qRT-PCR. As expected, the expression of *pUbi1*::*OsRNS1* lines was highly enhanced in both root and shoot tissues, whereas *pPer*::*OsRNS1* showed predominantly increased expression of the target gene in root tissues (Fig. 3e–f). In addition, all *OsRNS1*-overexpression plants using the *Per* promoter showed a more than 35% and 22% increase in root length and shoot length, respectively, when compared to the WT during the seeding stage after 10-day germination, whereas T4 *pUbi1*::*OsRNS1* plants showed either similar or weaker phenotypes than those of the WT (Fig. 3g–i). Our results indicated that expression of transgenes using *Per* dominantly works in the root, thus indicating the usefulness of this promoter to enhance root-related agronomic traits.

Overexpression of OsRNS1 under a root-preferred promoter enhances biomass and grain yield without side effects

The differences among WT, *pPer::OsRNS1,* and *pUbi1::OsRNS1* plants during the vegetative and reproductive stages are clearly shown in Fig. 4 and Table 1. The biomass and yield of transgenic plants into a certified genetically modified organism field in Kyung Hee University, Yongin, Korea (37° 14′ N) were evaluated for three cultivation seasons (2018–2019 and 2021) (Table 1; Supplemental Table S4). We planted 10 seedlings in each of two rows at 15 × 30 intervals. To eliminate the border effect, we planted wild type at both ends of the row. We used the 2019 dataset in Fig. 4 and the entire dataset in Table 1. Compared to the WT, the plant height of *Ubi1::RNS1-1* and *Ubi1::RNS1-2* decreased by 5.3% and 5.9%,

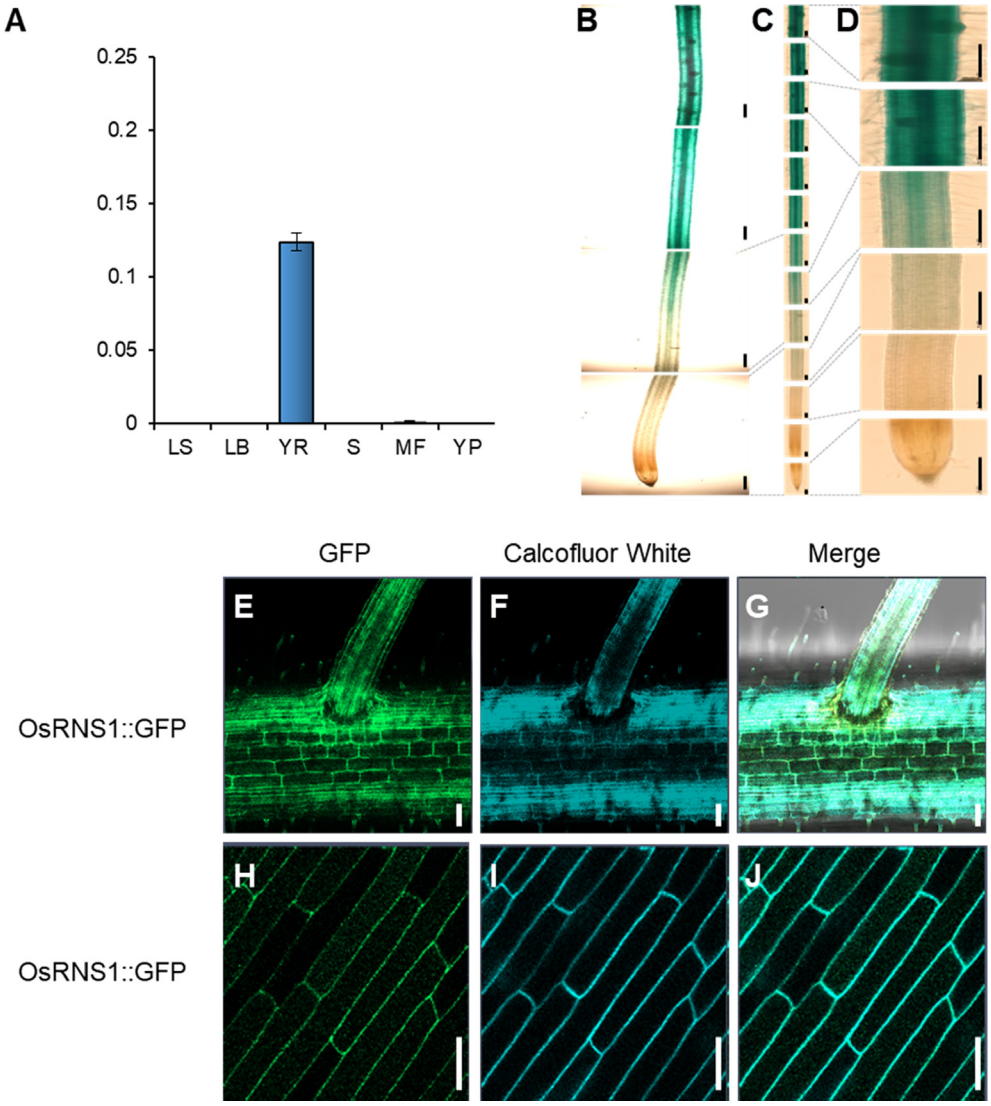

Fig. 2. Expression profiles of *OsRNS1* in anatomical tissues using RT-PCR analyses and GUS and sub-cellular localization of pOsRNS1::OsRNS1::GFP in rice transgenic plants. Anatomical expression from leaf sheath (LS), leaf blade (LB), young root (YR), seed (S), mature flower (MF), and young panicle (YP) by RT-PCR analysis was performed with four independent biological replicates (A). The *OsUbi5* gene was used as an internal control. Histochemical GUS staining in *pOsRNS1::GUS* transgenic plants: (B–D) root (scale bar: 500 μm). Transgenic plants expressing *pOsRNS1::OsRNS1::GFP* (E and H), calcofluor white strain (F and I) and overlay of the channels (G and J) showing fluorescent signals throughout the entire root and GFP expressed in the cell wall and intracellularly (A–G) (scale bar: 200 μm).

respectively, whereas that of *Per::RNS1-1* and *Per::RNS1-3* increased by 19.5% and 18%, respectively; and the number of panicles in *Ubi1::RNS1-1* and *Ubi1::RNS1-2* decreased by 7.4% and 12%, respectively, whereas that of *Per::RNS1-1* and *Per::RNS1-3* increased by 49.7% and 34.2%, respectively. However, when comparing the fertilization rate with that of the WT, *Ubi1::RNS1-1* and *Ubi1::RNS1-2* decreased by 26.1% and 45.4%, respectively, whereas that of *Per::RNS1-1* and *Per::RNS1-3* did not change significantly; when the total seeds were weighed, the weight of *Ubi1::RNS1-1* and *Ubi1::RNS1-2* decreased by 33.1% and 52.4%, respectively, whereas that of *Per::RNS1-1* and *Per::RNS1-3* increased by 42.6% and 24.9%, respectively (Table 1 and Fig. 4). In summary, the use of the root-preferred promoter for the *OsRNS1* gene showed an increase in the height, number of tillers and panicles, and total seed weight per plant compared to those of the WT. Comparatively, when using the *Ubi1* promotor, all measured agronomic traits decreased slightly, and the fertilization rate decreased significantly, which resulted in a considerable reduction in total seed weight per plant (Fig. 4). Overall, when a gene with root-preferred expression is used for overexpression analysis, the use of a root-preferred *Per* promoter is more effective in enhancing the root system and grain yield than the *Ubi1* promoter.

OsRNS1 might function as an RNase in roots

OsRNS1 encodes an RNase T2 class I enzyme in rice, which possesses RNA degradation capability [52]. To test the enzymatic role of OsRNS1, we conducted ribonuclease activity by SYTO® RNASelect™ Green Fluorescent Cell Stain (SYTO® RNASelect™) staining. SYTO® RNASelect™, which selectively labels total RNA and exhibits bright green fluorescence when bound to RNA was used in all *OsRNS1* transgenic plants. All plants were grown for 10 days, and the roots of the seedlings were stained with SYTO® RNASelect™. Total RNA levels were considerably reduced in *Ubi1::RNS1-1*, *Ubi1::RNS1-2*, *Per::RNS1-1*, and *Per::RNS1-3* compared with those of the WT, whereas two knockout mutants (*osrns1* and *OsRNS1-sgRNA*) increased the total RNAs, particularly in the root elongation region (Fig. 4i–p and Figure S4). Our result suggests that *OsRNS1* possesses RNase activity in the root.

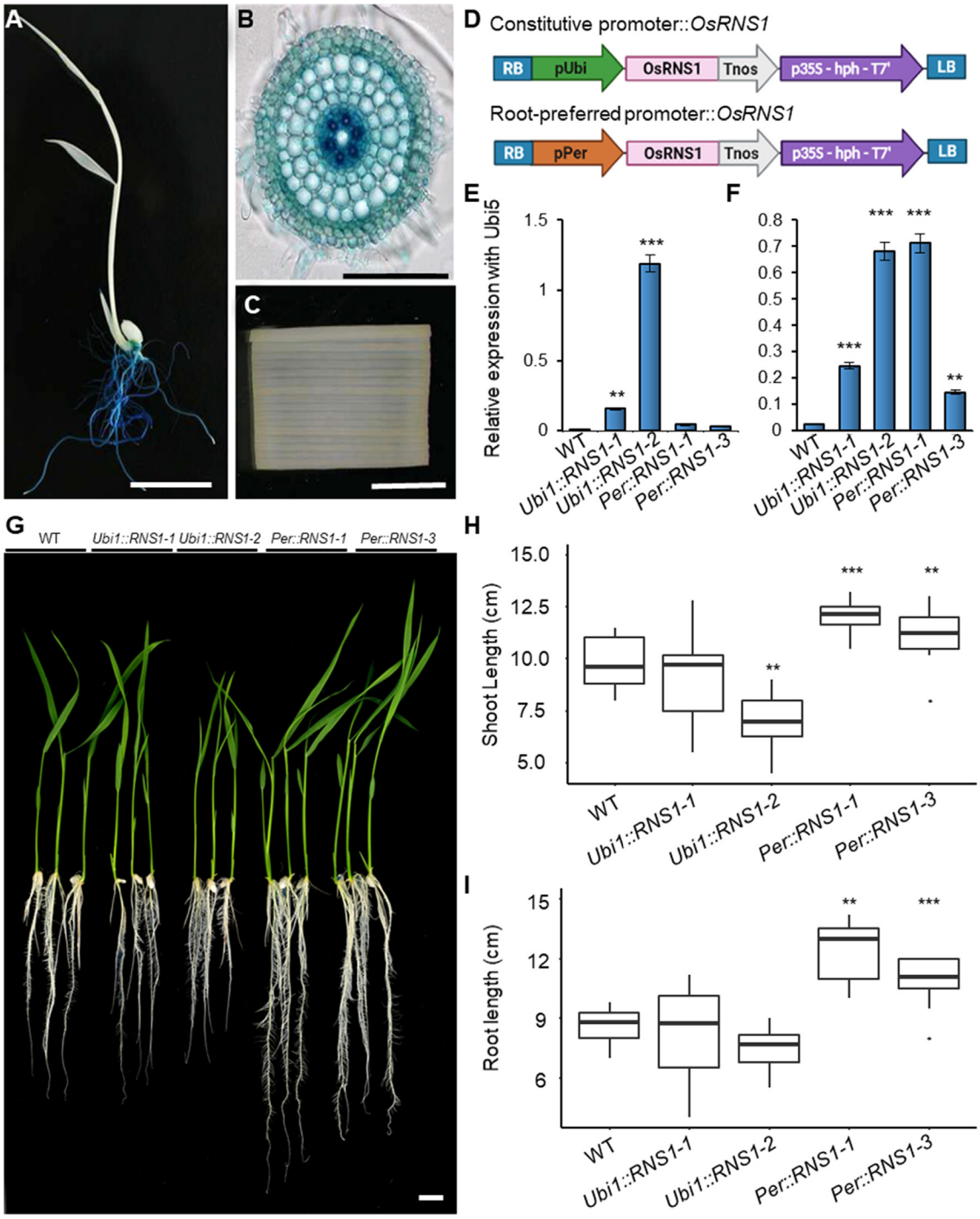

Fig. 3. Verification and application of a new root-preferred promoter expression and comparison of root dominance using two types of *OsRNS1* over-expressing transgenic plants. Histochemical staining of GUS activity under *pLOC_Os07g44440*(*pOs1-CysPrxB, Per*)::GUS transgenic plants: (A) root (scale bar: 1 cm), (B) root cross section (scale bar: 500 μm), and (C) leaf blade (scale bar: 5 mm). Schematic representation of the two types of *OsRNS1* overexpression vectors with the *Ubi1* promoter and *Per* promoter. (D) Confirmation of *OsRNS1* expression in the two types of *OsRNS1* over-expressing transgenic plants in shoot (E) and roots (F). Four biological replicates were prepared and analyzed separately. Phenotypic comparison between the WT and two types of *OsRNS1* over-expressing transgenic plants (*Ubi1::RNS1-1, Ubi1::RNS1-2, Per::RNS1-1*, and *Per::RNS1-3)* using (G) 10-day seedlings on MS media. Measurement of shoot (H) and root (I) lengths of the WT and two types of *OsRNS1* over-expressing-transgenic plants was performed with each 10 independent biological replicates. ** p-value < 0.01, and *** p-value < 0.001 were determined by the Student's *t*-test (scale bar, 1 cm).

OsRNS1 downstream genes identified through transcriptome analysis were differentially regulated by per and Ubi1 promoters

We found that the use of the root-preferred *Per* promoter for *OsRNS1* showed better performance in improving various agricultural traits than the use of the ubiquitously expressed promoter and WT. Therefore, we expect that the use of the *Per* promoter for *OsRNS1* might be differently regulated from that of the *Ubi1* promoter to enhance agronomic traits. To identify genes differentially affected by the use of the two promoters for *OsRNS1*, we performed RNA-sequencing (RNA-seq) analysis. In the analysis, we used young seedling roots after 10-day germination of the WT,

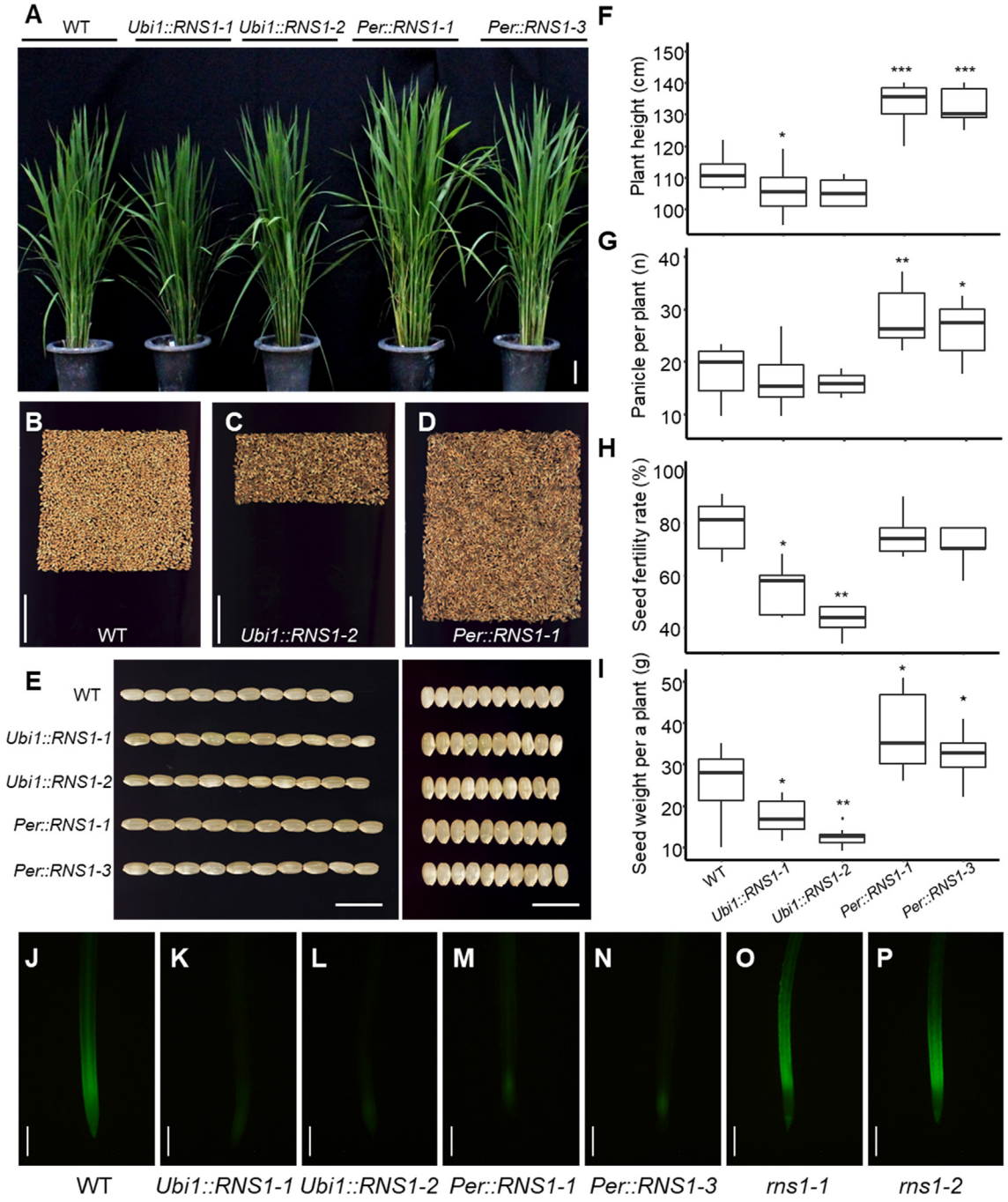

Fig. 4. Phenotype and quantitative validation of agronomic traits of *pUbi::OsRNS1* and *pPer::OsRNS1* plants grown in the paddy field and staining of total cellular RNA contents in all *OsRNS1* transgenic plants. Comparison among the phenotypes of the WT and two types of *OsRNS1* over-expressing transgenic plants (*Ubi1::RNS1-1, Ubi1::RNS1-2, Per::RNS1-1,* and *Per::RNS1-3)* at the maximum tillering stage (70 DAG) (A). Scale: 10 cm. Total seed per plant between the WT (B), *Ubi1::RNS1-2* (C), and *Per::RNS1-1* (D) plants. Scale: 10 cm. 10-seed size comparison between the WT and two types of *OsRNS1* over-expressing transgenic plants (E) (scale, 1 cm). Box plots of the agronomic traits of two independent homozygous T4 lines of *pUbi1::OsRNS1* and *pPer::OsRNS1* and corresponding WT controls in the paddy field (F–I). (F) Plant height, (G) number of panicles per plant, (H) seed fertility rate per plant, and (I) entire seed weight per plant. Measurements of the agronomic traits among the WT and two types of *OsRNS1* over-expressing transgenic plants was performed with 10 independent biological replicates. * p-value < 0.05, ** p-value < 0.01, and *** p-value < 0.001 were determined by the Student's *t*-test Box plot data samples were used from the 2019 data; the entire data region is presented in Table 1. Comparison among the total cellular RNA contents of the WT (J), two *pUbi1::OsRNS1* lines (K, L), *pPer::OsRNS1* lines (M, N), and two alleles of *osrns1* mutant plants (O and P). Whole seedlings after 10-day germination were stained with 5 μM SYTO® RNASelect™ Green Fluorescent Cell Stain, and observed using fluorescence microscopy (scale bar, 1 mm). Although we conducted experiments using five biological replicates with similar results, we presented only one dataset. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Ubi1::RNS1-2, and *Per::RNS1-1* with three biological replicates (Fig. 5a). We then identified up-regulated genes between *Ubi1::RNS1-2* vs. the WT, *Per::RNS1-2* vs. the WT, or *Per::RNS1-2* vs. *Ubi1::RNS1-2* under two criteria: >1 $\log_2$ foldchange and p-value < 0.05, and identified them using a Venn diagram. Compared to the WT, there were 1,267 and 1,598 up-regulated genes of *Per::RNS1-1* and *Ubi1::RNS1-2,* respectively, whereas 321 genes in *Per::RNS1-1* were up-regulated compared with *Ubi1::RNS1-2* (Fig. 5 and Supplemental Table S2). Transcriptomic analysis using the GO enrichment tool provides additional insights into the differential

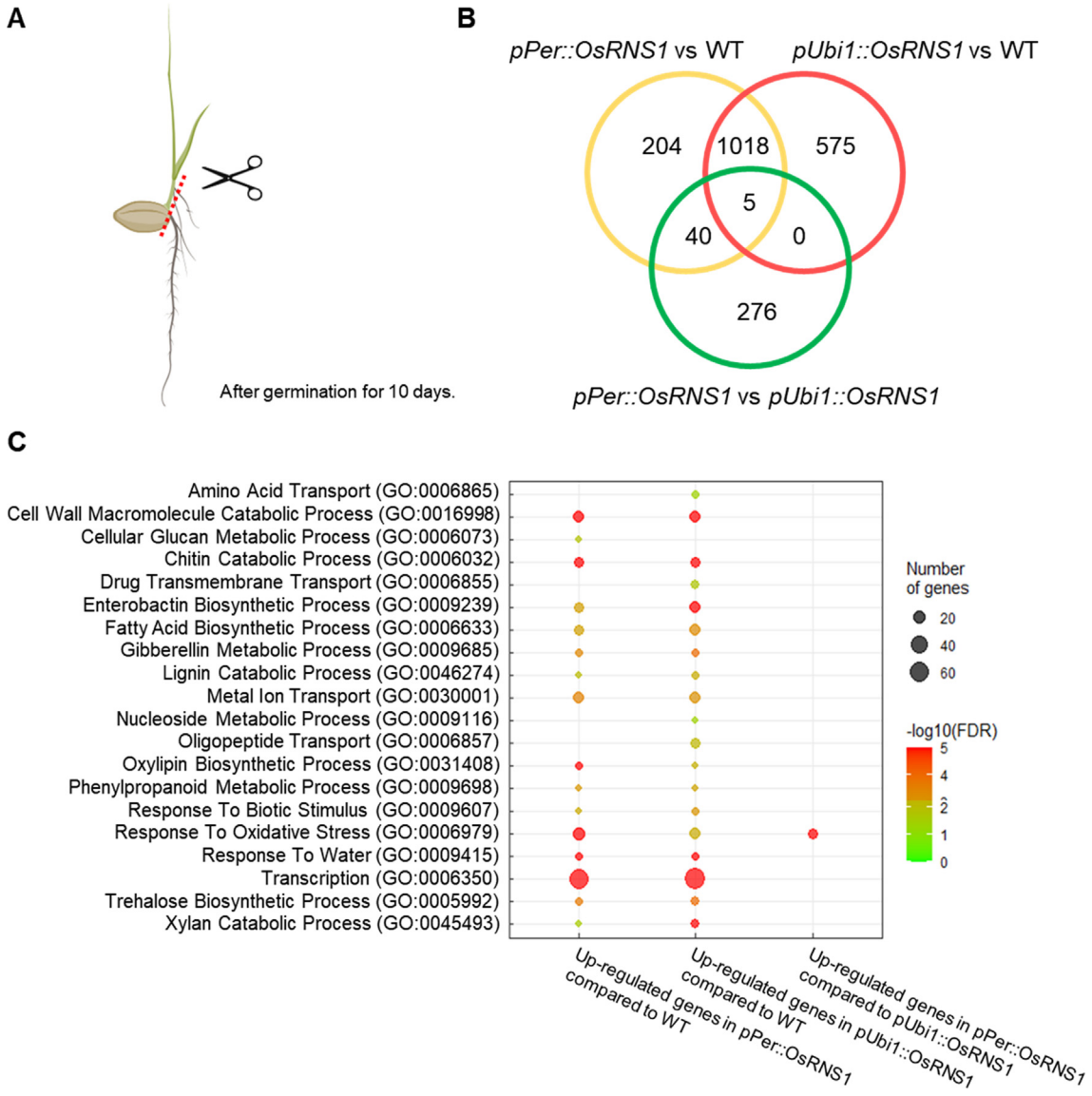

Fig. 5. Transcriptional changes between *pPer::OsRNS1*, *pUbi1::OsRNS1*, and WT roots. Schematic representation of the experimental design (A). In this analysis, we used the young seedling roots after 10-day germination of the WT, *pUbi1:OsRNS1 (Ubi1::RNS1-2),* and *pPer:OsRNS1(Per::RNS1-1)* with three biological replicates. Venn diagram representing the overlap of up-regulated genes between *Ubi1::RNS1-2* vs. WT or *Per::RNS1-1* vs. WT or *Per::RNS1-1* vs. *Ubi1::RNS1-2* under two criteria: >1 $\log_2$ fold change and p-value < 0.05 (B). The number in parentheses represents the number of up-regulated genes. Gene ontology (GO) enrichment analysis of differentially expressed genes (DEGs) (C). We selected the top 20 representative GO terms under two criteria: >4 gene number in a selected GO term and *P*-value < 0.05 and presented the resulting GO plot. The dot size indicates the number of DEGs associated with the process, and the dot color indicates the significance of enrichment (−log10 [false discovery rate corrected p-values]).

expression of genes by transcriptome analysis. In our study, we analyzed differences in GO enrichment by each promoter. We selected the top 20 representative GO terms under two criteria: >4 gene numbers in a selected GO term with a *p*-value < 0.05 and presented the resulting GO plot (Fig. 5c). From the selected 20 GO terms, "cell wall macromolecule catabolic process," "chitin catabolic process," "enterobactin biosynthetic process," "fatty acid biosynthetic process," "gibberellin metabolic process," "lignin catabolic process," "metal ion transport," "oxylipin biosynthetic process," "phenylpropanoid metabolic process," "response to biotic stimulus," "response to oxidative stress," "response to water," "transcription," "trehalose biosynthetic process," and "xylan catabolic process" are common GO terms enriched in up-regulated genes of the two over-expressing plants (*Ubi1::RNS1-2* and *Per::RNS1-1*) compared to those of WT. However, the "cellular glucan metabolic process" is only significantly enriched by *pPer::OsRNS1* in up-regulated genes, and "amino acid transport," "chitin catabolic process," "drug transmembrane transport," "nucleoside metabolic process," and "oligopeptide transport" are only enriched by *pUbi1::OsRNS1* in up-regulated genes. when we compared *Per::RNS1-1* and *Ubi1::RNS1-2*, "response to oxidative stress" was most significantly enriched in *Per::RNS1-1* compared to *Ubi1::RNS1-2* (Fig. 5c). Therefore, transcriptome data infers that "response to oxidative stress" is a common biological process up-regulated in both overexpression lines, whereas up-regulated genes of the root-preferred over-expressing lines are more strongly associated with "response to oxidative stress" than that of ubiquitously over-expressed lines.

ROS regulation is one of key factors contributing to enhanced yield in pPer::OsRNS1 lines

In the "response to oxidative stress" pathway, most of the genes encode class III peroxidases and antioxidant enzymes, which

participate in regulating ROS homeostasis. GO enrichment analyses for up-regulated genes clearly showed that ROS scavenging enzyme-related genes that were conceivably involved in ROS homeostasis regulation were significantly up-regulated in two types of *OsRNS1*-overexpression lines (*Ubi1::RNS1-2* and *Per::RNS1-1*), and more genes were up-regulated in *Per::RNS1-1* than in *Ubi1::RNS1-2* (Figure S5). To prove that *pPer::OsRNS1* regulates ROS homeostasis more strongly than *pUbi1::OsRNS1* and the WT, we used DAB, and CM-H2DCFDA, which are indicators of ROS. Although the results of DAB staining showed differences in ROS accumulation among the two over-expressing lines (*pUbi1::OsRNS1* and *pPer::OsRNS1*) and the WT, it was difficult to clearly show the difference between the two over-expressing plants (Fig. 6a–f). Examination of the ROS level using the dye in 10 DAG roots clearly revealed that signal intensities in the root epidermis of the two types of *OsRNS1*-overexpression lines indicated greatly reduced ROS levels when compared with those of the WT. In addition, the amount of ROS level decreased more in *Per:RNS1-1* and *Per::RNS1-3* than in the constitutive overexpression lines (*Ubi1::RNS1-1*and *Ubi1::RNS1-2*). We also stained the *osrns1* mutant (*osrns1-1*) with CM-H2DCFDA and found that ROS levels were slightly higher in the root epidermis than in that of the WT (Fig. 6g–r). These findings suggested that *OsRNS1* is likely to be involved in regulating ROS homeostasis and that the use of the *Per* promoter may further promote the expression of genes encoding ROS scavenging enzymes and enhance ROS regulation.

Discussion

One of the important factors to consider in developing biotech crops is the selection of a promoter to optimize the expression level and expression timing of the introduced gene. The selection of a promoter makes it possible to customize improvement of the agronomic trait according to tissue types—such as the stem, root, seed, flower—as well as plant differentiation and the growth period. Therefore, this study attempted to verify the usefulness of a new rice root-preferred promoter (*Per* promoter) to optimize the function of the target gene with root-preferred expression in rice.

OsRNS1 functions mainly in root development due to the shorter root phenotype in knockout mutants with root-preferred expression patterns (Figs. 1 and 2) [40,52]. We compared the effect of the root-preferred promoter (*pPer::OsRNS1*) with that of the ubiquitously expressed promoter (*Ubi1::RNS1*). As reported in some studies using ubiquitously overexpressed lines of other target genes, we found the similar side effects in the *pUbi1::OsRNS1* lines. However, *pPer::OsRNS1* lines showed enhanced grain yield in the normal field condition without side effects. Especially, *pPer::OsRNS1* lines displayed better performance than those using other root-preferred promoters (Figs. 3 and 4). Several recent studies have shown that the use of a root-specific promoter not only has better stress resistance but also improves agricultural traits than those of the constitutive promoters under drought [23–25,53]. The use of the *RCc3* promoter as a root-preferred promoter for applications of *OsERF71*, *OsNAC9*, and *OsNAC10* did not show significantly enhanced growth phenotypes compared to those of the WT and constitutive overexpression plants under the normal growth condition, whereas overexpression lines using the root-preferred promoters were more effective in enhancing grain yield under the drought condition compared with the constituted over-expressions [23–25,53]. However, recent studies using a similar strategy sometimes either does not work or causes growth defects. Several studies have used a root-specific promoter but have still shown growth defects or did not overcome the limitations that constitutive promoters retain [22,26,27]. For example,

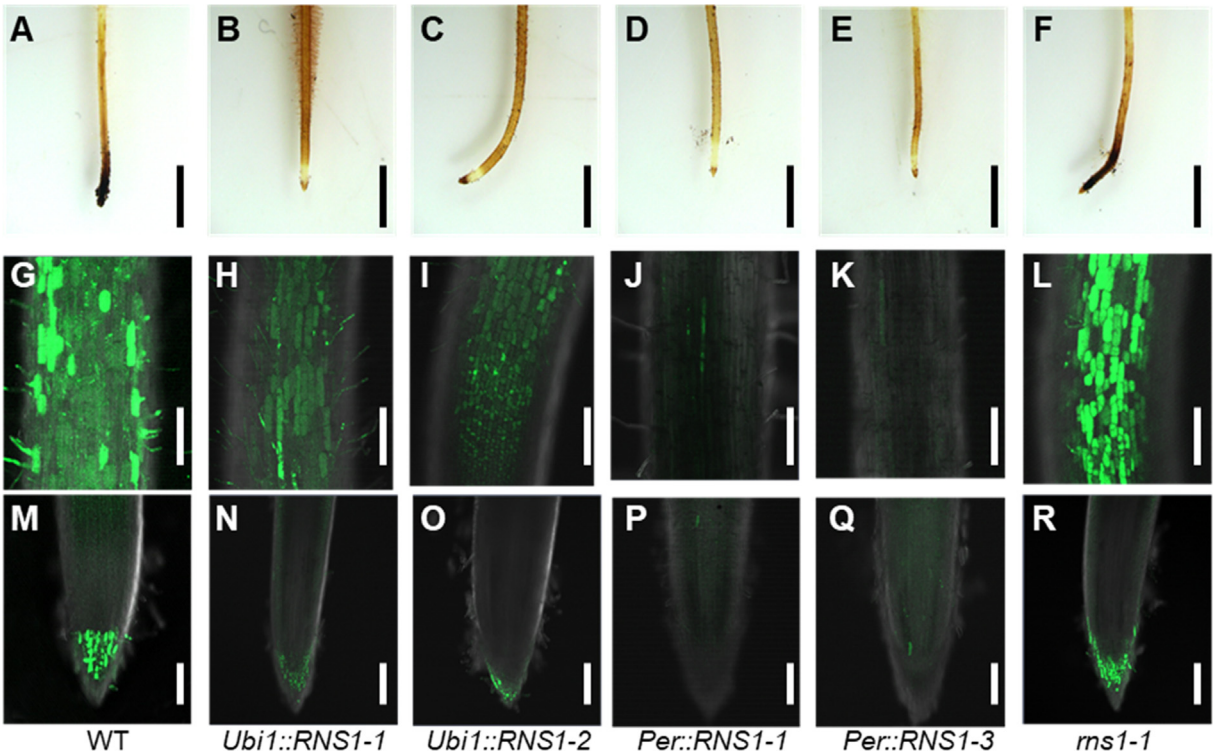

Fig. 6. Comparison of reactive oxygen species (ROS) accumulation in all *OsRNS1* transgenic plants roots. Representative images of 10-day old WT and all *OsRNS1* transgenic plants in the root epidermis stained with 3, 3′-diaminobenzidine (DAB) (A–F) and CM-H2DCFDA (G–R) to indicate reactive oxygen species (ROS) accumulation. Visualization of ROS in the primary roots of all seedlings using DAB (A–F) (scale bar: 5 mm). Visualization of ROS roots of all seedling root elongation zones (G–L) and root bottoms (M-R) using CM-H2DCFDA (scale bar: 500 μm). Although we conducted experiments using four biological replicates with similar results, we presented only one dataset.

OsCKX2, a *cytokinin oxidase/dehydrogenase*, is reported to cause plant development defects due to reduced cytokinin levels in the root when root-specific overexpression is performed using the *RCc3* promoter [27]. *OsC3H10*, a *CCCH-Zinc Finger gene*, is strongly expressed in seeds, and the expression is stimulated in response to drought, high salinity, and abscisic acid. Overexpression of *OsC3H10* with *pRCc3* showed no significant difference from the WT under drought stress, but overexpression of *OsC3H10* with *pPGD1* showed stronger drought resistance than the WT [26]. Therefore, we expect that use of the *Per* promoter showing root-preferred expression will be a useful option in future applications to enhance the agronomic traits of rice root.

Unlike previously reported promoters showing expression in part of the root region, the *Per* promoter in this study was strongly expressed throughout the entire roots and in the developing embryo, which retains an embryonic root [21] (Fig. 3a–b). Microdissection for seedling roots of *Per* promoter::GUS transgenic plants indicated that the *Per* promoter drives strong expression in the xylem and phloem as well as the exodermis, cortex, and endodermis of rice roots (Fig. 3b). The xylem and phloem are types of tissues that transport water and nutrients throughout the plant. Therefore, the use of the *Per* promoter might be a useful option for improving the function of genes with root-preferred expression involved in water absorption or nutrient absorption. On the other hand, *RCc3* promoters carry out root-preferred expressions of target genes in rice. Recent studies have shown that the *RCc3* promoter is not only strongly expressed in cortical cells and the endodermis of root but also in the phloem cell of the internode and node [54]. Therefore, the use of the *Per* promoter may limit side effects in other tissues or organs due to the specified expression patterns in the root. However, further analysis to directly compare the effects of various root-preferred promoters might give us a clearer guideline for their application.

OsRNS1 is one gene of RNase T2 class 1, and we estimated that it would play the role of an RNase in an RNA content assay (Fig. 4i–p and Figure S4). The use of a root-preferred promoter for *OsRNS1* has better agricultural traits than the WT and constitutive overexpression (Figs. 3 and 4). Thus, we tried to elucidate the molecular reason through transcriptional analysis (Fig. 5). Our transcriptomic data revealed that both of the two types of overexpression lines (*Ubi1::RNS1-2* and *Per::RNS1-1*) commonly retain several up-regulated genes belonging to *Class III Prxs,* and the transcriptome of root-preferred overexpression for *OsRNS1* additionally retains six *Class III Prxs* genes, one *peroxiredoxin* gene (*LOC_Os07g44440*), and one *glutaredoxin* gene (*LOC_Os10g34170*) as up-regulated genes (Figure S5). Of them, *Class III Prxs* plays various functions in H_2O_2 removal, cell wall hardening, cell wall component crosslinking, and defense against pathogen infection, and peroxiredoxin and glutaredoxin perform as scavengers of cellular ROS [55–59]. Thus, the use of a root-preferred promoter for *OsRNS1* was more effective in regulating ROS homeostasis.

In addition, *RNases T2* works in diverse processes, including abiotic stress, biotic stress, and oxidative stress response in various organisms [29,31,32,36,60–64]. *RNASET2* and *RNY1*, which are *RNase T2* genes of human and yeast, respectively, are involved in oxidative stress-mediated cell death and apoptosis [60,61,62]. In the case of plants, tomato T2 *RNaseLE* is induced by wounding; fungal infection; pathogen inoculation, including oxalic acid (OA) and H_2O_2; and phosphate starvation; *RNAi* lines of the T2 *RNaseLE* increased ROS and strong sensitivity through leaf necrosis during OA treatment [63,64]. Moreover, the ROS amount of the *AtRNS2* loss-of-function mutant is higher than that of the WT, and *AtRNS2* negatively controlled root hair growth by regulating RNA catabolism and ROS accumulation with RHS10 protein, which is a proline-rich receptor-like kinase [35,36]. In rice, there have been reports that ubiquitous overexpression of *OsRNS4* belonging to the same RNase T2 class 1 enhanced tolerance to high salinity and hyposensitivity to phytochrome-mediated light signals [32]. Furthermore, the expression of most *OsRNS* gene family members is up-regulated under phosphate starvation, and of them, *OsRNS1* showed root-preferred expression patterns under phosphate starvation [52]. Therefore, *OsRNS1* is likely involved in various stress responses by regulating ROS as well as playing a role as a ribonuclease.

Conclusion

Overall, when developing crops by using genetic engineering technology, the selection of the optimal promoter to determine the expression level and timing of the introduced gene seems to be very important. To optimize the function of *OsRNS1* with the root-preferred expression, we found that the use of the newly discovered *Per* promoter is more effective in improving root-related agricultural traits and grain yields than that of the constitutive promoter (*Ubi1*). Therefore, our strategy of using the *Per* promoter is a powerful tool to improve the function of root-preferred genes for enhancing crop yield. Additionally, the promoter discovered in this study has a potential for future applications for tissue specific gene editing using the CRISPR/Cas system. *OsRNS1* appears to serve as an RNase in roots and is a prime candidate for increasing the crop yield of rice in combination with the use of the root-preferred promoter.

Compliance with Ethics Requirements

All Institutional and National Guidelines for the care and use of plants were followed.

Credit Authorship Contribution Statement

Yun-Shil Gho: Conceptualization, Methodology, Data curation, Formal analysis, Investigation, Visualization, Funding acquisition, Project administration, Writing – original draft, Writing – review & editing. **Heebak Choi:** Methodology, Investigation, Visualization, Writing – original draft. **Sunok Moon:** Methodology. **Sung-Ryul Kim:** Methodology. **Sun-Hwa Ha:** Methodology. **Ki-Hong Jung:** Methodology, Investigation, Visualization, Funding acquisition, Project administration, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jare.2022.05.007.

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