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Identification and characterization of drought-induced long noncoding RNAs (*DRILs*) in rice

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Abstract

Long noncoding RNAs (lncRNAs) act as transcriptional regulators in plants and animals. To date, they have been reported to regulate various biological processes, such as phosphate homeostasis, grain yield, and fertility in rice (*Oryza sativa* L.). However, the lncRNAs involved in abiotic stress responses remain poorly identified in rice. In this study, we analyzed the expression profiles of lncRNAs using public rice transcriptome datasets derived from abiotic stress-treated samples. We found that the expression of thousands of rice lncRNAs was significantly altered in the shoot and root tissues under different abiotic stresses (drought, high salinity, low temperature, and abscisic acid). We selected six novel drought-induced lncRNAs (*DRIL*s, specifically *DRIL1* to *DRIL6*) for further study. Real-time polymerase chain reaction analysis revealed the differential expression patterns of these *DRIL*s under various stress conditions. The expression of abiotic stress-responsive genes was upregulated in the protoplasts by transiently overexpressed *DRIL1* and *DRIL4*. Therefore, *DRIL*s may be involved in the transcriptional regulation of abiotic stress-responsive genes in rice.

Keywords: Long noncoding RNA, Abiotic stress response, Drought, Abscisic acid, High salinity, Low temperature, Rice, Protoplasts

Introduction

Innovative sequencing technologies have revealed numerous unidentified transcripts in plants. To date, thousands of noncoding RNAs (ncRNAs) in plants have been annotated, and described ncRNA loci have increased continually in the plant genome [1]. ncRNAs do not code for proteins but have key regulatory roles in various biological processes [2]. Recent studies have demonstrated the broad range of long noncoding RNA (lncRNA) functions, including chromatin remodeling and transcriptional regulation at specific genomic loci [3]. The lncRNAs are ncRNAs that are longer than 200 nucleotides and have low protein-coding potential (< 100 amino acids); they modulate the expression of neighboring genes by *cis*-action and the expression of distant genes by trans-action [4, 5].

The functions of lncRNAs in rice (*Oryza sativa* L.) have been experimentally characterized in a few studies, which demonstrated that lncRNAs are involved in critical biological processes in rice [6]. Among them, *LRK Antisense Intergenic RNA* (*LAIR*) is a natural antisense transcript (NAT) transcribed from the antisense strand of the adjacent *Leucine-rich Repeat Receptor Kinase* (*LRK*) gene cluster in rice. *LAIR* overexpression increases grain yield and upregulates the expression of *LRK* genes [7]. Additionally, *cis-NAT*$_{PHO1;2}$ regulates phosphate homeostasis and plant fitness by promoting *Phosphate 1;2* (*PHO1;2*) translation [8]. Overexpression of *NAT*$_{PHO1;2}$ leads to an increase in PHO1;2 protein levels under conditions of sufficient phosphate. Other rice NATs such as *Twisted Leaf* and *Early Flowering-Completely Dominant* regulate

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leaf development and flowering, respectively [9, 10]. Moreover, intergenic lncRNAs, including *Long-Day–Specific Male-Fertility–Associated RNA*, *An Leaf Expressed and Xoo-induced lncRNA 1,* and *Mis-shapen Endosperm*, are involved in photoperiod-sensitive male sterility, disease resistance, and endosperm development, respectively [11–13]. However, the lncRNAs related to abiotic stress responses have not yet been elucidated in rice.

Drought, high salinity, and low temperature are common abiotic stressors that adversely affect crop development and productivity. Plants have evolved molecular systems that coordinate gene expression to protect them from abiotic stress and boost their chances of survival in locations with abiotic stressors [15]. Intensive research has focused on protein-coding genes to understand the mechanisms of these stress responses and has provided a valuable platform for stress-tolerant crop development.

Here, we sought to identify abiotic stress-responsive lncRNAs via transcriptome analysis [16]. We then selected a group of unknown noncoding transcripts that were responsive to abiotic stresses and designated six of them as drought-induced long noncoding RNAs (*DRIL*s) whose induced expressions were further validated under long-term drought stress. We aimed to investigate whether *DRIL*s regulate the expression of stress-responsive genes and demonstrate that lncRNAs play essential roles in abiotic stress responses in rice.

Results and discussion

Transcriptome-wide re-analysis of abiotic stress-related lncRNAs

We re-analyzed public RNA-sequencing data to identify lncRNAs that responded to diverse abiotic stresses. Overall, 3,425 lncRNAs were induced or repressed under abiotic stress. Among them, 1,794 lncRNAs were commonly up-regulated and 1,631 were down-regulated in shoot and root samples (Fig. 1a). The heat map analysis in Fig. 1b shows the expression patterns of lncRNAs under different abiotic stress conditions. We considered the six most upregulated lncRNAs under four different abiotic stress conditions as *DRIL*s (Table 1). Five of the lncRNAs (*DRIL*1 to *DRIL*5) are intergenic, and *DRIL6* is a NAT. Schematic diagrams of the genomic locations of the *DRIL*s are shown in Additional file 1: Figure S1; the *DRIL*s had various transcript lengths. Predicted gene structures of the *DRIL*s were diagramed using the Integrative Genomics Viewer browser (Additional file 1: Figure S2). We evaluated the protein-coding potential of the *DRIL*s using the Coding Potential Calculator 2 to predict the coding potential of each transcript [17]. Five *DRIL* transcripts showed a very low coding potential score (0–0.2), similar to that of *ELENA1*, an lncRNA that has demonstrated no protein-coding potential [18]. *DRIL6* showed a high coding potential score, comparable to that of the known protein-coding gene *OsNAC14* (Additional file 1: Figure S3) [19]. Although there are no reports of NATs with protein-coding capacity, examining *DRIL6* using mutated putative ORF is necessary to prove that it is an authentic lncRNA.

*DRIL*s are abiotic stress-inducible lncRNAs

The expression patterns of *DRIL*s under abiotic stress were investigated using quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Total RNA was extracted from the leaves and roots of 15-day-old rice seedlings (*O. sativa* L. cv. Kitaake) after exposure to drought, high salinity, abscisic acid (ABA), and low temperature. Each *DRIL* responded differentially to abiotic streses. *DRIL1* expression was significantly upregulated in shoots by ABA, whereas it was highly induced in the roots by salt. *DRIL5* expression was induced by drought, ABA, and salt stress in shoots, whereas it was strongly induced in roots, especially under cold conditions. The expression of *DRIL2*, *DRIL3*, and *DRIL4* was induced under all stress conditions. To distinguish *DRIL6* expression from the sense gene, the expression of *DRIL6* and sense genes was verified using strand-specific qRT-PCR analysis. The results showed that *DRIL6* expression was induced under drought conditions. Thus, the expression of *DRIL*s was induced by various abiotic stressors (Fig. 2). Next, we examined the expression patterns of the *DRIL*s under long-term drought conditions. After drought treatment, the time-course transcript levels of *DRIL*s in the leaves were investigated using qRT-PCR analysis. *Dehydration stress-inducible protein 1* (*OsDip1*, *Os02g0669100*) was used as a marker gene for the drought response. Our results showed that the expression of all *DRIL*s gradually increased during long-term drought treatment, suggesting that all six *DRIL*s were involved in drought stress responses in rice (Fig. 3).

*DRIL*s positively regulate the expression of stress-responsive genes

To understand the transcription network that is regulated by *DRIL*s, we performed transient expression analysis using a rice protoplast system. *DRIL*s were overexpressed via the *GOS2* constitutive promoter (Fig. 4a). We performed qRT-PCR analysis using the total RNA extracted from the rice protoplasts. The results showed that the expression of stress marker genes, such as *OsWRKY71* (*Os02g0181300*), *WIH2* (*Os08g0205800*), *OsABA45* (*Os12g0478200*), and *RBBI2-3* (*Os01g0124650*), was increased by *DRIL1* overexpression (Fig. 4b). Additionally, cells that overexpressed *DRIL4* showed enhanced expression of stress marker genes, such as *WIH2*, *RGLG2* (*Os12g0288400*), *OsGDH2* (*Os04g0543900*), and *OsTPP1*

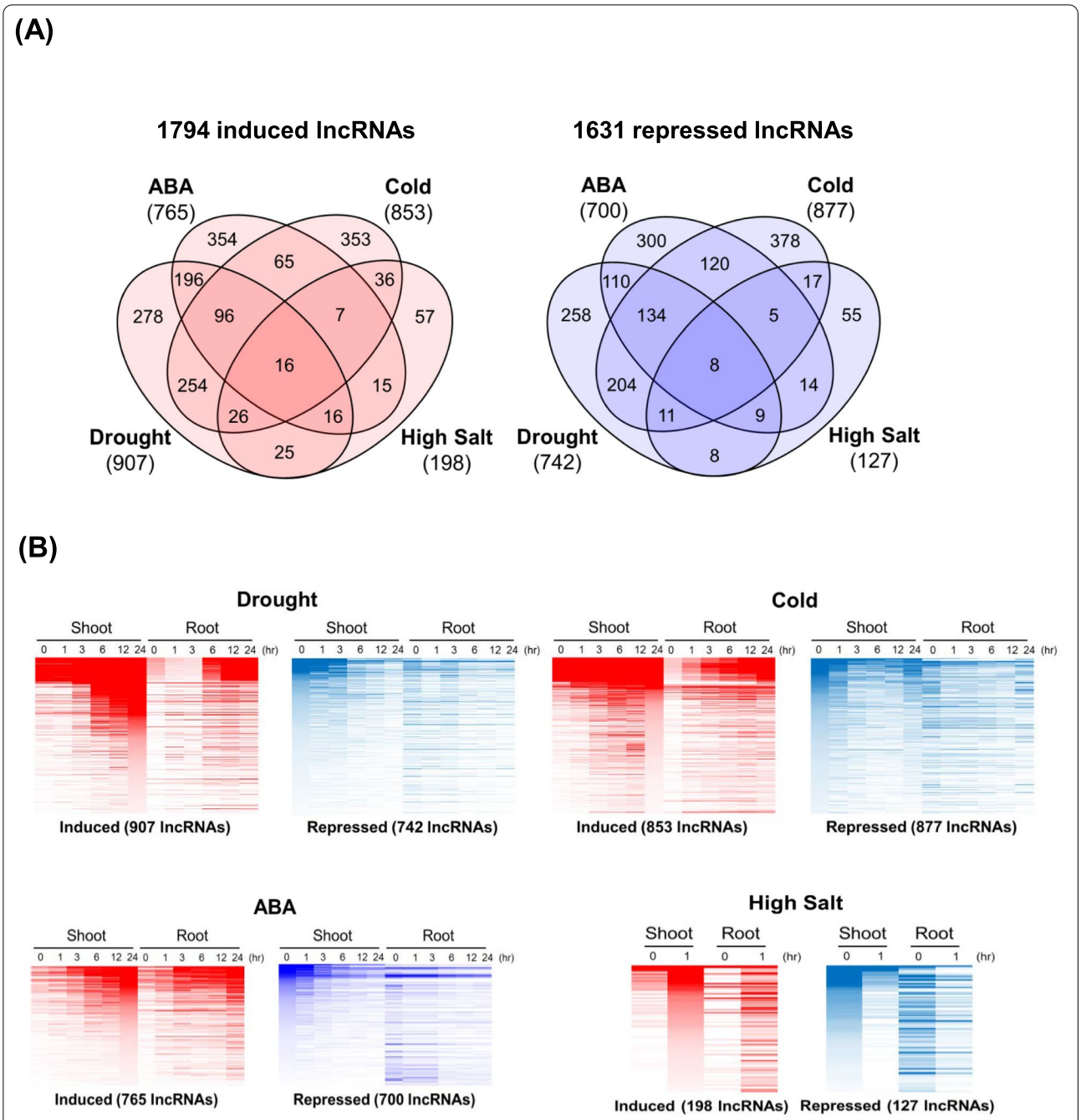


Fig. 1 Genome-wide screening of abiotic stress-related lncRNAs. **A** Venn diagrams show the number of commonly expressed lncRNAs in shoot and root samples treated with drought, cold, abscisic acid (ABA), and salt. **B** Heat map analysis shows the differential expression of lncRNAs after drought, cold (4 °C), ABA (100 μM), and high salt (150 mM NaCl) treatments

(*Os02g0661100*), compared to the control (empty vector) (Fig. 4d). These stress marker genes were noted by previously reported RNA gel blot analysis results [20] and were confirmed by qRT-PCR analysis (Additional file 1: Figure S4). *OsGDH2*, *RBBI2-3*, and *RGLG2* were expressed under drought, ABA, salt, and cold stress conditions. *OsWRKY71* and *WIH2* expression was induced by drought, ABA, and salt stress. *GEM* expression was induced under drought and low temperature, and *OsTPP1* was induced only by cold stress. We then selected a downstream gene that responded to multiple abiotic stresses as a marker gene for various patterns. To

Table 1 Six most up-regulated lncRNAs under abiotic stress conditions

Name	Genomic location	Size (bp)	Strand	Type	Flanking genes
DRIL1	Chr08:4,543,173..4,544,172	1000	−	lincRNA	Os08g0177300, Os08g0177550
DRIL2	Chr10:12,121,875..12,125,997	4123	−	lincRNA	Os10g0378450, Os10g0379100
DRIL3	Chr02:8,735,040..8,740,967	5928	−	lincRNA	Os02g0254700, Os02g0255000
DRIL4	Chr02:17,320,371..17,323,522	3152	+	lincRNA	Os02g0494000, Os02g0494400
DRIL5	Chr04:24,983,361..24,986,221	2861	−	lincRNA	Os04g0500600, Os04g0500700
DRIL6	Chr01:8,949,585..8,950,987	1403	+	NAT	

confirm whether *DRIL1* and *DRIL4* have *cis*-target genes, the expression of two neighboring genes was examined. Our results showed that there was no change in the expression levels of these neighboring genes, suggesting that *DRIL1* and *DRIL4* are trans-acting lncRNAs (Fig. 4c, e). Subsequently, we investigated the spatiotemporal expression of *DRIL1* and *DRIL4* corresponding to each developmental stage. lncRNAs exhibit tissue and cell type specificity similarly to protein-coding genes [21]. Hence, *DRIL1* showed specific expression in root tissue; however, *DRIL4* did not significantly differ, regardless of the tissue and the developmental stages (Additional file 1: Figure S5). To assess whether *DRIL6* expression changed that of the sense gene *OsDof2*, we analyzed their relative expression levels using strand-specific qRT-PCR. Our results indicated that there was a mutual activation between *DRIL6* and *OsDof2* (Fig. 4f) because *DRIL6* was the antisense transcript of the *OsDof2* transcription factor. A previous study showed that *OsDof2* has a circadian rhythm and is regulated by phytochrome signaling, which is associated with grain size in rice [22]. It has been reported that lncRNAs, as pivotal regulators, are involved in every step of gene expression. In general, lncRNAs act in *cis* or *trans*, regulating neighboring or distal target genes. For instance, lncRNAs alter gene transcription by changing chromatin modifications and guiding or sequestering transcriptional activators or repressors. Also, lncRNAs control post-transcriptional processing, such as splicing, editing, and stability of messenger RNAs. Besides, lncRNAs affect translation, localization, and stability of proteins [2, 3, 5, 6]. In this study, we demonstrated that *DRIL1*, *DRIL4*, and *DRIL6* activate the expression of stress marker genes and *cis*-target gene, respectively. Therefore, we assume that these three *DRIL*s might activate their target genes through the regulatory mechanisms mentioned above. Although the precise mechanism and function of *DRIL*s are still unknown, we expect that *DRIL*s could affect abiotic stress responses in plants. Further research should focus on functional characterization of *DRIL*s to understand the molecular mechanisms of lncRNAs in these stress responses.

Materials and methods

Plant growth conditions and stress treatments

The *O. sativa* L. *japonica* rice cultivar Kitaake was used in this study; its genetic sequence was obtained from the Phytozome v. 13 genomics resource data. For germination, dehusked rice seeds were surface-sterilized by soaking in a 70% ethanol solution for 1 min, followed by washing with a 50% sodium hypochlorite solution for 30–40 min. The seeds were then washed ten times with sterile distilled water and sown on Murashige and Skoog media. After three days, the rice was transferred to seedling culture containers (Phytohealth, 120 × 80 mm; SPL, Korea), which were used to grow the seedlings. All rice materials were grown in a growth chamber at 28 °C under a 12 h light/12 h dark photoperiod. For the stress treatment, 14-day-old seedlings were adapted to water for one day and treated for each stress condition.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from wild-type plants using a total RNA purification kit (Hybrid-R, Geneall, Korea), following the manufacturer's instructions. For cDNA synthesis, 1 μg of total RNA was reverse-transcribed using an improved reverse transcriptase (SuperiorScript II Reverse Transcriptase, Enzynomics, Korea) for 5 min at 37 °C and 60 min at 50 °C. Subsequently, the reaction

(See figure on next page.)

Fig. 2 Relative expression patterns of the *DRIL*s in response to abiotic stresses. Fifteen-day-old rice seedlings were exposed to drought (air-drying), salt (400 mM NaCl), ABA (100 μM abscisic acid), and cold (4 °C). Leaves from the rice plants were harvested at the indicated times after treatment. The left graph shows expression in the shoot, and the right graph shows expression in the root. *OsUBIQUITIN1* (*OsUbi1*) was used as an internal control for normalization. Data represent the mean value and standard deviation (n = 3). Significant differences from the mock 0 h control are indicated by asterisks (unpaired Student's *t*-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001)

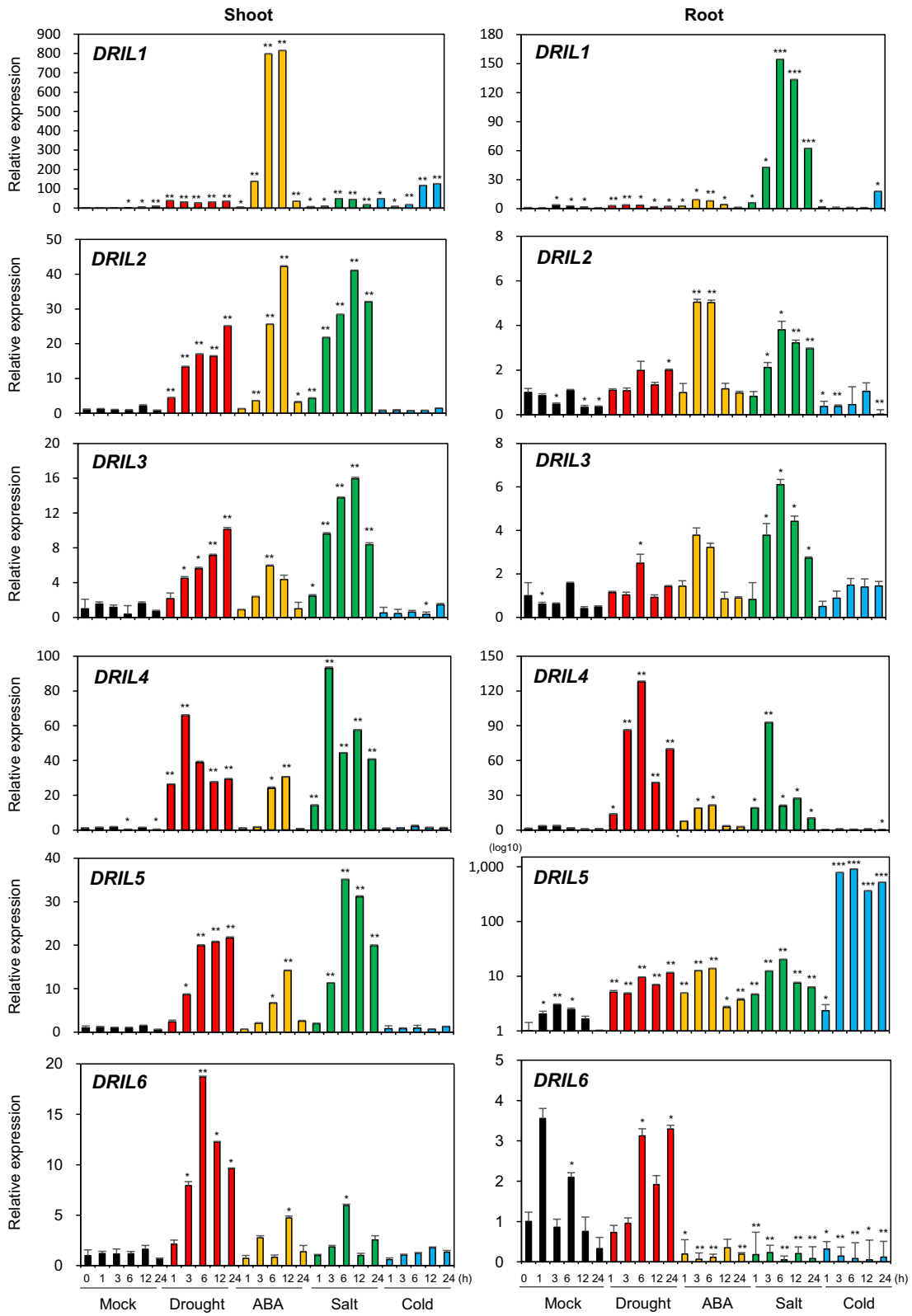


Fig. 2 (See legend on previous page.)

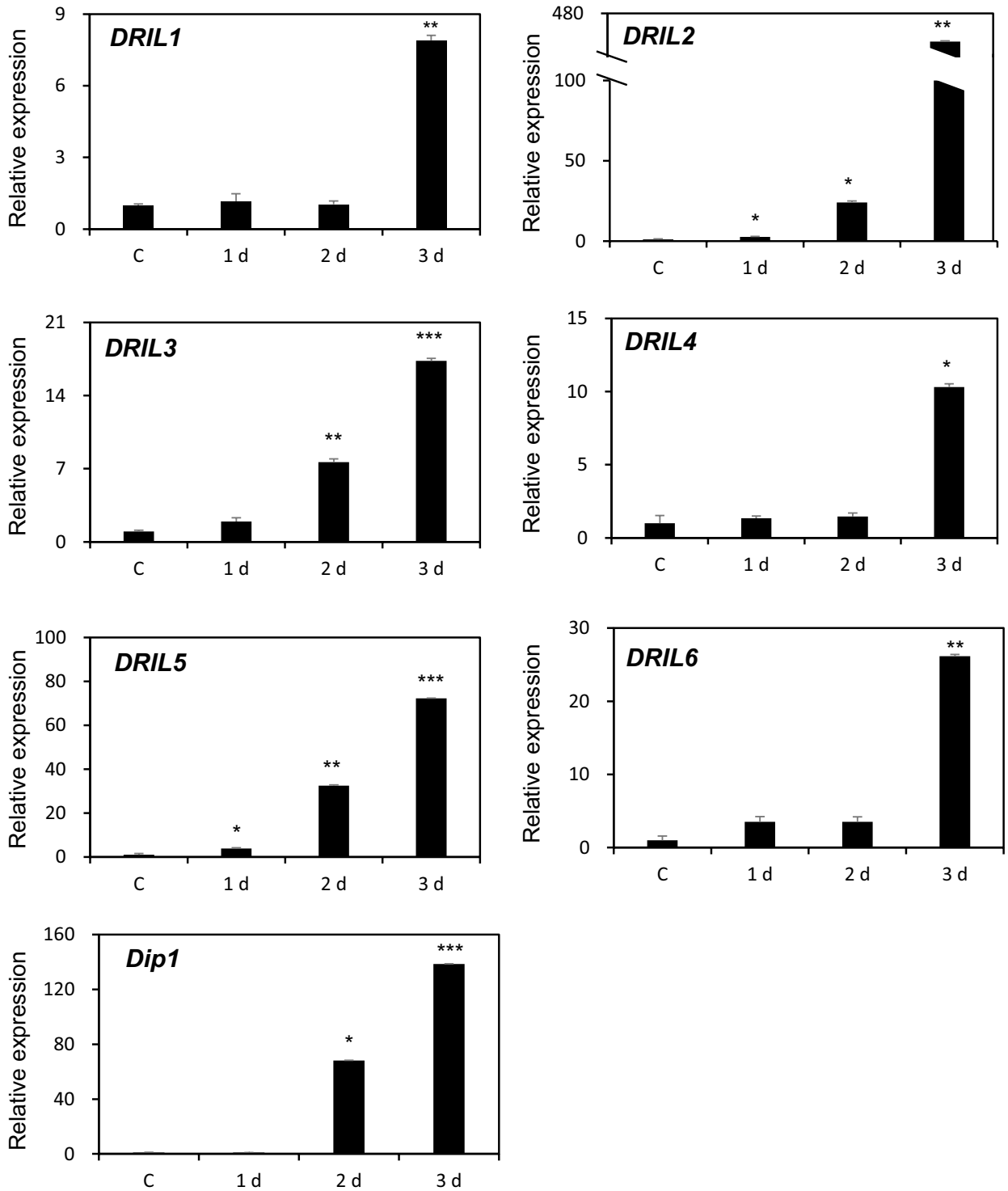
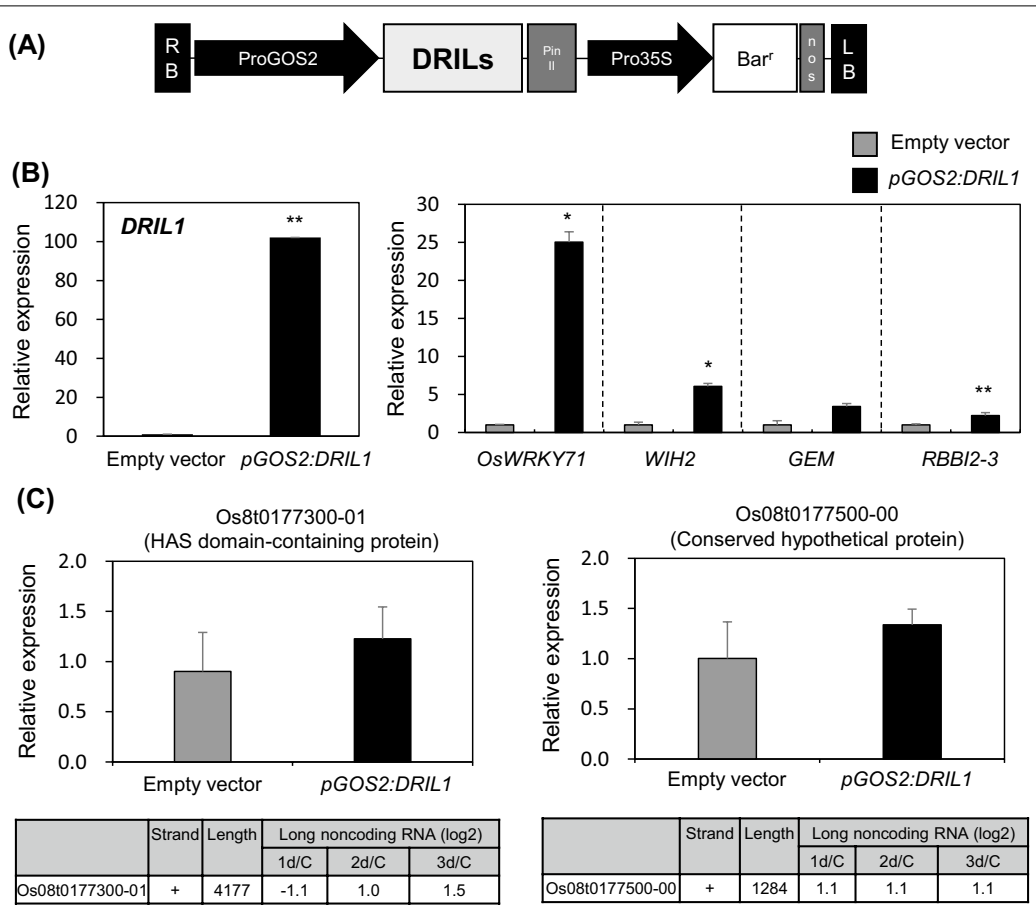


Fig. 3 Expression patterns of the *DRIL*s during long-term drought conditions. One-month-old rice seedlings were exposed to drought conditions for 3 d. Relative expression of *DRIL*s was determined via qRT-PCR analysis. *OsUbi1* was used as an internal control for normalization. Data represent the mean value and standard deviation (n = 3). Significant differences from the control are indicated by asterisks (unpaired Student's *t*-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001)



	Strand	Length	Long noncoding RNA (log2)		
			1d/C	2d/C	3d/C
Os08t0177300-01	+	4177	-1.1	1.0	1.5

	Strand	Length	Long noncoding RNA (log2)		
			1d/C	2d/C	3d/C
Os08t0177500-00	+	1284	1.1	1.1	1.1

Fig. 4 Overexpression of the *DRIL*s in rice protoplasts. **A** Schematic diagram of the transient overexpression constructs of the *DRIL*s. In the constructs, the *DRIL*s are under the *GOS2* promoter and *Pin*II terminator. The binary vector was generated through the gateway system. **B** *DRIL1* overexpression and the expression levels of stress marker genes. *OsUbi1* was used as an internal control for normalization. **C** The expression of the putative *cis*-target genes of *DRIL1*. Numeric values in Tables indicate fold changes derived from RNA-sequencing with long-term drought stress-treated samples. d, drought; C, control. **D** *DRIL4* overexpression and the expression levels of stress marker genes. **E** The expression of the putative *cis*-target genes of *DRIL4*. Numeric values in Tables indicate fold changes derived from RNA-sequencing with long-term drought stress-treated samples. d, drought; C, control. **F** Overexpression of *DRIL6* and *OsDof2*. The green bars represent *OsDof2* overexpression, and the yellow bars represent *DRIL6* overexpression (unpaired Student's *t*-test, *P ≤ 0.05, **P ≤ 0.01)

was terminated by incubating for 10 min at 70 °C. qRT-PCR was performed (using the 2X Real-Time PCR Smart Mix, SRH72-M10h, SolGent, Korea). The reaction was performed at 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 20 s, and 70 °C for 30 s, using an qRT-PCR machine (AriaMx, Agilent, USA). *OsUbi1* (*Os06g0681400*) was used as an internal control for normalization. Three replicates were analyzed for quantitative experiments. The primer sequences used for qRT-PCR are listed in Additional file 2: Table S1.

Strand-specific reverse transcription

An adapter-mediated qRT-PCR assay was used to distinguish strand-specific expression, as stated previously [23]. We used synthesized cDNAs with a antisense *DRIL6- or Dof2-* specific reverse primer and including a tag sequence (Additional file 2: Table S1). Subsequently, qRT-PCR was performed with the tag-specific primer and *DRIL6- or Dof2*-specific forward primer.

Protoplast isolation and transient gene expression

A polyethylene glycol (PEG)–mediated protoplast transformation method was used to transiently express *DRIL*s and verify the relationship between *DRIL*s and stress marker genes [24, 25]. Leaf sheaths of 100 rice seedlings were cut into 0.5 mm pieces using a sharp blade on glass. The pieces were transferred into 0.6 M mannitol solution and incubated for 20 min at room temperature in the dark. After removing the mannitol solution, the pieces were soaked in enzyme solution consisting of 1.5%

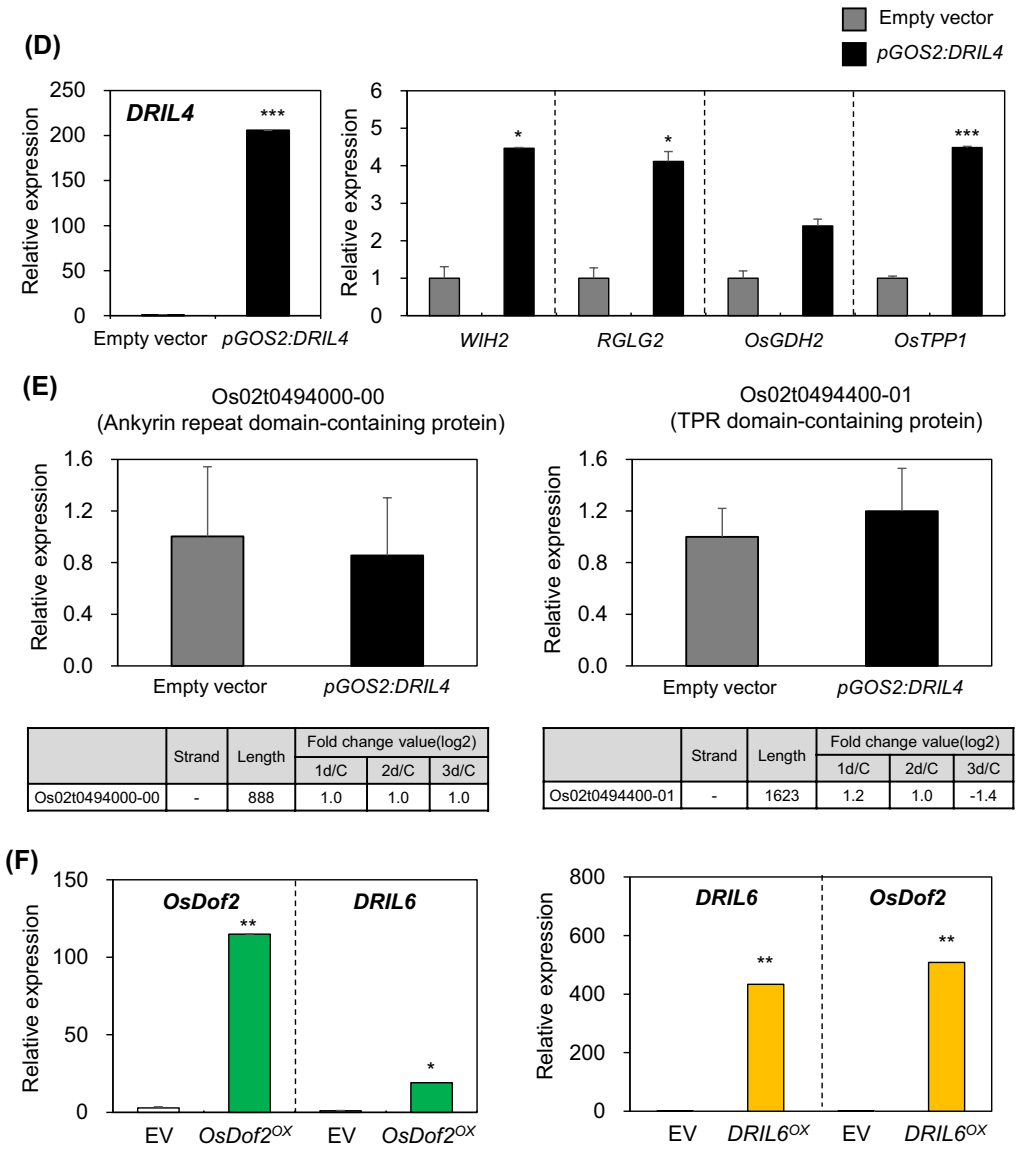


Fig. 4 continued

Cellulase R-10 (Yakult, Japan), 0.75% Macerozyme R-10 (Yakult, Japan), 0.5 M mannitol, 10 mM MES (pH 5.7), 0.1% BSA, 10 mM $CaCl_2$, and 5 mM β-mercaptoethanol for cell wall degradation. Vacuum infiltration was repeated thrice for 15 min to apply the enzyme solution. Digestion was performed in a dark chamber with gentle shaking for 4 h. The solution was filtered twice through 70 μm and 40 μm nylon meshes (Falcon, USA) and centrifuged at $150 \times g$ for 3 min. The protoplast pellet was resuspended in W5 solution, which contained 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES (pH 5.7). The protoplast concentration was measured under a microscope using a hemocytometer and was adjusted to 7×10^7 protoplasts/mL. To transfect DNA into cells, 50 μL of the protoplast solution (2×10^6 cells) was mixed with 15 μL of plasmid (1 μg) and 130 μL of PEG solution, which contained 0.2 M mannitol, 100 mM $CaCl_2$, and 40% w/v PEG4000. The mixture was incubated for 15 min in the dark; subsequently, 1 mL of W5 solution was added. The mixture was then centrifuged at $300 \times g$ for 2 min to collect protoplasts, which were resuspended in an incubation solution containing 0.5 M mannitol, 20 mM KCl, and 4 mM MES (pH 5.7) for 12 h. Protoplasts were harvested by centrifugation at $300 \times g$ for 2 min and used for RNA extraction and qRT-PCR.

Abbreviations
ABA: Abscisic acid; *DRIL*s: Drought-induced long noncoding RNAs; lncRNAs: Long noncoding RNAs; NAT: Natural antisense transcript; ncRNAs: Noncoding RNAs; PEG: Polyethylene glycol; qRT-PCR: Quantitative real-time polymerase chain reaction.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-022-00751-5.

Additional file 1: Figure S1. Schematic diagram of the genomic locations of the *DRIL*s. **Figure S2.** Visualizations of the RNA-sequencing reads in the Integrative Genomics Viewer browser. These show an increasing pattern as time elapsed. **Figure S3.** Coding potential scores of the *DRIL*s. This was analyzed by using the Coding Potential Calculator 2 to predict the coding potential of each transcript. *ELENA1* was used as the control for lncRNA, and *OsNAC14* was used as the control for protein-coding RNAs. **Figure S4.** Relative expression patterns of stress marker genes in response to abiotic stressors. Fifteen-day-old seedlings (*Oryza sativa L*. japonica cv. Kitaake) were exposed to drought (air-drying), salt (400 mM NaCl), ABA (100 μM abscisic acid), and cold (4 °C). *OsUbi1* was used as an internal control for normalization. Data represent the mean value and standard deviation (n=3). Significant differences from the mock 0 h control are indicated by asterisks (unpaired Student's t-test, *P ≤ 0.05, **P ≤ 0.01, ***P ≤0.001). **Figure S5.** *DRIL1* and *DRIL4* expression at each developmental stage. qRT-PCR analysis of *DRIL* expression in rice tissues at different developmental stages.

Additional file 2: Table S1. List of primer sequences.

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Author contributions
NO and CJ designed the study. NO performed the experiments. PJ assisted with bioinformatics analysis. JL, JKS, JHK, and HSC interpreted and discussed the data. NO, JSS, and CJ wrote the manuscript and prepared the figures. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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