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Research article

The rice ethylene response factor OsERF83 positively regulates disease resistance to *Magnaporthe oryzae*

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ABSTRACT

Rice blast caused by *Magnaporthe oryzae* is one of the most destructive diseases of rice (*Oryza sativa*) worldwide. Here, we report the identification and functional characterization of a novel ethylene response factor (ERF) gene, *OsERF83*, which was expressed in rice leaves in response to rice blast fungus infection. *OsERF83* expression was also induced by treatments with methyl jasmonate, ethephon, and salicylic acid, indicating that multiple phytohormones could be involved in the regulation of *OsERF83* expression under biotic stress. Subcellular localization and transactivation analyses demonstrated that OsERF83 is a nucleus-localized transcriptional activator. A gel-shift assay using recombinant OsERF83 protein indicated that, like other ERFs, it binds to the GCC box. Transgenic rice plants overexpressing *OsERF83* exhibited significantly suppressed lesion formation after rice blast infection, indicating that OsERF83 positively regulates disease resistance in rice. Genes encoding several classes of pathogenesis-related (PR) proteins, including PR1, PR2, PR3, PR5, and PR10, were upregulated in the *OsERF83*ox plants. Taken together, our findings show that OsERF83 is a novel ERF transcription factor that confers blast resistance by regulating the expression of defense-related genes in rice.

1. Introduction

Rice (*Oryza sativa* L.) is a staple crop for more than half of the world's population (Khush, 2005). Rice blast, caused by the filamentous ascomycete fungus *Magnaporthe oryzae*, severely diminishes rice production across a wide area of the globe, and is considered to be among the ten most important fungal plant pathogens based in terms of its economic relevance (Dean et al., 2012). The enhancement of blast resistance in rice will therefore contribute to sustainably enhancing global food security.

M. oryzae infection occurs in the aerial tissues, including the leaves, stems, nodes, and panicles, throughout rice development (Wilson and Talbot, 2009). This hemibiotroph forms a specialized infection structure called an appressorium, a dome-shaped cell responsible for the mechanical penetration of the rice cuticle to facilitate its entry into the plant cells (Howard et al., 1991). Colonization within the host progresses by the cell-to-cell movement of hyphae through the plasmodesmata, and lesions become visible between 72 and 96 h after infection (Kankanala et al., 2007). The fungus sporulates from the disease lesions under conditions of high humidity, enabling its rapid spread to neighboring plants by wind and dewdrop splash (Talbot, 2003).

Upon rice blast infection, the host plant recognizes conserved pathogen-associated molecular patterns (PAMPs) and activates an immune response to inhibit the colonization of the invading organism (Liu et al., 2013). This PAMP-triggered immunity (PTI) controls the deposition of lignin, the biosynthesis of diterpene phytoalexins, and the expression of pathogenesis-related (*PR*) genes (Kawano et al., 2014) through complex signal transduction mechanisms such as MAPK cascades (Kishi-Kaboshi et al., 2010; Lieberherr et al., 2005). The biosynthesis of salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are also activated to regulate the defense responses upon PAMP perception (Dong, 1998; Pieterse and Van Loon, 1999; Reymond and Farmer, 1998). SA is generally involved in the defense against biotrophs

Abbreviations: EDTA, ethylenediaminetetraacetic acid; ET, ethylene; ERF, ethylene response factor; JA, jasmonic acid; MeJA, methyl jasmonate; PAMP, pathogen-associated molecular pattern; PTI, PAMP-triggered immunity; PR, pathogenesis related; SA, salicylic acid; TBE-PAGE, tris-borate-EDTA/polyacrylamide gel electrophoresis; TF, transcription factor

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or hemibiotrophs, while JA and ET play important roles in the defense against necrotrophs (De Vos et al., 2005; Glazebrook, 2005; Rojo et al., 2003).

During blast infection, rice cells undergo a wide range of transcriptional changes (Li et al., 2006; Mosquera et al., 2009). Previous studies have shown that different types of transcription factors (TFs), such as bHLHs (Kim et al., 2012), NACs (Sun et al., 2013), and WRKYs (Pandey and Somssich, 2009), are involved in rice defense responses. Ethylene response factors (ERFs) are a subfamily of the plant-specific AP2/ERF TF superfamily and each possess a single AP2/ERF domain, a DNA-binding domain containing 60 to 70 amino acid residues (Riechmann et al., 2000). The first ERF was identified in tobacco (*Nicotiana tabacum*) as a protein that binds to the promoter region of ethylene-responsive genes (Ohme-Takagi and Shinshi, 1995). In rice, 163 AP2/ERF family genes have been identified, of which 77 belong to the ERF subfamily (Sharoni et al., 2011). Several *OsERF* genes are involved in responses to abiotic stresses such as drought, salt, and submergence (Hattori et al., 2009; Jung et al., 2010; Lee et al., 2016; Schmidt et al., 2013; Wan et al., 2011; Yu et al., 2017; Zhang et al., 2013). Although many reports have associated ERFs with defense responses based on their expression patterns (Cao et al., 2006; Li et al., 2006; Mosquera et al., 2009), very few rice ERFs have been shown to contribute to disease resistance in vivo (Liu et al., 2012).

In this study, we isolated a novel ERF gene, *OsERF83*, expressed in blast-infected rice leaves, and revealed that it functions as a transcriptional activator. *PR* genes were upregulated in transgenic plants overexpressing *OsERF83*, which enhanced their resistance to *M. oryzae*.

2. Material and methods

2.1. Phylogenetic analysis

Full-length amino acid sequences of *Arabidopsis thaliana* and *O. sativa* ERF proteins were collected from TAIR (https://www.arabidopsis.org/) and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/), respectively. The accession numbers of the gene models described in the phylogenetic tree are: At4g18450.1 (*AtERF91*), At3g23240.1 (*ERF1*), At2g31230.1 (*AtERF15*), At1g06160.1, (*ORA59*), At3g23220.1 (*AtERF95*), At5g43410.1 (*AtERF96*), At1g04370.1 (*AtERF14*), At3g23230.1 (*TDR1*), LOC_Os03g64260.1 (*OsERF83*), LOC_Os05g49010.1 (*OsERF84*), LOC_Os05g37640.1 (*OsERF85*), LOC_Os07g22770.1 (*OsERF86*), LOC_Os09g39850.1 (*OsERF87*), LOC_Os03g05590.1 (*OsERF88*), LOC_Os10g30840.1 (*OsERF89*), LOC_Os08g44960.1 (*OsERF90*), LOC_Os09g39810.1 (*OsERF123*), LOC_Os04g18650.1 (*OsERF128*), and LOC_Os07g22730.1 (*OsERF136*). The amino acid sequence alignment and phylogenetic analysis were performed using ClustalW (http://clustalw.ddbj.nig.ac.jp/). The neighbor-joining method was used to create the phylogenetic tree.

2.2. Preparation of spore suspension

M. oryzae (race: 007) which is compatible with rice cultivars with *Pia* was obtained from Dr. Nakashita at Fukui Prefectural University (Yasuda et al., 2017). The strain was cultured for three weeks on a potato dextrose agar (Difco) plate in the dark at 25 °C, then resuspended in distilled water using a paint brush and inoculated onto an oatmeal agar plate (Difco). After incubation for 3 d in the dark at 25 °C, the plate was irradiated with a blacklight blue lamp for 2 d at 25 °C to induce sporulation. A 0.05% Tween 20 solution was poured onto the oatmeal agar plate, and the spores were collected using a paint brush. The concentration of the spore suspension was adjusted to 1.0×10^5 spore/mL with 0.05% Tween 20 before being used to inoculate the rice.

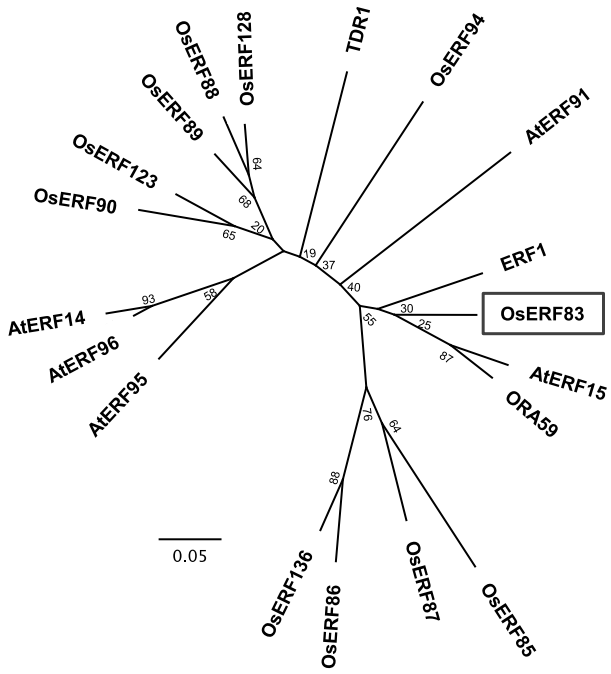


Fig. 1. Phylogenetic analysis of the subgroup IXc ERFs in *Arabidopsis* and rice. The amino acid sequences of the AP2/ERF domains were aligned using ClustalW, and the phylogenetic tree was created using the neighbor-joining method. The bar represents the evolutionary distance between the proteins expressed as the number of substitutions per amino acid residue. The bootstrap values (1000 replicates) are shown in percentage.

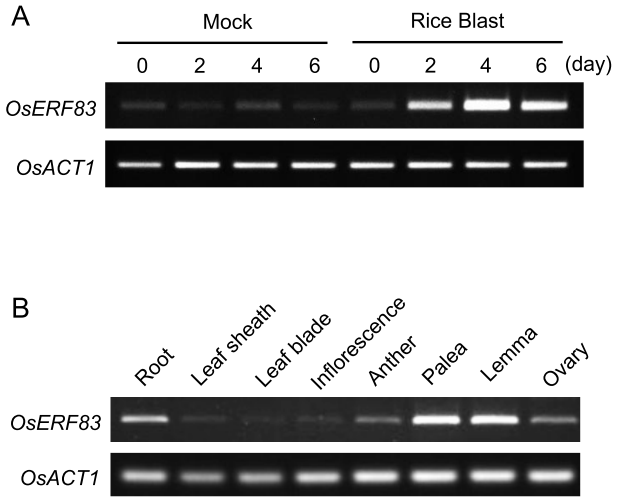


Fig. 2. Expression profile of *OsERF83*. (A) Expression of *OsERF83* under rice blast infection. Hydroponically grown seedlings were sprayed with a rice blast spore suspension (in 0.05% Tween 20) or 0.05% Tween 20 (mock). The total RNA was extracted from the shoots at the indicated time points after inoculation. (B) Organ-specific expression of *OsERF83*. Total RNA was isolated from the root, leaf sheath, leaf blade, inflorescence, anther, palea, lemma, and ovary. For (A) and (B), semi-quantitative RT-PCR was performed using *OsERF83*-specific primers. *OsACT1* expression was used as the endogenous control.

2.3. Plant materials and growth conditions

Oryza sativa (L.) cultivar Yukihikari was used in this study. Yukihikari carries the *Pia* resistance gene and is susceptible to *M. oryzae* (race: 007). The seeds were surface-sterilized with 70% ethanol for

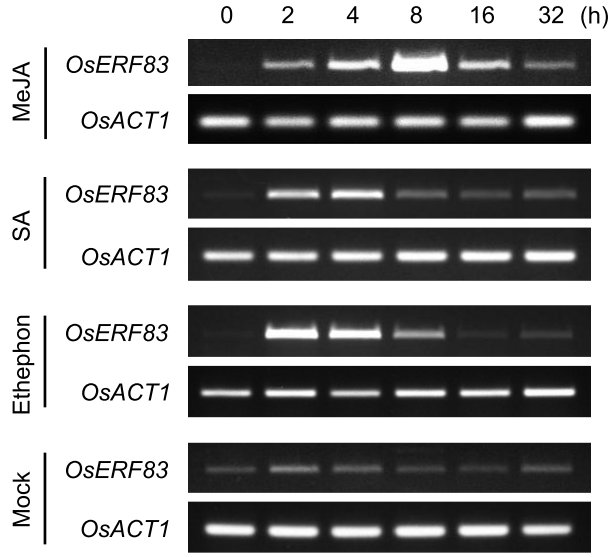


Fig. 3. Induction of *OsERF83* in response to exogenous phytohormones. Hydroponically grown 14-d-old seedlings were sprayed with methyl jasmonate (MeJA; 100 μM), salicylic acid (SA; 5 mM), ethephon (1 mM), or a mock solution (0.05% Tween 20). Total RNA was extracted from the shoot tissue at the indicated time points. Semi-quantitative RT-PCR was performed using *OsERF83*-specific primers. *OsACT1* was used as the endogenous control.

5 min and with 2% sodium hypochlorite for 30 min, then germinated for 1 d in the dark at 25 °C. The germinated seeds were evenly distributed onto a plastic mesh grid supported by a plastic container filled with sterilized water and grown in a growth chamber at 25 °C under continuous illumination. For the inoculation assay, the 12-d-old hydroponically grown rice seedlings were sprayed with the spore suspension of *M. oryzae*, and then placed in a plastic box to maintain a high level of humidity.

2.4. Phytohormone treatments

The 14-d-old hydroponically grown seedlings were sprayed with 100 μM methyl jasmonate (MeJA) (Wako, Osaka, Japan), 5 mM SA (Nacalai Tesque, Kyoto, Japan), or 1 mM ethephon (Sigma-Aldrich, St Louis, MO, USA), all of which were dissolved in a 0.05% Tween 20 solution. The shoots were sampled 0 h, 2 h, 4 h, 8 h, 16 h, and 32 h after the treatment.

2.5. Semi-quantitative RT-PCR

Total RNA was extracted from the shoot tissues using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA was synthesized from 500 ng total RNA using the High Capacity RNA-to-cDNA kit (Thermo Fisher Scientific, Waltham, MA, USA). Semi-quantitative RT-PCR was performed using ExTaq (Takara Bio). A partial *OsERF83* fragment was amplified using primers 5′-CATGTCGCTTCATCCTCACC-3′ and 5′-AGGTAGTCAGGTCCCAGGTC-3'. The following conditions were used: 98 °C for 30 s, followed by 33 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 30 s. The housekeeping gene *OsACTIN1* (*OsACT1*) was amplified with primers 5′-TCCATCTTGGCATCTCTCAG-3′ and 5′-GTACCCTCATCAGGCATCTG-3′ under the conditions: 98 °C for 30 s, followed by 26 cycles of 98 °C for 10 s, 55 °C for 15 s, and 68 °C for 30 s.

2.6. Subcellular localization analysis

The 35S promoter region and a synthetic DNA sequence encoding green fluorescent protein with a S65T mutation (sGFP) were cut out from the GATEWAY binary vector pGWB5 (Nakagawa et al., 2007) using *Hind*III and *Sac*I and cloned into pUC19 (Takara Bio, Kusatsu, Japan). The nopaline synthase terminator region from pAct-ZH2 was also cloned into pUC19 using the *Sac*I and *Eco*RI restriction sites. The resulting plasmid, pUC-GWGFP, contained the *sGFP* gene located downstream of the *attR* sequence from the GATEWAY cassette. The open reading frame (ORF) of *OsERF83* without the stop codon was amplified using primers 5′-AAAAAGCAGGCTATGCATTGCTGCATGTCG-3′ and 5′-AGAAAGCTGGGTAGATGGAGTGGTGGCTTG-3′, cloned into pDONR221 (Thermo Fisher Scientific), then introduced into the expression vector pUC-GWGFP using a LR reaction. The resulting plasmid, pUC-ERF83GFP, expresses the OsERF83 protein fused with sGFP at its C-terminus. pUC-ERF83GFP and pUC-GWGFP were transformed into onion (*Allium cepa* L.) epidermal cells using a PDS-1000 biolistic delivery system (Bio-Rad Laboratories, Hercules, CA, USA) and transiently expressed. The GFP signals were monitored using a fluorescence microscope (Leica DM6000B; Leica Microsystems, Wetzlar, Germany) equipped with a GFP filter.

2.7. Transactivation assay in yeast cells

The *OsERF83* ORF was amplified and cloned into the pEG202 plasmid (Gyuris et al., 1993) using primers 5′-GAATTCATGCATTGCTGCATGTCGC-3′ and 5′-CTCGAGTCAGATGGAGTGGTGGCTTG-3'. The resulting vector facilitates the constitutive expression of *LexA*-fused *OsERF83* under the control of the *ALCOHOL DEHYDROGENASE 1* (*ADH1*) promoter. $B42_{AD}$ and *OsACT1* were also introduced into pEG202 as the positive and negative control, respectively. *Saccharomyces cerevisiae* EGY48 (*MATα*, *ura3*, *his3*, *trp1*, *LexA*(op(x6))-*LEU2*) containing pJK103, a *LacZ* reporter plasmid, was used as the host strain. All plasmids were transformed into the host strain using the lithium acetate method (Schiestl and Gietz, 1989). The transactivation activity was evaluated in terms of leucine auxotrophy and β-galactosidase activity on an SD medium lacking uracil, histidine, and leucine.

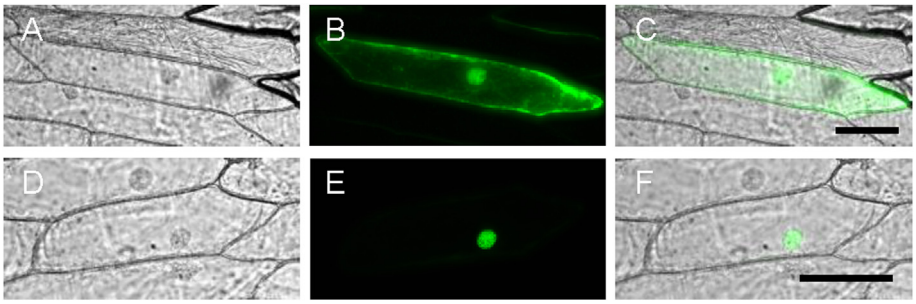


Fig. 4. Subcellular localization of OsERF83 in onion epidermal cells. Micrographs of onion epidermal cells transiently expressing free *GFP* (A, B) or *OsERF83-GFP* (D, E). (C, F) Merged bright field and fluorescence images. Scale bars represent 50 μm.

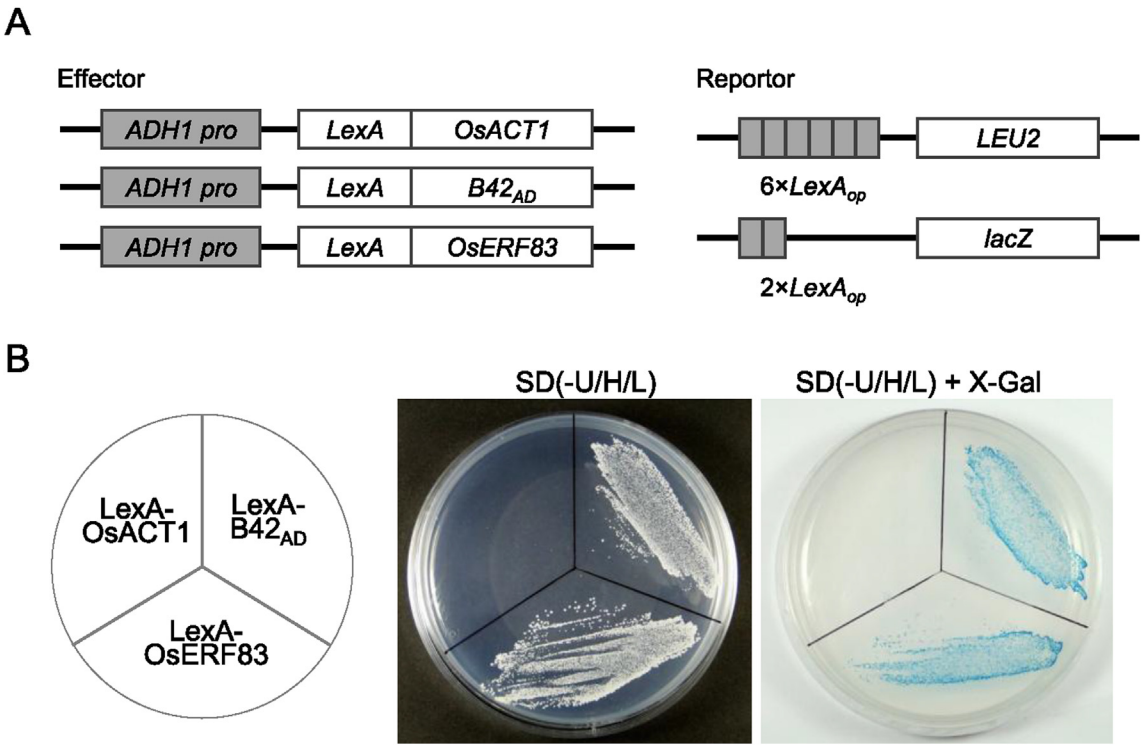


Fig. 5. Transactivation activity of OsERF83. (A) Schematic models of the effector and reporter genes. LexA was used as a DNA-binding domain. *OsACT1* and $B42_{AD}$ were used as negative and positive controls, respectively. (B) Transactivation activity of OsERF83, performed using a yeast two-hybrid system. Yeast cells carrying each effector plasmid were grown on SD medium lacking uracil, histidine, and leucine. The β-galactosidase activity was evaluated in yeast cells grown on SD medium supplemented with X-gal.

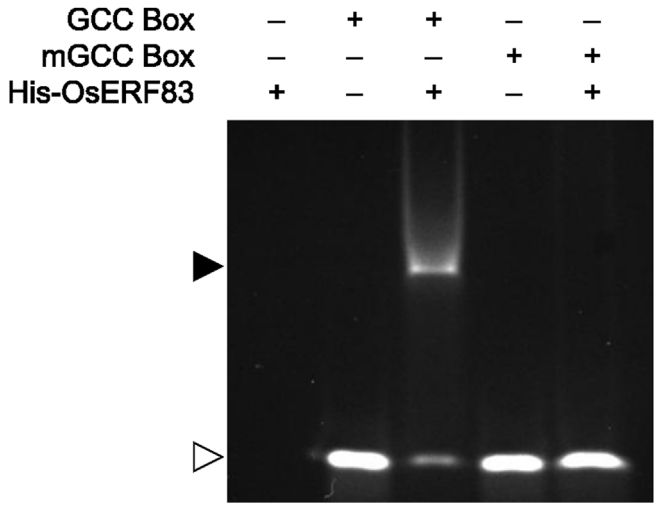


Fig. 6. Electrophoretic mobility shift assays using recombinant OsERF83. Purified His-OsERF83 was incubated with GCC or mGCC probes. Protein-probe complexes were detected using TBE-PAGE followed by ethidium bromide staining. The shifted band and free-probe band are indicated by the black and white triangles, respectively.

2.8. Electrophoretic mobility shift assays

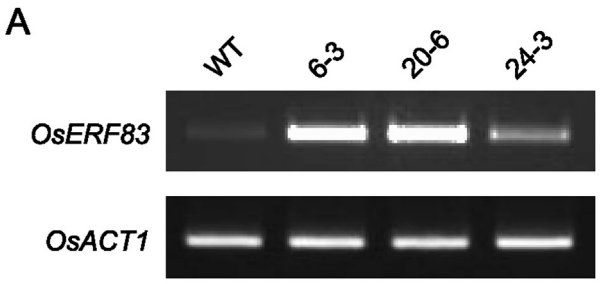
The ORF of *OsERF83* was amplified using primers 5′-GGATCCATG CATTGCTGCATGTCGC-3' (underlined nucleotides: *Bam*HI restriction site) and 5′-CTCGAGAGATCAGATGGAGTGGTGG-3' (underlined nucleotides: *Xho*I restriction site) and ligated into pET28b (Novagen; Merck, Kenilworth, NJ, USA) to create an N-terminal His-tag fusion. The resulting plasmid was introduced into *Escherichia coli* BL21(DE3). The expression of the recombinant gene was induced with a 16-h treatment of 0.4 mM isopropyl-β-d-thiogalactopyranoside at 4 °C and purified using Ni-NTA agarose (Qiagen). The GCC probe oligo (GCC: 5′-TAAGAGCCGCCTAAGAGCCGCC-3′) and the mutant GCC probe oligo (mGCC: 5′- TAAGATCCTCCTAAGATCCTCC-3′) were annealed to their respective pair strands. The binding reaction was performed on ice for 60 min in binding buffer [20 mM Hepes (pH 7.6), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM $(NH_4)_2SO_4$, 1 mM dithiothreitol, 2% Tween 20, 30 mM KCl] with 2.5 μM DNA oligomer and 1 μg purified protein. The protein-probe complexes were separated from the free probes using tris-borate-EDTA/8% polyacrylamide gel electrophoresis (TBE-PAGE). The mobility of the probes was detected with ethidium bromide staining.

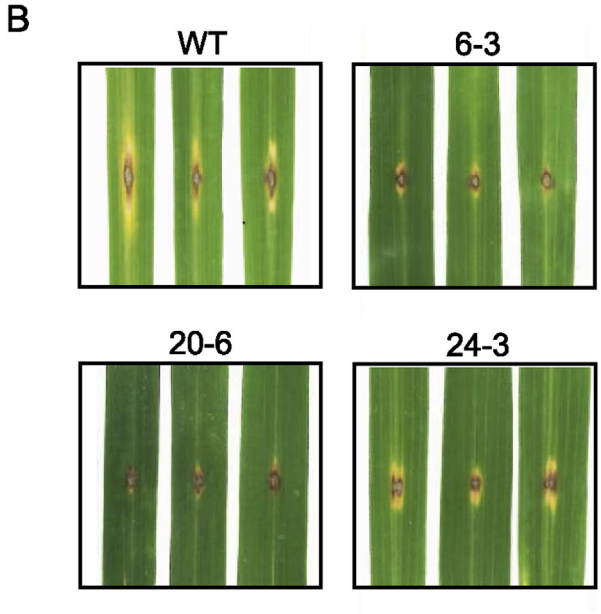
2.9. Generation of transgenic rice lines overexpressing OsERF83

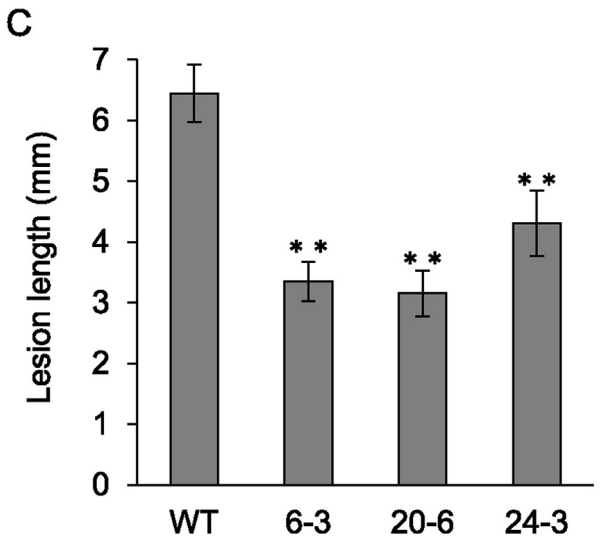
The ORF of *OsERF83* was amplified using primers 5′-TCTAGATCT ATTATTCGGCGGCGGC-3' (underlined nucleotides: *Xba*I restriction site) and 5′-GGTACCACATGTTAACCACTGCTAG-3' (underlined nucleotides: *Kpn*I restriction site). After digestion with *XbaI* and *KpnI*, the amplified fragments were introduced into a pAct-ZH2 binary vector (Sentoku et al., 2000). The transgenic plants (referred to as *OsERF83*ox) were generated using an *Agrobacterium tumefaciens*-mediated transformation (Hiei et al., 1994).

2.10. Detached leaf inoculation assay

Wild-type and transgenic plants were grown on soil for 26 d at 25 °C in a 16-h light/8-h dark photoperiod. The 5th leaf of each plant was detached and placed on wet paper towels in a plastic box. Each leaf was inoculated with four 3-μL spots of the spore suspension (1.0×10^5 spore/mL) and incubated in the box for 5 d at 25 °C. During this period, distilled water was supplied daily to maintain a high humidity level in the box. After this incubation, the lesion lengths were measured from







digital images of the leaves using Image J software. The lesion sizes difference between wild-type and each transgenic line were statistically analyzed using a Student's t-test.

Fig. 7. *M. oryzae* resistance of in *OsERF83*-overexpressing (*OsERF83*ox) plants. (A) The expression levels of *OsERF83* in the transgenic overexpression plants (lines 6-3, 20-6, and 24-3). Three independent lines were analyzed. Semi-quantitative RT-PCR was performed using *OsERF83*-specific primers. *OsACT1* was used as an endogenous control. Total RNA was extracted from 5th leaves of wild type (WT) and transgenic lines. (B) Disease resistance assay against *M. oryzae*. The 5th leaves of the wild-type and *OsERF83*ox plants were inoculated with a spore suspension and incubated for 5 d, after which the disease lesions were observed. (C) Measurement of the lesion lengths shown in (B). The data represent the mean ± SE (n = 24). Asterisks indicate statistically significant differences compared with the wild-type (*t*-test, $p < 0.01$).

2.11. Expression analysis using qRT-PCR

Total RNA was extracted from the 5th leaf of wild-type and transgenic rice plants using the RNeasy Plant Mini Kit (Qiagen). First-strand cDNA was synthesized from 500 ng total RNA using a PrimeScript RT reagent kit (Takara Bio) and used for a qRT-PCR analysis. The qRT-PCR was performed using an ABI7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific) and SYBR Premix Ex TaqII (Takara Bio). The following conditions were used: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The transcripts were normalized relative to the transcript levels of the *OsACT1* housekeeping gene. The specificity of the PCR was automatically determined using a melting curve analysis of the amplified products performed by the PCR system. The primers used for the qRT-PCR are listed in Supplementary Table 1.

3. Results

3.1. OsERF83 is a disease-inducible gene expressed in rice shoots

To identify genes with pivotal roles in defense signaling, we searched for pathogen-inducible TFs in young rice leaves infected with *M. oryzae*. We identified an uncharacterized ERF, *OsERF83*, which was found to encode a 226-amino-acid protein with a high sequence similarity to the *Arabidopsis* ERFs, AtERF15, ORA59, and ERF1 (Fig. 1). *OsERF83* was expressed at relatively low levels in the leaf under non-stress conditions, but was drastically induced two days after blast inoculation and peaked at four days after inoculation (Fig. 2A). In addition, expression analysis using various tissues revealed prominent levels of *OsERF83* expression in the root, palea, and lemma compared with leaf sheath, leaf blade, and inflorescence (Fig. 2B).

3.2. Multiple phytohormones regulate the expression of OsERF83

Phytohormones such as JA, SA, and ET are known to activate defense responses. To determine whether *OsERF83* is involved in phytohormone-mediated signaling pathways, its expression was analyzed in rice seedlings treated with exogenous MeJA, SA, and ethephon. In the MeJA-treated plants, the *OsERF83* transcripts gradually accumulated from 2 h after the treatment and peaked at 8 h, but remained relatively high at 16 h (Fig. 3). In contrast, SA treatment induced the rapid up-regulation of *OsERF83* expression for the first 4 h, which then declined sharply to a near-basal level after 8 h. Similar results were also observed in the ethephon-treated plants. These differing expression patterns suggest that the regulation of *OsERF83* expression in the leaves upon blast infection is complex and may involve multiple signaling pathways.

3.3. Subcellular localization of OsERF83

As an ERF family TF, OsERF83 was expected to localize to the nucleus. The cNLS Mapper software (http://nls-mapper.iab.keio.ac.jp/) predicted that OsERF83 contains a monopartite nuclear localization sequence in the vicinity of the AP2/ERF domain, and that it localizes to nucleus. To confirm this, *OsERF83-GFP* was transiently expressed in

Table 1
Transcriptome comparison of WT versus transgenic plants.

RAP ID	MSU ID	Description	Fold change (#20–6/WT)	
Hormone associated gene				
Os10g0419400	LOC_Os10g28350	Submergence induced protein 2	17.18	13.74
Os01g0757200	LOC_Os01g55240	Gibberellin 2-oxidase	19.22	19.30
Os08g0508700	LOC_Os08g39830	EIN3-like protein	7.34	8.70
Os06g0216300	LOC_Os06g11290	OPDA reductase	8.03	7.24
Os03g0180900	LOC_Os03g08320	ZIM domain containing protein	3.33	2.28
Os03g0402800	LOC_Os03g28940	ZIM domain containing protein	4.34	3.59
Os07g0153000	LOC_Os07g05830	ZIM domain containing protein	69.93	72.52
Os10g0392400	LOC_Os10g25290	ZIM domain containing protein	4.30	6.14
Transcription factor				
Os04g0301500	LOC_Os04g23550	bHLH domain containing protein	10.56	4.63
Os03g0327800	LOC_Os03g21060	NAC domain containing protein	8.97	5.50
Os07g0683200	LOC_Os07g48450	NAC domain containing protein	8.84	5.46
Os07g0684800	LOC_Os07g48550	NAC domain containing protein	8.97	6.37
Os08g0200600	LOC_Os08g10080	NAC domain containing protein	17.56	21.35
Os11g0126900	LOC_Os11g03300	NAC domain containing protein	5.49	8.36
Os01g0734000	LOC_Os01g53260	WRKY transcription factor	3.36	2.51
Os03g0657400	LOC_Os03g45450	WRKY transcription factor	8.25	3.86
PR gene				
Os07g0129200	LOC_Os07g03710	PR1/Pathogenesis-related protein/OsPR1#074	3.98	3.66
Os01g0940800	LOC_Os01g71350	PR2/Beta-1,3-glucanase/Gns6	8.20	2.69
Os01g0287600	LOC_Os01g18400	PR3/Chitinase/cht10	3.14	3.27
Os01g0860500	LOC_Os01g64110	PR3/Chitinase	4.78	4.91
Os04g0494100	LOC_Os04g41680	PR3/Chitinase/cht5	7.19	2.60
Os03g0663500	LOC_Os03g46070	PR5/Thaumatin/TLP	4.58	3.35
Os03g0300400	LOC_Os03g18850	PR10/Pathogenesis-related protein	10.74	15.30
Os12g0555200	LOC_Os12g36850	PR10/Pathogenesis-related protein/PR10b	3.47	3.91
Other difence-related gene				
Os04g0112100	LOC_Os04g02120	NB-ARC domain containing protein	5.50	5.89
Os12g0281600	LOC_Os12g18374	NB-ARC domain containing protein	3.57	3.42
Os08g0155900	LOC_Os08g05960	Pathogen-induced defense-responsive protein	5.79	3.09
Os02g0626400	LOC_Os02g41650	Phenylalanine ammonia-lyase	3.49	4.45
Os02g0627100	LOC_Os02g41680	Phenylalanine ammonia-lyase	3.28	3.27
Os04g0518400	LOC_Os04g43800	Phenylalanine ammonia-lyase	4.11	11.62
Os05g0427400	LOC_Os05g35290	Phenylalanine ammonia-lyase	11.40	14.87
Os10g0398100	LOC_Os10g25870	Plant disease resistance response protein family protein	8.35	5.77
Os11g0214400	LOC_Os11g10800	Plant disease resistance response protein family protein	3.68	4.51
Os10g0569400	LOC_Os10g41980	Rice pathogen-related protein/RIR1b	6.45	16.74
Os10g0569600	LOC_Os10g41999	Rice pathogen-related protein/RIR1b-like	5.84	12.43
Os10g0569800	LOC_Os10g42020	Rice pathogen-related protein	6.18	2.08
Os11g0559200	LOC_Os11g35500	Xanthomonas oryzae pv. oryzae resistance 21	3.83	2.51

onion epidermal cells transformed using particle bombardment. As shown in Fig. 4A–C, fluorescence signals were distributed evenly throughout the cytoplasm and nuclei of cells transformed with a free *GFP* construct. By contrast, the fluorescence signals were observed exclusively in the nucleus when the *OsERF83-GFP* construct was expressed (Fig. 4D–F), indicating that OsERF83 is localized to the nucleus.

3.4. OsERF83 is a positive transcriptional regulator

To determine whether OsERF83 is a positive or negative regulator of transcription, a transactivation assay was performed using a yeast two-hybrid system. An effector construct was prepared by fusing OsERF83 downstream of the DNA-binding domain LexA and was then introduced into host yeast cells expressing *LEU2* and *lacZ* as reporter genes (Fig. 5A). As shown in Fig. 5B, the yeast cells expressing *LexA-OsERF83* exhibited the same growth as the positive controls, LexA-B42$_{AD}$, on the SD medium lacking leucine. In addition, the blue coloration indicated that β-galactosidase activity was present in the LexA-OsERF83 strain (Fig. 5B). By contrast, the negative control LexA-OsACT1 showed no growth in the absence of leucine. These results indicate that OsERF83 functions as a positive regulator of transcription.

3.5. OsERF83 interacts with the GCC box in vitro

The AP2/ERF domain specifically binds to the GCC box consensus sequence (Ohme-Takagi and Shinshi, 1995); therefore, we performed an electrophoretic mobility shift assay to check whether this was also true for OsERF83 using a recombinant protein produced in *E. coli*. The purified His-OsERF83 protein was incubated with either the GCC box probe (TAAGAGCCGCC) or the mutant GCC box probe (TAAGATCC TCC) and separated using TBE-PAGE. A probe-protein complex was detected when the GCC box was used as a probe, whereas no shifted bands were detected when the binding of His-OsERF83 to the mGCC box probe was assessed (Fig. 6). These data indicate that OsERF83 specifically binds to the GCC box.

3.6. OsERF83 confers resistance against rice blast

To investigate whether OsERF83 contributes to the acquisition of disease resistance, transgenic plants overexpressing *OsERF83* under the control of the *OsACT1* promotor were generated. Three independent *OsERF83*ox transgenic lines were selected (Fig. 7A), and their resistance to *M. oryzae* was determined using a leaf inoculation assay. The 5th leaf of each transgenic plant was detached and inoculated with a spore suspension of *M. oryzae*. After a 5-d incubation, the lesion lengths on the inoculated leaves were measured. All transgenic lines showed a

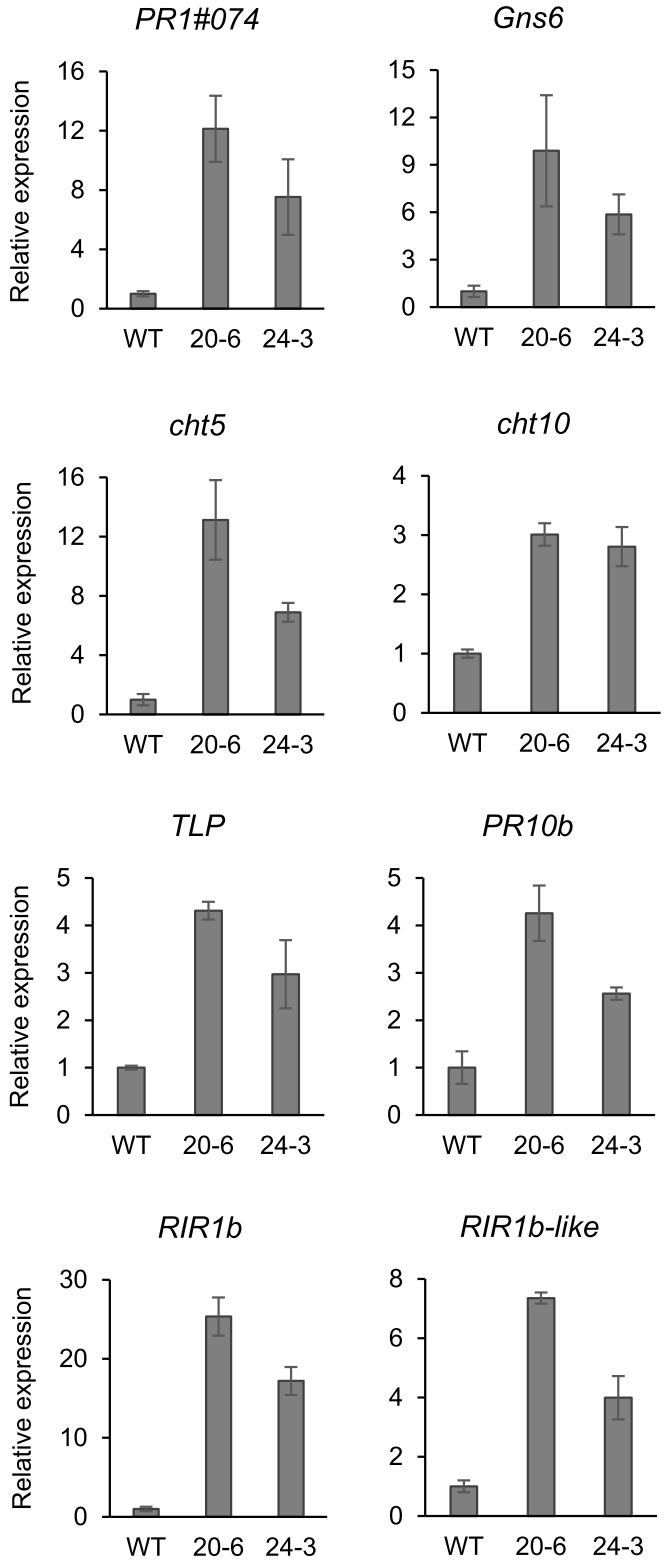


Fig. 8. Expression analysis of defense-related genes in *OsERF83*-overexpressing (*OsERF83*ox) plants. qRT-PCR was performed using total RNA isolated from the 5th leaves of the wild-type (WT) and transgenic (overexpression lines 20-6 and 24-3) plants grown under non-stress conditions. The expression level of each defense-related gene was normalized to the expression of *OsACT1*. The data represent the means of three independent experiments ± SE.

remarkable decrease in lesion length compared with the wild-type (Fig. 7B), with 52%, 49%, and 67% reductions in the lesions on the 6-3, 20-6, and 24-3 lines, respectively (Fig. 7C). The degree of resistance against *M. oryzae* appeared to be related to the expression level of *OsERF83* in these transgenic lines (Fig. 7A and C). These data suggest that OsERF83 positively regulates resistance against rice blast.

3.7. Identification of OsERF83 regulon genes

Our data suggested that OsERF83 functions as a transcriptional activator that confers disease resistance; therefore, it was speculated that the OsERF83-mediated defense mechanism is constitutively activated in the transgenic plants. To identify the candidate downstream targets of OsERF83, we performed a differential expression analysis using a microarray. The transcriptomes of the *OsERF83*ox (line 20-6) and wild-type plants were compared, revealing the upregulation of several defense-related genes in the *OsERF83*ox plants (Table 1

). We focused on the *PR* genes and validated their expression patterns using quantitative RT-PCR. In addition to line 20-6, the low-expression line 24-3 was also analyzed. The genes encoding the PR proteins PR1 (LOC_Os07g03710: *PR1#074*), PR2 (LOC_Os01g71350: *Gns6*), PR3 (LOC_Os04g41680: *cht5*, LOC_Os01g18400: *cht10*), PR5 (LOC_Os03g46070: *TLP*), and PR10 (LOC_Os12g36850: *PR10b*) were upregulated in two independent transgenic lines (Fig. 8). Two other defense-related genes known to confer disease resistance, *RIR1b* (LOC_Os10g41980) and *RIR1b-like* (LOC_Os10g41999) (Schaffrath et al., 2000), were also upregulated in the transgenic lines. Together these data suggest that the upregulation of these defense-related genes may result in the disease resistance displayed by the *OsERF83*ox lines.

4. Discussion

ERFs play a vital role in the growth and environmental adaptation of plants, and have been shown to function in the defense against biotic stresses in several species, including *Arabidopsis*, tomato (*Solanum lycopersicum*), and tobacco (Berrocal-Lobo et al., 2002; Gu et al., 2002; Guo et al., 2004). In rice, many reports have implicated *OsERF*s in defense responses based on their upregulation upon pathogen infection (Cao et al., 2006; Li et al., 2006; Mosquera et al., 2009). However, direct evidence for OsERFs conferring disease resistance is rare, with a few examples reported to date (Liu et al., 2012). In the present study, we isolated *OsERF83* and characterized its function in disease resistance. OsERF83 acts as a transcriptional activator that binds to the GCC core sequence and regulates *PR* gene expression, contributing to the enhancement of blast resistance.

Arabidopsis and rice ERFs can be divided into 15 groups based on the amino acid sequences of their AP2/ERF domains (Nakano et al., 2006). OsERF83 is categorized as a group IXc ERF, which include eight *Arabidopsis* ERFs and 11 rice ERFs (Nakano et al., 2006) (Fig. 1). Our phylogenetic analysis indicated that OsERF83 forms a clade with three *Arabidopsis* ERFs, ERF1, AtERF15, and ORA59 (Fig. 1). The expression of these *Arabidopsis* ERFs are induced by pathogen infection, and their overexpression enhances disease resistance in the transgenic plants via the upregulation of the *PR* genes (Berrocal-Lobo et al., 2002; Pre et al., 2008; Solano et al., 1998; Zarei et al., 2011; Zhang et al., 2015). This insight suggests that OsERF83 is orthologous to these *Arabidopsis* ERFs and that group IXc ERFs play crucial roles in disease resistance in not only *Arabidopsis* but also rice. Moreover, a recent study revealed that OsERF87 and OsERF136, which belong to a different clade of group IXc ERFs to OsERF83, directly bind to the promoter region of *RSOsPR10*, a PR10-class gene, and activate its expression, while OsERF83 failed to show this activity (Yamamoto et al., 2018). Therefore, rice group IXc ERFs might have acquired functional diversity related to disease responses over the course of evolution.

The group IXc ERFs function as transcriptional activators by binding to the eukaryotic transcriptional complex through their EDLL motifs in

the C-terminus (Huang et al., 2016). In the *OsERF83*ox lines, the upregulation of the *PR* genes was presumed to contribute to their enhanced resistance to rice blast (Figs. 7 and 8). Using a genome database search, we identified the GCC core sequence (GCCGCC) in the promoter regions of the upregulated *PR* genes *PR1#074*, *Gns6*, *cht5*, and *RIR1b*, suggesting that OsERF83 may regulate their expression directly. By contrast, other *PR* genes upregulated in *OsERF83*ox (*cht5*, *TLP*, *PR10b*, and *RIR1b*-like) did not contain a GCC core sequence in their promoter region (up to −3000 bp). OsERF83 might regulate the expression of these *PR* genes indirectly by upregulating the expression of other TFs. Our microarray analysis showed that some non-ERF-type TFs were upregulated in *OsERF83*ox (Table 1), which might in turn regulate the other upregulated *PR* genes.

We showed that *OsERF83* is expressed in a tissue-specific manner. In the leaf, *OsERF83* expression is maintained at low levels under non-stress conditions but is highly upregulated in response to the blast inoculation and phytohormone treatments (Figs. 2 and 3). In contrast, higher levels of *OsERF83* expression were observed in the root, palea, and lemma (Fig. 2B), which could indicate that *OsERF83* plays a different physiological role in these tissues. In fact, several transcriptome studies have shown that iron deficiency induces *OsERF83* expression in the roots (Wu et al., 2011; Zheng et al., 2009). Iron starvation induces the expression of ferric reductase, iron transporters, and H^+-ATPases following the biosynthesis of ET (Romera and Alcántara, 2004). Therefore, in roots, OsERF83 might be involved in iron absorption. Moreover, some *OsERF*s, *FRIZZY PANICLE* and *MULTI-FLORET SPIKELET1*, have been identified as key TFs regulating the formation of florets, which comprise one palea and one lemma (Komatsu et al., 2003; Ren et al., 2013). Future research should therefore investigate other possible functions of *OsERF83*.

Fusarium fujikuroi causes bakanae disease, the most significant seed-borne disease of rice (Desjardins et al., 1997). A recent study showed that the *OsERF83* expression is associated with bakanae disease resistance (Matic et al., 2016), as *OsERF83* expression was induced by the infection in a bakanae-resistant cultivar, Selenio, but not in a bakanae-sensitive cultivar, Dorella (Matic et al., 2016). This pattern suggests that OsERF83 plays a vital role in the acquisition of bakanae resistance in rice. In addition to pathogens, *OsERF83* is also highly induced by the feeding of the brown planthopper (*Nilaparvata lugens*), one of the most serious insect pests in rice (Wang et al., 2012). These observations, together with our data, strongly suggest that *OsERF83* contributes to rice resistance to a wide range of biotic stresses.

CRediT authorship contribution statement

Daisuke Tezuka: Writing – original draft, Data curation. **Aya Kawamata:** Data curation. **Hideki Kato:** Data curation. **Wataru Saburi:** Formal analysis. **Haruhide Mori:** Formal analysis. **Ryozo Imai:** Formal analysis, Writing – original draft.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.017.

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