Ectopic Expression of a Cell-Wall-Degrading Enzyme-Induced *OsAP2/ERF152* Leads to Resistance against Bacterial and Fungal Infection in *Arabidopsis*

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ABSTRACT

Pathogen secreted cell-wall-degrading enzymes (CWDEs) induce plant innate immune responses. The expression of rice transcription factor *APETALA2/ethylene response factor-152* (*OsAP2/ERF152*) is enhanced in leaves upon treatment with different CWDEs and upon wounding. Ectopic expression of *OsAP2/ERF152* in *Arabidopsis* leads to induction of immune responses such as callose deposition and upregulation of both salicylic acid- and jasmonic acid/ethylene-responsive defense genes. *Arabidopsis* transgenics expressing *OsAP2/ERF152* exhibited resistance to infections caused by both bacterial and

fungal pathogens (*Pseudomonas syringae* pv. *tomato* DC3000 and *Rhizoctonia solani* AG1-IA, respectively). Ectopic expression of *OsAP2/ERF152* results in transient activation of mitogen-activated protein kinases 3/6 (MPK3/6), which could be leading to the induction of a broad range immunity in *Arabidopsis*.

Keywords: AP2/ERF transcription factor, callose deposition, cell-wall-degrading enzymes, MPK3/6, *OsAP2/ERF152*, *Pseudomonas syringae*, *Rhizoctonia solani*.

Plant cell-wall-degrading enzymes (CWDEs) are important components of the virulence repertoire of microbial pathogens. However, the damage that they cause results in release of cell wall degradation products which serve as damage-associated molecular patterns (DAMPs) and elicit plant immune responses. *Xanthomonas oryzae* pv. *oryzae*, the bacterial pathogen that causes bacterial blight disease in rice, secretes a diverse arsenal of CWDEs such as cellulaseA (ClsA), cellobiosidaseA (CbsA), lipase/esteraseA (LipA), and xylanase (XynB). Prior treatment of rice leaves with CWDEs enhances tolerance to subsequent infection with *X. oryzae* pv. *oryzae* (Jha et al. 2007). Transcriptome analyses performed in rice leaves indicated that a number of transcription factors are differentially expressed at 12 h after treatment with either ClsA (26 of 152 differentially expressed genes) or LipA (68 of 720 differentially expressed genes) (Jha et al. 2010; Ranjan et al. 2015). We initiated this study to characterize the *APETALA2/ethylene response factor-152* (*OsAP2/ERF152*) transcription factor because it was highly upregulated in the transcriptome analysis performed upon treatment of rice leaves with ClsA (Jha et al. 2010).

The members of the AP2/ERF gene family regulate functions related to development and environmental stress responses in plants (Licausi et al. 2013). Most of the AP2/ERF proteins are expressed in response to the ethylene (ET) hormone (Licausi et al. 2013; Shari

et al. 2011); however, members of this transcription factor family are also reported to be expressed in response to jasmonic acid (JA) and abscisic acid. Several AP2/ERF transcription factors are known to function as positive or negative regulators of plant immune responses (Licausi et al. 2013). We have identified two AP2/ERF transcription factors, LOC_Os08g36920.1 and LOC_Os01g21120.1, that were upregulated at 12 h after treatment of rice leaves with either ClsA or LipA enzymes (Supplementary Table S1). Among the two transcription factors, the expression of *OsAP2/ERF152* (LOC_Os08g36920.1) was found to be upregulated to a higher extent. In our previous report, the transient overexpression of *OsAP2/ERF152* was found to induce callose deposition and programmed cell death as well as enhance tolerance to subsequent infection with *X. oryzae* pv. *oryzae* (Jha et al. 2010). In the present study, we wanted to assess the effect of ectopic expression of *OsAP2/ERF152* in the induction of immune responses in a heterologous system, *Arabidopsis thaliana*. Our results demonstrate that the ectopic expression of *OsAP2/ERF152* leads to enhanced callose deposition, expression of both the salicylic acid (SA)- and JA/ET-responsive defense genes, and transient activation of MPK3 and -6 in *Arabidopsis*. This results in resistance to infections caused by a hemibiotroph, *Pseudomonas syringae* pv. *tomato* and a necrotroph, *Rhizoctonia solani* AG1-IA. Our results suggest that ectopic overexpression of *OsAP2/ERF152* can be a way of enhancing tolerance to hemibiotrophic and necrotrophic pathogens in plants.

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MATERIALS AND METHODS

Plant materials and growth conditions. All of the experiments in rice were performed in bacterial leaf-blight-susceptible rice cultivar Taichung Native-1 (TN-1) grown in the greenhouse. *OsAP2/ERF152* transgenic lines were generated in *A. thaliana* Columbia ecotype (Col-0). For all of the experiments, *Arabidopsis* seed were sterilized and germinated on 0.5× Murashige and Skoog (MS) agar medium (Murashige and Skoog 1962). Seven-day-old

Arabidopsis seedlings were transferred to soil (vermiculite/perlite/tissue culture mix, 1:1:2 ratio) and grown in a plant growth chamber maintained at 22°C with a 12-h day-and-night cycle.

CWDEs and elicitor treatment in rice leaves. The expression levels of *OsAP2/ERF152* gene transcript were studied in rice upon treatment with various CWDEs and elicitors, as described earlier (Pillai et al. 2018). Briefly, leaves ($n = 15$ to 20) of 2-week-old TN-1 rice seedlings were infiltrated with one of the following using a needleless syringe: xylanase (2 U/ml), pectinase (2 U/ml), flagellin 22 (Flg22; 100 μ M), lipopolysaccharide (LPS; 100 μ M), or sterile water. For studying response to wounding, leaves of the rice seedlings were pin pricked 8 to 10 times and sprayed with water. Leaves were collected after 12 h of the treatment and further processed for RNA extraction and quantitative PCR (qPCR) analysis for studying the expression levels of *OsAP2/ERF152*. Each experiment had three biological replicates and three such independent experiments were performed.

Localization studies in onion epidermis. For localization studies, the *OsAP2/ERF152* gene was cloned (excluding stop codon) into the pH7FWG2 plant expression vector using Gateway cloning. The gene was expressed constitutively under the CaMV 35S promoter and with a green fluorescent protein (GFP) tag at the C-terminal region of the protein. For localization studies, *Agrobacterium* strain AGL1 carrying pH7FWG2::*OsAP2/ERF152* was syringe infiltrated into onion epidermal cells (Xu et al. 2014). Briefly, an overnight-grown culture of *Agrobacterium* was diluted to an optical density at 600 nm (OD_{600}) of 0.01 in infiltration buffer (42 mM dextrose, 100 mM $CaCl_2$, 0.01 mM 6-benzylaminopurine [BAP], 0.01% Silwet L-77, 0.05 mM $MgCl_2$, 12.5 mM acetosyringone, and 100 mM morpholineethanesulfonic acid [pH 5.6] solution). For transformation, 100 to 200 μ l of the above suspension was injected into the onion scales between adaxial epidermis and mesophyll tissue using a needled syringe. The onion bulb was incubated at 28°C for 48 h. The epidermis was peeled off and placed on a slide over a drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Fluorshield; Calbiochem). The samples were imaged under GFP, DAPI, and phase contrast filters using an ECLIPSE Ni-E epifluorescence microscope (Nikon).

Microbial growth conditions. *P. syringae* pv. *tomato* DC3000 culture was grown on King's basal agar medium (HiMedia). For all assays, *Agrobacterium* strains and *P. syringae* pv. *tomato* were grown in Luria-Bertani (LB) broth (HiMedia) at 28°C for 18 h. The fungal pathogen *R. solani* AG1-IA was grown and maintained on potato dextrose agar medium (HiMedia) at 28°C.

Construction of plant expression vector. The cDNA was synthesized from the total RNA isolated from ClsA enzyme-treated rice leaves (12 h posttreatment) using Superscript III reverse transcription (Invitrogen). The *OsAP2/ERF152* (LOC_Os08g36920.1) gene without the stop codon was cloned into the 17- β -estradiol-inducible plant-expression vector pMDC7 using Gateway cloning as per the manufacturer's instructions. The pMDC7::*OsAP2/ERF152* vector was transformed into *Agrobacterium tumefaciens* strain LBA4404 by electroporation and the transformed clones were selected on a medium containing the appropriate antibiotics (Supplementary Table S2). The LBA4404/pMDC7::*OsAP2/ERF152* clones were confirmed for the construct by colony PCR and subsequent sequencing of the PCR amplicons by using pMDC7-specific primers (Supplementary Table S3).

Generation of stable transgenic *Arabidopsis* plants. *Arabidopsis* Col-0 plants (wild type) were transformed with *A. tumefaciens* LBA4404 carrying pMDC7::*OsAP2/ERF152* by the floral dip method (Clough and Bent 1998). The transformed seedlings were selected on MS agar with hygromycin (25 μ g/ml). The presence of the *OsAP2/ERF152* gene in the putative T_1 transgenic plants was assessed by genotyping using direct PCR (Terra PCR direct polymerase kit; Clontech) with T-DNA-specific primers (Supplementary Table S3) followed by sequencing of the amplified product. All functional studies were performed on T_2

progenies of two independent transgenic lines (numbers XVE:: *OsAP2/ERF152*-5 and -15), in which high levels of *OsAP2/ERF152* expression were observed after estradiol treatment.

Callose deposition assay. Wild-type and transgenic *Arabidopsis* plants that were at the rosette stage (4 weeks old) were used for callose deposition assays. The leaves were infiltrated either with estradiol (20 μ M), which is the inducer of the XVE promoter, or with water using a 1-ml needleless syringe. At 12 h postinfiltration, the leaves were harvested and processed for callose staining by fixing in ethanol/acetic acid (3:1) solution to remove the chlorophyll completely. The leaves were then rehydrated by 2 h of 70% ethanol treatment, followed by 2 h of water treatment. The callose deposits were stained by soaking the leaves for 16 to 18 h in 0.01% (wt/vol) aniline blue dye dissolved in 150 mM potassium-phosphate buffer (pH 9.5) (Millet et al. 2010). The leaves were observed for callose deposits under 10 $\times$ magnification of an epifluorescence microscope (ECLIPSE Ni-E; Nikon) using the blue filter (excitation wavelength = 365 nm and emission wavelength > 420 nm). The number of callose deposits per field of view for a minimum of five leaves with three different fields was counted. Three such independent experiments were performed in two independent lines.

Quantitative real-time PCR analysis. RNA was extracted from either rice or *Arabidopsis* tissues using TRIzol reagent (Thermo Fisher Scientific). The total RNA sample was treated with DNaseI (NEB). Total RNA (1 μ g) was used for cDNA synthesis using cDNA EcoDry premix kit-Oligo dT (Clontech, Takara). The diluted cDNA sample (1:10 fold) was used for qPCR analysis using Power SYBR green/ROX Master Mix (Thermo Fisher Scientific) on the 7900 HT sequence detection system (Applied Biosystem). For rice and *Arabidopsis* samples, *OsGAPDH* and *AtUBQ5* genes, respectively, were used as internal controls. All of the primers used for qPCR (Supplementary Table S3) in this study were designed using Quantprime (Arvidsson et al. 2008). The fold change in expression for various genes between water-treated (control) and estradiol-treated samples was calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

***P. syringae* pv. *tomato* infection assay in *Arabidopsis*.** The *P. syringae* pv. *tomato* infection assay was performed in 4-week-old transgenic *Arabidopsis* (XVE::*OsAP2/ERF152*) plants. In order to induce *OsAP2/ERF152* expression, the pots were treated with either only water or water containing 40 μ M estradiol 24 h prior to infection. For bacterial infection, the *P. syringae* pv. *tomato* culture was grown for 16 to 18 h in LB broth containing rifampicin (50 mg/liter). The culture was pelleted, washed, and resuspended in sterile 10 mM $MgCl_2$ solution with an OD_{600} of 0.01. The bacterial cell suspension was infiltrated into leaves of pretreated plants. Leaf samples were collected on the 0th and 2nd day postinfiltration for measuring the bacterial growth (Starkey and Rahme 2009). Briefly, the leaves were crushed in sterile 10 mM $MgCl_2$ solution and serially diluted to obtain countable bacterial colonies. The same procedure was repeated with five different plants in two independent lines and the experiments were replicated thrice.

***R. solani* AG1-IA infection assay in *Arabidopsis*.** *R. solani* infection assay was performed on 4-week-old rosette-stage transgenic *Arabidopsis* plants (XVE::*OsAP2/ERF152*) by inducing the expression of *OsAP2/ERF152* as mentioned above. For *R. solani* infection, the sclerotium was germinated by growing in liquid potato dextrose broth for 24 h at 28°C. The fungal culture was resuspended in sterile water by pipetting thoroughly. The induced and uninduced transgenic plants were infected by adding 200 μ l of the culture suspension at the center of the rosette. The infected plants were monitored for disease symptoms and photographed until the 10th day postinfection. For the estimation of fungal growth in the infected plants, the entire plant was uprooted and frozen in liquid nitrogen. Genomic DNA was isolated from the plants using a plant DNA isolation kit (Sigma). The DNA concentration was measured spectrophotometrically and different dilutions of the total DNA (0.1 to 100 ng) were subjected to fungal DNA

quantification. The level of fungal *18S rRNA* gene was quantitated in three independent samples by qPCR using *R. solani*-specific primers (Okubara et al. 2008). Normalization was done using the *Arabidopsis ubiquitin 5 (AtUBQ5)* gene.

Seedling growth inhibition assay. Seven-day-old seedlings ($n = 15$) of either XVE::*OsAP2/ERF152* transgenic lines or wild-type *Arabidopsis* plants were germinated on MS agar and grown vertically on the agar surface in either the presence or absence of 20 μ M estradiol. The fresh weight of each seedling was measured on the 10th day. The experiment was repeated thrice with two independent transgenic lines.

Western blot analysis. For mitogen-activated protein kinase (MAPK) assays, 2-week-old wild-type and transgenic *Arabidopsis* seedlings ($n = 10$ to 15) were submerged in sterile water with or without 20 μ M estradiol. The samples were collected after 0, 10, 15, 30, and 60 min of estradiol treatment and frozen in liquid nitrogen. The crude protein was extracted using protein extraction buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 250 mM mannitol, 5 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1% Triton X100, 1 mM phenylmethylsulfonyl fluoride, and 1 mM NaF) along with a plant protease inhibitor cocktail (Sigma-Aldrich). The protein concentration was estimated spectrophotometrically and an equal amount of protein was resolved on 10% polyacrylamide gel. The MPK6 and -3 proteins were probed using antiphospho p44/42 MAPK (Thr202/Tyr204) antibody (1:2000; catalog number CST9101S; Cell Signaling Technology). Antirabbit peroxidase conjugated secondary antibody (1:50000; AbCAM) was probed and the protein band was viewed using Luminata Forte HRP substrate (Millipore). The signal was captured using a Vilber Lourmat chemiluminescence imaging system.

RESULTS

Expression of *OsAP2/ERF152* is induced upon cell wall damage. The previous microarray analyses of rice leaves indicated that the expression of *OsAP2/ERF152* was induced by 2 to fourfold at 12 h posttreatment with either cellulase (ClsA) or esterase (LipA) enzymes (Jha et al. 2010; Ranjan et al. 2015). In order to determine whether *OsAP2/ERF152* expression is induced with other kinds of CWDEs, we treated rice leaves with commercially available pectinase and xylanase. The expression of *OsAP2/ERF152* was induced four- and twofold after treatment with pectinase and xylanase, respectively (Fig. 1). Wounding of rice leaves also induced the expression of *OsAP2/ERF152*. However, treatment

Fig. 1. Expression of *OsAP2/ERF152* is induced upon treatment with cell-wall-degrading enzymes. Young leaves of 2-week-old Taichung Native-1 rice seedlings ($n = 15$ to 20) were infiltrated with one of the following: control (water), pectinase (2 U/ml), xylanase (2 U/ml), flagellin 22 (Flg22; 100 μ M), or lipopolysaccharide (LPS; 100 μ M). Leaves ($n = 15$) were also injured 10 times using a 1-ml syringe needle for wound treatment. At 12 h after the treatment, leaves were harvested and processed for quantitative PCR. The relative fold change was calculated over water-treated or untreated (for wound treatment) samples. *OsGAPDH* was used as internal control. The graph represents the average from three biological replicates and the error bar denotes standard deviation.

with pathogen-associated molecular patterns (PAMPs) such as Flg22 and LPS failed to induce *OsAP2/ERF152* expression in rice. *OsAP2/ERF152* transcription factor has a nuclear localization sequence (from 132 to 140 amino acids) (Jha et al. 2010); hence, we studied its localization in plant cells. GFP-tagged *OsAP2/ERF152* was transiently expressed in onion epidermal cells for localization studies. *OsAP2/ERF152*-GFP merges with DAPI-stained nucleus and confirms localization into the nucleus (Supplementary Fig. S1).

Ectopic expression of *OsAP2/ERF152* leads to the induction of callose deposition in *Arabidopsis*. Jha et al. (2010) reported that the transient overexpression of *OsAP2/ERF152* led to enhanced callose deposition in rice seedlings. In order to determine whether ectopic expression of *OsAP2/ERF152* induces callose deposition in *Arabidopsis*, we generated stably transformed transgenic lines expressing *OsAP2/ERF152* under an estradiol-inducible promoter (XVE::*OsAP2/ERF152*). The T₂ progeny of two independently transformed XVE::*OsAP2/ERF152* transgenic lines that expressed higher levels of *OsAP2/ERF152* (3- to 10-fold) in a qPCR analysis were used for all studies (Supplementary Fig. S2). Leaves of the wild type and XVE::*OsAP2/ERF152* were infiltrated with either estradiol or water. Leaves were collected after 12 h and stained for callose deposition. Ectopic expression of *OsAP2/ERF152* was found to induce callose deposition (approximately fivefold higher) as compared with the control (water-treated) leaves (Fig. 2A and B). Similar results were obtained in three different experiments performed in two independently transformed transgenic lines. Treatment with estradiol alone did not induce callose deposition in the wild-type *Arabidopsis* plants (Kachewar et al. 2019; Pillai et al. 2018) (Fig. 2B).

Ectopic expression of *OsAP2/ERF152* resulted in enhanced expression of SA- and JA/ET-responsive defense genes in *Arabidopsis*. Plant immune responses are controlled by plant defense hormones such as SA, JA, and ET. These hormones regulate the expression of many defense response genes which actually act to inhibit growth of the invading pathogens. The expression of defense response genes regulated by different phytohormones was analyzed in XVE::*OsAP2/ERF152* plants in the presence or absence of estradiol. Upon induction of *OsAP2/ERF152* expression, the transcript levels of *Pathogenesis related-1 (PR1)*, *PR2*, *PR3*, *Phytoalexin deficient-3 (PAD3)*, and *avrPphB susceptible-3 (PBS3)* were significantly upregulated when the average cycle threshold (Ct) values were compared with that of the uninduced samples using Student's *t* test ($P < 0.05$) (Fig. 2C). Both SA-responsive (*PR1*, *PR2*, *PAD3*, and *PBS3*) as well as JA-responsive (*PR3*) defense genes were upregulated on induction of expression of *OsAP2/ERF152* in the *Arabidopsis* transgenic lines. We further tested the expression of other biosynthetic and responsive genes whose levels are regulated by either SA or JA/ET. Interestingly, *OsAP2/ERF152* expression induced the expression of genes that are regulated by the SA hormone (*Isochorismate synthase* and *SA glycosyl transferase*), JA hormone (*Allene oxide synthase-1*, *coronatine-insensitive protein-1*, and *Jasmonate zim domain*), or ET hormone (*Ethylene response factor 1 [ERF1]*, *ethylene insensitive-2*, and *1-aminoacyclop propane 1-carboxylate synthase*) (Fig. 2D). We found that the expression of *OsAP2/ERF152* results in the upregulation of SA- as well as JA/ET-responsive genes in *Arabidopsis*. Treatment with estradiol alone did not induce expression of either SA- or JA/ET-responsive genes in the wild-type *Arabidopsis* plants (Kachewar et al. 2019) (Supplementary Fig. S3).

Expression of *OsAP2/ERF152* provides resistance to *P. syringae* infection in *Arabidopsis*. In a previous study, transient overexpression of *OsAP2/ERF152* was found to lead to resistance against subsequent *X. oryzae* pv. *oryzae* infection in rice (Jha et al. 2010). In order to determine whether ectopic expression of *OsAP2/ERF152* would enhance tolerance to bacterial infection in another plant, we inoculated estradiol-inducible XVE::*OsAP2/ERF152* transgenic lines with *P. syringae* pv. *tomato* DC3000 in the

presence and absence of estradiol. Induction of *OsAP2/ERF152* expression resulted in a two- to threefold reduction in the total average bacterial count (Fig. 3A) as compared with the uninduced condition. Thus, ectopic expression of *OsAP2/ERF152* in *Arabidopsis*

led to resistance to *P. syringae* pv. *tomato* infection, which is a hemibiotrophic pathogen. Treatment with estradiol alone did not affect *P. syringae* pv. *tomato* disease progression in the wild-type *Arabidopsis* plants (Kachewar et al. 2019).

Fig. 2. Ectopic expression of *OsAP2/ERF152* induces plant defense response. **A**, Leaves of 4-week-old of 17- β -estradiol-inducible XVE::*OsAP2/ERF152* transgenic lines of T₂ generations were infiltrated with either the XVE promoter inducer (20 μ M estradiol) or water (with dimethyl sulfoxide) (MQ) using a 1-ml needleless syringe. Leaves were harvested after 12 h and processed for aniline blue staining. Callose was observed as white spots under an epifluorescence microscope. The scale bar in the images represent 50 μ m. **B**, Bars represent the mean value of number of callose deposits per field of view ($n = 5$). All of the data were analyzed using Student's *t* test (* indicates significant difference with $P < 0.05$). Leaves from 3- to 4-week-old XVE::*OsAP2/ERF152* plants treated with either estradiol or water were harvested and processed for quantitative PCR analysis after 12 h of the treatment. WT = wild type. **C** and **D**, Expression of *OsAP2/ERF152* induced various defense response genes regulated by either salicylic acid (SA) or jasmonic acid/ethylene: *Pathogenesis related-1* (*PR1*), *PR2*, *PR3*, *Phytoalexin deficient-3* (*PAD3*), *avrPphB* susceptible-3 (*PBS3*), *Allene oxide synthase-1* (*AOS1*), *Coronatine-insensitive protein-1* (*COI1*), *Jasmonate zim domain* (*JAZ10*), *1-aminoacyclopentane 1-carboxylate synthase* (*ACC*), *Ethylene insensitive-2* (*EIN2*), *Ethylene response factor-1* (*ERF1*), *SA glycosyl transferase* (*SAGT*), and *Isochorismate synthase* (*ICST*). The *Arabidopsis UBQ5* gene was used as internal control for quantitative real-time PCR analysis. The graph represents the relative fold change ($2^{-\Delta\Delta C_t}$) calculated using expression values of induced over uninduced plants after 12 h of induction. The average from three replicates is plotted in the graph and error bars denote standard deviation. Similar results were obtained in three separate experiments with two independent XVE::*OsAP2/ERF152* transgenic lines.

Fig. 3. Ectopic expression of *OsAP2/ERF152* leads to resistance against *Pseudomonas syringae* pv. *tomato* and *Rhizoctonia solani* infection in *Arabidopsis* transgenic plants. A day prior to the microbial infection, 4-week-old *Arabidopsis* transgenic plants (XVE::*OsAP2/ERF152*) were treated with either only water (MQ) or water containing 40 μ M estradiol. For the *P. syringae* pv. *tomato* infection assay, leaves were infiltrated with an overnight-grown culture (optical density at 600 nm = 0.01). Samples (three leaves per plant) were collected on the 0th and 2nd days postinfection (dpi) and processed for total bacterial count. **A**, Average number of CFU calculated using five different plants with two independent lines. For *R. solani* infection, 200 μ l of an overnight-grown fungal culture was dropped at the center of the *Arabidopsis* rosette. The number of infected leaves was counted on 10th dpi. The average percent of infected leaves per plant was plotted for **B**, induced and **C**, uninduced plants. **D**, Fungal growth was quantified by quantitative real-time PCR analysis for the fungal *18S rRNA* gene from the total DNA extracted from infected plants. Plant DNA was normalized using *Arabidopsis AtUBQ5*. The bar represents average $2^{-\Delta Ct}$ values. All of the data were analyzed using Student's *t* test (* indicates significant difference with $P < 0.05$). Error bars represent standard deviation. Similar results were obtained in three separate experiments with two independent XVE::*OsAP2/ERF152* transgenic lines.

Expression of *OsAP2/ERF152* provides resistance against *R. solani* infection in *Arabidopsis*. We then tested whether *OsAP2/ERF152* expression would lead to resistance to the necrotrophic pathogen *R. solani*. A suspension of an overnight-grown *R. solani* culture was inoculated at the center of the rosette of XVE::*OsAP2/ERF152* transgenic plants pretreated with either estradiol or water. The number of leaves with disease symptoms was counted on the 10th day postinfection. In the transgenic plants that were pretreated with water (uninduced condition), *R. solani* infection caused either partially necrotic lesions or the entire leaf was dead (Fig. 3B). However, the younger leaves remained green and appeared to be healthy. The number of such diseased leaves was significantly lower in *OsAP2/ERF152*-expressing plants (induced condition) (Fig. 3C). Fungal growth was quantitated by checking the amplification of a fungal gene by qPCR. A significant ($P < 0.05$) reduction in fungal load was observed following induction of *OsAP2/ERF152* expression (Fig. 3D), revealing that the expression of this rice transcription factor leads to resistance to infection caused by a necrotrophic pathogen in *Arabidopsis*. Treatment with estradiol alone did not affect *R. solani* disease progression in the wild-type *Arabidopsis* plants (Kachewar et al. 2019).

Constitutive expression of *OsAP2/ERF152* results in the reduction of seedling growth in *Arabidopsis*. Treatment with elicitors such as Flg22, oligogalacturonides, chitin, and cellobiose leads to growth inhibition in *Arabidopsis* seedlings (Denoux et al. 2008; Souza et al. 2017). The continuous activation of immune responses has adverse effects on plant growth (de Vries et al. 2017; Yang et al. 2012). Because *OsAP2/ERF152* induces plant immune responses such as callose deposition and expression of defense genes, we tested the effect of *OsAP2/ERF152* expression on plant growth. In order to study the effect of expression of *OsAP2/ERF152* on growth, the seedlings of XVE::*OsAP2/ERF152* transgenic lines and wild-type plants were grown on MS agar medium in the presence and absence of estradiol. After 10 days of estradiol treatment, the fresh weight of individual seedlings was measured. Continuous induction of *OsAP2/ERF152* resulted in a significant reduction in the fresh weight. (Fig. 4). The rosette diameter of the seedlings remained unchanged while the root branching was significantly reduced. However, the inducer (estradiol) had no effect on the growth of wild-type seedlings. Similar results were obtained in two independently transformed transgenic lines across three independent experiments.

Fig. 4. Constitutive expression of *OsAP2/ERF152* has an inhibitory effect on plant growth. For assaying the effect on growth, seedlings of wild type (WT) and XVE::*OsAP2/ERF152* were grown vertically on Murashige and Skoog agar medium in the absence (MQ) and presence of estradiol. On the 10th day, individual seedlings ($n = 15$) were weighed and the average fresh weight was plotted. The error bar represents standard deviation. Data were analyzed using Student's t test (* indicates significant difference with $P < 0.05$). Similar results were obtained in two separate experiments with two independent XVE::*OsAP2/ERF152* transgenic lines.

Ectopic expression of *OsAP2/ERF152* activates MAPK signaling in *Arabidopsis*. MAPK signaling is induced in response to external as well as internal signals in plant cells. The activation of MAPKs is the early signaling event observed during PAMP-triggered immunity as well as DAMP-triggered immunity (Meng and Zhang 2013). Because MAPK signaling is an upstream signaling event, we tested its involvement in *OsAP2/ERF152*-induced immunity. For this, phosphorylation of MPK3 and MPK6 were tested in 2-week-old wild-type and XVE::*OsAP2/ERF152* transgenic lines. The seedlings were treated with estradiol for various durations (0, 10, 15, 30, and 60 min), followed by total protein extraction and Western blot analysis. The expression of *OsAP2/ERF152* resulted in the transient activation of MPK3 and MPK6, which was initiated at 15 min and reached a maximum at 30 min post estradiol treatment (Fig. 5). The levels of phosphorylated MPK6 and -3 returned to the basal levels at 60 min in *OsAP2/ERF152*-expressing seedlings. Tsuda et al. (2013) reported that estradiol treatment did not induce MPK3 and -6 activation in wild-type *Arabidopsis*. In the present study, a basal level of MPK3 and -6 was observed in wild-type seedlings which remained unchanged upon estradiol treatment (Fig. 5). We used Flg22 as a positive control for locating phosphorylated MPK3 and -6 (Supplementary Fig. S2). Similar results were obtained in two independent lines across two experiments.

DISCUSSION

The action of CWDEs releases cell wall degradation products which induce plant immune responses (Walton 1994). We have shown earlier that treatment with CWDEs induces a strong immune response potent enough to provide enhanced tolerance to bacterial infection in rice (Jha et al. 2007). Transcriptional reprogramming is a key step in the induction of an immune response and this process is generally executed by transcription factors. We initiated this study to characterize the *OsAP2/ERF152* transcription factor because it was highly upregulated in the transcriptome analysis performed upon treatment of rice leaves with ClsA (Jha et al. 2010). In rice, expression of *OsAP2/ERF152* is induced by a variety of CWDEs but not by Flg22 and LPS, suggesting that it may be specific for cell wall damage or CWDE-induced immune responses. Ectopic expression of *OsAP2/ERF152* leads to resistance against hemibiotrophic and necrotrophic infection in *Arabidopsis*. It is well reported that SA plays an active role in controlling the growth of a hemibiotrophic pathogen while the JA/ET-mediated plant defense response suppresses necrotrophic infection in *Arabidopsis* (Tsuda et al. 2009; Xin and He 2013). The resistance to *P. syringae* pv. *tomato* infection observed upon *OsAP2/ERF152* expression could be due to the expression of SA-responsive defense genes. Independent studies show that the increased tolerance to *R. solani* infection in rice is associated with plant responses induced by either

Fig. 5. Ectopic expression of *OsAP2/ERF152* leads to transient activation of MPK3 and MPK6 in *Arabidopsis*. For testing activation of MPK3 and -6, 2-week-old seedlings ($n = 10$ to 15) of the wild type and XVE::*OsAP2/ERF152* were treated with estradiol for different time points (0, 10, 15, 30, and 60 min) and processed for protein extraction followed by Western blotting using antiphospho p44/42 (Thr202/Tyr204) antibodies. MAPK = mitogen-activated protein kinase and CBB = Coomassie Brilliant Blue. Both of the above experiments were repeated twice in two independent transgenic lines.

SA, JA, or ET (Kouzai et al. 2018). There are many studies reported where activation of a broad immune response is associated with the activation of both SA and JA response pathways. Treatment with Flg22, *Bacillus cereus* AR156 (a nonpathogenic rhizobacterium), or chitosan induce both the SA- and JA/ET-mediated immune response pathway in *Arabidopsis* (Denoux et al. 2008; Jia et al. 2018; Jiang et al. 2016). A similar result was reported in strawberry plants, where *Colletotrichum acutatum* (a fungal pathogen) partially activated the SA and JA/ET response pathways (Amil-Ruiz et al. 2016). Thus, the resistance to hemibiotrophic and necrotrophic infection in *OsAP2/ERF152*-expressing *Arabidopsis* lines may be due to the enhanced callose deposition and induction of various defense genes involved in the SA and JA/ET responses.

Induction of the DAMP-triggered immune response involves activation of MAPKs as one of the earliest events, and MPK3 and -6 are activated upon treatment with oligogalactourans and cellobiose in *Arabidopsis* plants (Meng and Zhang 2013; Souza et al. 2017). Ectopic expression of *OsAP2/ERF152* led to transient activation of MPK3 and MPK6 in *Arabidopsis*. Activation of MPK3 and MPK6 leads to induction of the ET response pathway in *Arabidopsis*. MPK3 plays an important role in activating the SA response while MPK6 partially induces expression of JA-responsive and biosynthetic genes (Meng and Zhang 2013). Previous studies show that activation of MPK3 and MPK6 leads to resistance to infection by *P. syringae* pv. *tomato* and a fungal pathogen, *Botrytis cinerea*, in *Arabidopsis* (Asai et al. 2002). Thus, we propose that the *OsAP2/ERF152*-mediated activation of a broad-range immune response may be due to the transient activation of MPK3 and -6, which may lead to upregulation of SA- and JA/ET-responsive genes.

Overall, the present study demonstrates that ectopic expression of *OsAP2/ERF152* activates a broad range of immunity that leads to resistance to fungal and bacterial infection in *Arabidopsis*. This immunity involves activation of MPK3 and -6, which may lead to enhanced expression of plant defense genes regulated by both SA and JA/ET hormones. Previous work by Jha et al. (2010) showed that transient expression of *OsAP2/ERF152* leads to resistance to *X. oryzae* pv. *oryzae* infection in rice. The fact that overexpression of *OsAP2/ERF152* leads to resistance to bacterial infection in rice and in *Arabidopsis* suggests that its overexpression may be helpful in providing tolerance to infection in different plants. However, a continuous expression of *OsAP2/ERF152* leads to growth inhibition, suggesting that it must be used with a regulatable promoter; specifically, a defense-inducible promoter. Also, overexpression of *OsAP2/ERF152* provides a moderate level of tolerance, suggesting that it must be used in conjunction with other defense genes. Future work would be directed toward expressing *OsAP2/ERF152* with a defense-inducible promoter in crop plants. Also, we would be looking to determine the exact role of *OsAP2/ERF152* in CWDE-induced immune responses in rice through development of null mutants using genome editing techniques.

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