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 were used for enhancing function of root preferential genes.
- Root preferential promoters in rice were 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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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, AtFDP, ArFD, 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 HvM51 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. sq/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/AACG overhangs for ligation, was annealed to at 37 °C for 60 min, denatured at 95 °C 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 (BamHI and SpeI) 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, 18509-100ML-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 Zen II 8000 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−ΔΔCt and 2−ΔΔCt 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 log2-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-
mments 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 \( P < 0.05 \) 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 \( \mu \)M SYTO\(^{\text{TM}}\) RNASelect\(^{\text{TM}}\) Green Fluorescent Cell Stain (SYTO\(^{\text{TM}}\) RNASelect\(^{\text{TM}}\)) 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 \( \mu \)M 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.

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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::OsRNS1 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 cm 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%, respectively.
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 promoter, 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).

**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 (osrs1 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.
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.
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 log₂ fold change 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
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 “xylose catabolic process” are common GO terms enriched in up-regulated genes of the two over-expressing plants (\( \text{Ubi}1::\text{RNS}1-2 \) and \( \text{Per}::\text{RNS}1-1 \)) compared to those of WT. However, the “cell wall glucan metabolic process” is only significantly enriched by \( \text{Per}::\text{OsRNS}1 \) in up-regulated genes, and “amino acid transport,” “chitin catabolic process,” “drug transmembrane transport,” “nucleosome metabolic process,” and “oligopeptide transport” are only enriched by \( \text{Ubi}1::\text{OsRNS}1 \) in up-regulated genes. when we compared \( \text{Per}::\text{RNS}1-1 \) and \( \text{Ubi}1::\text{RNS}1-2 \), “response to oxidative stress” was most significantly enriched in \( \text{Per}::\text{RNS}1-1 \) compared to \( \text{Ubi}1::\text{RNS}1-2 \) (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.

**Fig. 5. Transcriptional changes between \( \text{pPer}::\text{OsRNS}1 \), \( \text{pUbi}1::\text{OsRNS}1 \), 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, \( \text{pUbi}1::\text{OsRNS}1 \) (\( \text{Ubi}1::\text{RNS}1-2 \)), and \( \text{pPer}::\text{OsRNS}1 \) (\( \text{Per}::\text{RNS}1-1 \)) with three biological replicates. Venn diagram representing the overlap of up-regulated genes between \( \text{Ubi}1::\text{RNS}1-2 \) vs. WT or \( \text{Per}::\text{RNS}1-1 \) vs. WT or \( \text{Per}::\text{RNS}1-1 \) vs. \( \text{Ubi}1::\text{RNS}1-2 \) under two criteria: >1 log2 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 (\(-\log10 \text{false discovery rate corrected p-values}\)).

ROS regulation is one of key factors contributing to enhanced yield in \( \text{pPer}::\text{OsRNS}1 \) 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 constitutively 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 contains 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 H2O2 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 [29,31,32,60–64]. 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 H2O2; 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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