

Submergence Confers Immunity Mediated by the WRKY22 Transcription Factor in *Arabidopsis*^W

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Transcriptional control plays an important role in regulating submergence responses in plants. Although numerous genes are highly induced during hypoxia, their individual roles in hypoxic responses are still poorly understood. Here, we found that expression of genes that encode members of the WRKY transcription factor family was rapidly and strongly induced upon submergence in *Arabidopsis thaliana*, and this induction correlated with induction of a large portion of innate immunity marker genes. Furthermore, prior submergence treatment conferred higher resistance to the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis*. Among the WRKY genes tested, WRKY22 had the highest level of induction during the early stages of submergence. Compared with the wild type, WRKY22 T-DNA insertion mutants *wrky22-1* and *wrky22-2* had lower disease resistance and lower induction of innate immunity markers, such as *FLG22-INDUCED RECEPTOR-LIKE KINASE1 (FRK1)* and *WRKY53*, after submergence. Furthermore, transcriptomic analyses of *wrky22-2* and chromatin immunoprecipitation identified several potential targets of WRKY22, which included genes encoding a TIR domain-containing protein, a plant peptide hormone, and many *OLIGO PEPTIDE TRANSPORTER* genes, all of which may lead to induction of innate immunity. In conclusion, we propose that submergence triggers innate immunity in *Arabidopsis* via WRKY22, a response that may protect against a higher probability of pathogen infection either during or after flooding.

INTRODUCTION

Due to their immobility, plants have evolved complex sensing mechanisms and response pathways that help them adapt to diverse environmental conditions and ensure their survival (Bailey-Serres and Voeseenek, 2008). Transcriptional regulation is one of the mechanisms used by plants to protect against biotic and abiotic stresses. Multiple signaling pathways are triggered transcriptionally in response to flooding (Klok et al., 2002; Fukao and Bailey-Serres, 2008; Hattori et al., 2009). Functional studies of plant flooding and the effects of low oxygen have mainly focused on altering the expression of the genes encoding the enzymes for fermentation and glycolysis (Ellis et al., 1999; Kürsteiner et al., 2003; Bieniawska et al., 2007; Dolferus et al., 2008). Transcriptomic studies have revealed that expression of huge numbers of genes (~10% genes assayed) is altered upon flooding (Klok et al., 2002; Liu et al., 2005; Kreuzwieser et al., 2009; van Dongen et al., 2009; Hsu et al., 2011), suggesting that global transcriptional regulatory networks play important roles in response to flooding.

Determining the roles of transcription factors (TFs) is key to understanding transcriptional regulatory networks. Several flooding-responsive TFs are known to be involved in hypoxia signaling and/or anoxic tolerance in *Arabidopsis thaliana*, including a myeloblastosis (MYB) TF, a NAM/ATAF/CUC TF,

and several APETALA2/ethylene-responsive factors (AP2/ERFs) (Hoeren et al., 1998; Christianson et al., 2009; Hinz et al., 2010; Licausi et al., 2010a; Yang et al., 2011). In rice (*Oryza sativa*), several AP2/ERFs play central roles in modulating ethylene signaling in response to submergence (Xu et al., 2006; Fukao and Bailey-Serres, 2008; Hattori et al., 2009). Although the roles of these TFs in flooding tolerance have been characterized (Xu et al., 2006; Fukao and Bailey-Serres, 2008; Hattori et al., 2009), their roles cover only a fraction of the transcriptional regulation seen in transcriptomic data. Therefore, it is important to identify and investigate more TFs and transcriptional regulatory pathways that are associated with submergence at the level of the transcriptome.

In *Arabidopsis*, the WRKY TF superfamily consists of 74 members (Eulgem, 2005). Members of the WRKY TF family contain at least one conserved DNA binding region, the WRKY domain that comprises the highly conserved WRKYGQK peptide sequence, and a zinc finger motif (Eulgem et al., 2000). WRKY TFs are involved in the regulation of gene expression during biotic stress, abiotic stress, senescence, and several developmental processes (Rushton et al., 2010). Current transcriptomic data indicate that several genes that encode WRKY TF family members are induced to high levels upon flooding (Klok et al., 2002; Liu et al., 2005; Kreuzwieser et al., 2009; Hsu et al., 2011), but the functions of these flood-responsive WRKY TFs during flooding are unclear. Furthermore, WRKY-mediated pathways are also known to have a major role underpinning immune responses in plants (Eulgem and Somssich, 2007; Pandey and Somssich, 2009).

Plant innate immunity is triggered by microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) and/or

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effector-triggered immunity. In PAMP-triggered immunity, attack by pathogens is recognized by groups of proteins termed pattern recognition receptors (PRRs) that trigger a defense response (Boller and Felix, 2009). Recognition of the molecular signatures of pathogens activates mitogen-activated protein kinases and calcium-dependent protein kinases to induce innate immunity marker genes, such as *FLG22-INDUCED RECEPTOR KINASE1* (At2g19190) (Asai et al., 2002; Boudsocq et al., 2010). These responses can eventually lead to systemic acquired resistance (SAR), which confers immunity against subsequent infections. The SAR defense responses, which are activated at different sites of pathogen attack, are mediated mainly by jasmonic acid, salicylic acid, and ethylene (Thomma et al., 2001). The development of SAR is associated with pathogenesis-related (PR) proteins, proteins encoded by the host plant but specifically induced in pathology-related situations (Van Loon and Van Strien, 1999; van Loon et al., 2006).

In this work, we found that many *WRKY* and innate immunity marker genes are strongly induced during submergence and that submergence treatment conferred higher resistance to the bacterial pathogen *Pseudomonas syringae* in *Arabidopsis*. The correlation between the inductions of these two groups of genes led us to speculate that innate immunity may be induced by submergence. T-DNA insertion mutant studies showed that *WRKY22* mediates the bacterial resistance conferred by submergence treatment. Downstream targets of *WRKY22* were identified based on differential expression of targets in *WRKY22* mutants, and the protein–DNA interactions between *WRKY22* protein and promoters of targets. Finally, our results reveal a glimpse of the possible signaling pathways that underlie higher bacterial resistance in plants that have undergone submergence treatment. We speculate that these mechanisms protect plants from pathogen infection that is more likely to occur during or after flooding.

RESULTS

Many *WRKY* Genes and Marker Genes of Innate Immunity Are Strongly Induced during Submergence

To identify submergence-responsive TFs that had not been previously studied, we dissected transcriptomic profiles in *Arabidopsis* during submergence via microarray analysis. Using a fourfold induction threshold to filter our microarray data, we found that 20 out of a total of 74 *WRKY* TF genes in the *Arabidopsis* genome were strongly induced during submergence (Figure 1A, left). Among these, seven were rapidly induced after 1 h of submergence treatment, implying early roles upstream of signaling pathways. The transcript levels of these *WRKY* genes were validated by quantitative real-time RT-PCR (qRT-PCR) (Figure 1A, right). Of the *PR* and stress-related genes tested, only *PR-3* and *PR-4* were slightly induced within 6 h of submergence, whereas most of the *PR* genes as well as *PLANT DEFENSIN1.2* (*PDF1.2*; At5g44420) were not dramatically induced (Figure 1B). By contrast, many of the innate immunity marker genes were strongly activated by submergence (Figures 1B to 1D; see Supplemental Table 1 online for gene information). *PEP1 RECEPTOR*, *ELICITOR PEPTIDE PRECURSOR*

(*PROPEP*), *CELL WALL-ASSOCIATED KINASE* (*WAK*), and *RAPID ALKALINIZATION FACTOR1* are considered to be damage-associated molecular pattern (DAMP) marker genes (Boller and Felix, 2009; Tör et al., 2009; De Lorenzo et al., 2011; Ranf et al., 2011; Ma et al., 2012; Ferrari et al., 2013; Logemann et al., 2013). Of the DAMP marker genes tested, *PROPEP2* and *PROPEP3* were induced upon submergence (see Supplemental Figure 1 online). These results indicate that plant innate immunity may be induced during submergence. We also found that these *WRKY* genes were coexpressed with innate immunity marker genes following drought, hypoxia, and salt treatments (see Supplemental Figure 2 online; Hruz et al., 2008). These results raise the possibility that the *WRKY* TFs may mediate immunity in response to various abiotic stresses.

Submergence Treatment Conferred Higher Resistance to *P. syringae*

As submergence upregulated many innate immunity markers and *WRKY* genes, we speculated that plant immunity may be increased by submergence. To determine whether plant immunity was induced, we first submerged *Arabidopsis* plants and then assayed their immunity through inoculation with the virulent bacterial pathogen *P. syringae* pv *tomato* DC3000 (*Pst* DC3000). To minimize interference from damage caused by submergence, the length of time plants were submerged was carefully chosen to ensure that submergence was long enough to induce innate immunity markers and *WRKY* genes, but not so long as to damage plants. A recent report defined the median lethal time of complete submergence in the dark to be around 8 d for *Arabidopsis* ecotype Columbia-0 (Vashisht et al., 2011). We found that submergence treatment for 12 h followed by 1 h of recovery under light in air induced high levels of transcripts for innate immunity marker genes *FRK1*, *PEROXIDASE62*, *CYP81F2*, *WRKY22*, and *WRKY30* (Figure 2). We therefore used these parameters for submergence prior to inoculation for subsequent experiments. Next, presubmerged *Arabidopsis* were dip inoculated with *Pst* DC3000 and disease progression was evaluated. The presubmerged plants developed fewer disease symptoms and had lower bacterial populations than did nonsubmerged control plants (Figure 3). Since some innate immunity markers and *WRKY* genes were induced rapidly (Figure 1), such rapid induction could contribute to certain level of submergence-triggered plant immunity. Thus, we also tested whether shorter period of submergence triggered plant immunity. The plants treated with 2 h of presubmergence developed fewer disease symptoms than did nonsubmerged control plants (see Supplemental Figure 3 online), suggesting that those rapidly induced innate immunity markers and *WRKY* genes contribute to part of the submergence-triggered immunity. Collectively, these molecular and phenotypic data indicate that prior submergence conferred higher resistance to pathogens in plants than that found in nonsubmerged plants.

WRKY22 Is Rapidly Induced by Submergence

As we had found that submergence could induce expression of innate immunity markers and *WRKY* genes and confer higher

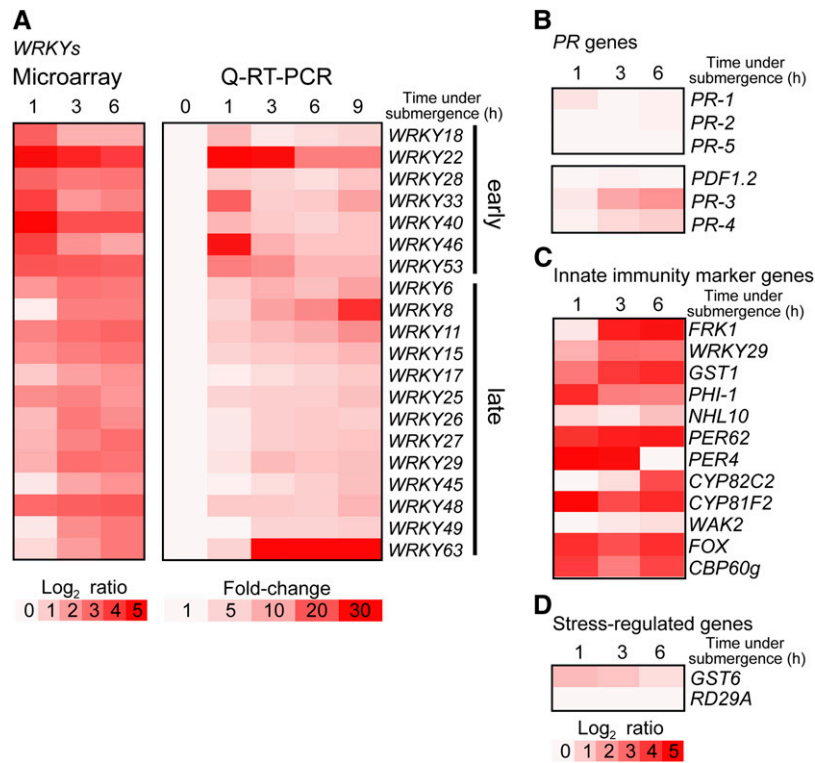


Figure 1. Induction of Innate Immunity Markers and *WRKY* Genes in Response to Submergence.

(A) Expression of *WRKY* genes is induced by submergence. Gene expression was determined by microarray analysis and validated by qRT-PCR in 9-d-old wild-type *Arabidopsis* (Columbia) seedlings from at least four independent biological replicates.

(B) to (D) Innate immunity marker genes were responsive to submergence **(C)**, but *PR* genes **(B)** and stress-regulated genes **(D)** were not (see Supplemental Table 1 online for gene information). The color scale indicates treatment-to-control ratio of expression in log₂ or in fold induction.

resistance to a virulent bacterial pathogen, we next investigated whether the induction of innate immunity markers and activation of disease resistance during submergence was mediated by the *WRKY* TFs. We thus tested whether mutations in *WRKY* could affect submergence-triggered resistance and induction of innate immunity markers upon submergence. We selected *WRKY22* for functional characterization, as it was rapidly induced after 1 h of submergence treatment and had the highest raw intensity fold induction of all the submergence-responsive *WRKY* genes in our microarray data (Figure 1A, left). As determined by qRT-PCR, *WRKY22* was upregulated 30-fold after 1 h of submergence treatment, and this upregulation gradually decreased to >10-fold after 6 h (Figure 1A, right). Although *WRKY22* is known to be induced by dark treatment (Zhou et al., 2011), our qRT-PCR data indicated that *WRKY22* was induced threefold after 1 h of dark treatment and eightfold after 1 h of hypoxic (0.5% of oxygen) treatment (Figure 4A), whereas it was induced >30-fold after 1 h of submergence treatment (Figure 1A, right; see Supplemental Figure 4C online). This strong and rapid induction of *WRKY22* suggests that *WRKY22* may play a role upstream of submergence signaling. Two independent mutant lines, designated *wrky22-1* (SALK_047120) and *wrky22-2* (SALK_098205), carrying T-DNA insertions in the first and third exons of the *WRKY22* gene, respectively, were obtained (see Supplemental Figures 4A

and 4B online). Levels of T-DNA disrupted *WRKY22* transcript in these knockout lines were reduced to ~50% of intact *WRKY22* transcript in Columbia plants (see Supplemental Figure 4C online), suggesting that in addition to disrupting the coding sequences, the T-DNA insertions also affected the mRNA levels.

Submergence-Triggered Resistance Is Affected in *WRKY22* Mutants

To determine whether *WRKY22* is important for submergence-triggered resistance, *wrky22-1* and *wrky22-2* were subjected to submergence followed by inoculation with *Pst* DC3000. After submergence, *wrky22-1* and *wrky22-2* developed more disease symptoms and higher bacterial populations than did wild-type plants (Figures 4B to 4D), suggesting that *WRKY22* mediates submergence-triggered resistance. In nonsubmerged plants, disease symptoms in *wrky22-1* and *wrky22-2* could not be visually distinguished by the naked eye (see Supplemental Figure 5A online), but the degrees of the symptoms in *wrky22-1* and *wrky22-2*, as measured by the damage index that used in Figure 3B, were statistically higher than in the wild type (see Supplemental Figure 5B online). This result suggested that *WRKY22* also mediates plant basal innate immunity. To further characterize the molecular function of *WRKY22* in

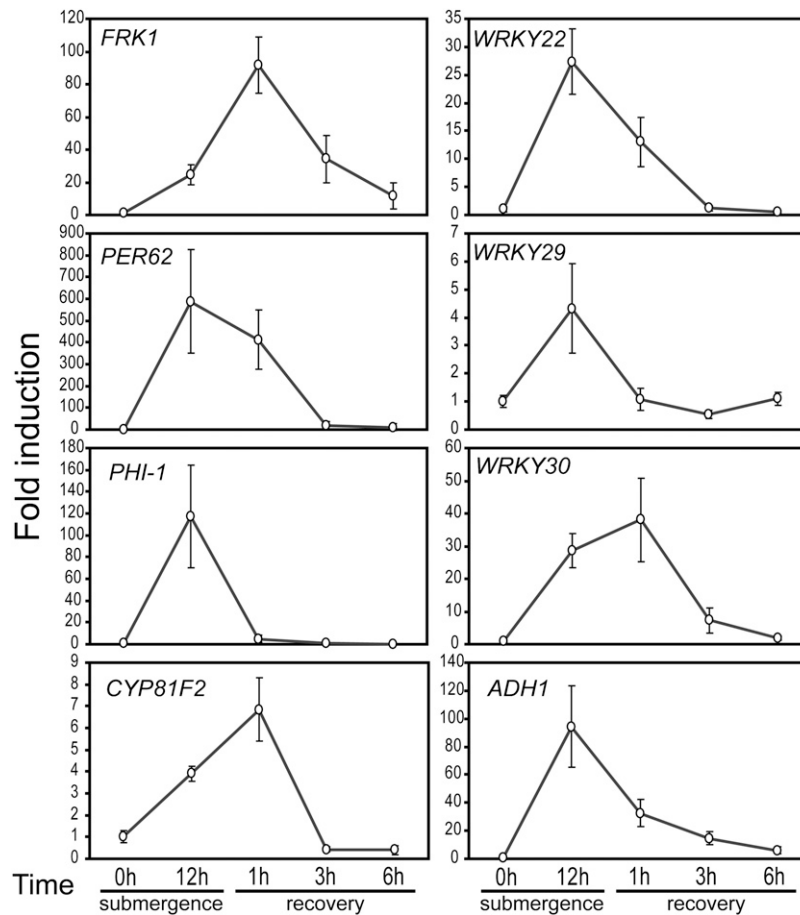


Figure 2. Expression of Innate Immunity Marker Genes after Submergence.

Five-week-old Columbia plants were submerged for 12 h and allowed to recover for up to 9 h, with samples collected at the indicated time points. Transcript levels were detected by qRT-PCR using specific primers. *TUBULIN* mRNA was used as an internal control. The data represent means \pm SD from four to seven independent biological replicates.

submergence-triggered resistance, we examined the effects of *WRKY22* mutations on the submergence-responsive innate immunity markers. Expression of two defense-related *WRKY* genes, *WRKY29* and *WRKY53*, and two innate immunity markers, *FRK1* and *CAM-BINDING PROTEIN60-LIKE G*, were examined in *wrky22-1* and *wrky22-2* with qRT-PCR. Induction of *WRKY53* and *FRK1* transcripts were significantly lower in *wrky22-1* and *wrky22-2* (Figure 5). This result indicates that *WRKY22* mediates a portion of the signaling pathway activating innate immunity in response to submergence.

Identification of *WRKY22* Targets in Response to Submergence

To investigate the *WRKY22*-mediated signaling pathway in response to submergence, we used two approaches to identify *WRKY22* target genes: (1) a time course study using Agilent *Arabidopsis* arrays to identify differentially expressed genes in *wrky22-2* lines, and (2) chromatin immunoprecipitation followed

by microarray hybridization (ChIP-chip) to screen for *WRKY22* direct targets.

To identify differential expression of submergence-responsive genes, we first generated a list of submergence-responsive genes that showed more than twofold induction in expression with raw intensity >100 at any one time point from 1 to 6 h under submergence in the wild-type Columbia strain. The expression of the selected submergence-responsive genes in the wild type was then compared with the expression in the *wrky22-2* line. Genes with raw intensities or normalized ratios >1.5 (Columbia/*wrky22-2* ratio) were identified as genes downregulated in the *wrky22-2* line (see Supplemental Data Set 1 online), among which were potential targets of *WRKY22*. The top 20 genes according to the ratios from each time point were taken. These genes were then categorized based on functional classification. Differentially regulated genes not included in the top 20 lists but within the same family or classification were also taken (see Supplemental Table 2 online). Expression of many submergence-responsive genes was affected in *wrky22-2* line. These genes were functionally categorized as immunity related,

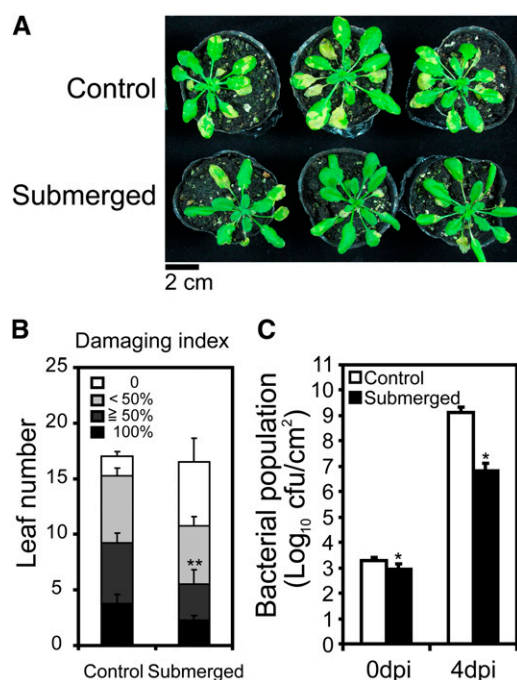


Figure 3. Plant Immunity Is Triggered by Submergence.

(A) Disease symptoms assessed 4 d postinoculation (dpi) with *P. syringae* in submerged and control Columbia plants. Bar = 2 cm.

(B) The levels of resistance were defined using a damage index based on the necrotic and chlorotic area of leaves (black, 100% leaf area; dark gray, equal to or >50% leaf area; light gray, <50% leaf area; white, no damage observed). The data represent means \pm SD from four independent repeats. Statistical differences between submerged and control were determined using Student's *t* test for the sum of 100% and \geq 50% indexes. ***P* < 0.01.

(C) Bacterial population at 0 and 4 d postinoculation in submerged and control Columbia plants. The data represent means \pm SD from five independent repeats. Statistical differences between submerged and control plants were determined by Student's *t* test. **P* < 0.05. cfu, colony-forming units.

TFs, carbohydrate and amino acid metabolism related, redox related, transporters, heat shock proteins, protein kinases, U-box proteins, and hormone biosynthesis related, suggesting that these functional categories were mediated by WRKY22 in response to submergence.

To identify direct targets of WRKY22, we created a transgenic *Arabidopsis* line that expresses a c-myc epitope-tagged WRKY22 and used ChIP-chip to screen for candidates and validate the in vivo protein–DNA interactions with ChIP followed by quantitative PCR (ChIP–qPCR). The WRKY22 and c-myc epitope tag fusion construct was generated and transformed into *wrky22-2* plants. The resulting transgenic lines should have better ChIP efficiency than the wild-type background, due to the reduced competition for WRKY22 binding sites from endogenous WRKY22. RT-PCR and immunoblot analyses were performed to validate that the induction patterns of transcript and protein levels of c-myc–tagged WRKY22 during hypoxia were similar to those of endogenous WRKY22 (see Supplemental

Figure 6 online). ChIP-enriched DNA fragments were identified using criteria of a window of +300 to –1200 of a gene for a promoter, a width of four probes or more, and a false discovery rate < 0.1. The ChIP-chip experiments were repeated six times (i.e., six biological replicates). Candidates were defined by the presence of the promoter in three out of six biological replicates. Candidates were then classified based on their hypoxic responsiveness with a positive response defined as gene expression levels exhibiting more than twofold or <0.5-fold induction in any time point under submergence treatments in expression array data. Three candidate lists of upregulated, downregulated, and unchanged targets in response to submergence were generated (see Supplemental Table 3 online). Among 569 candidates, 29 were upregulated, 22 were downregulated, and 518 were not significantly regulated. To determine the submergence induction of gene expression that is directly mediated by WRKY22, we focused on the 29 upregulated candidates (see Supplemental Table 3 online). Twenty-two out of 29 promoters in the list harbor a canonical W-box, which is defined by the sequence TTGACY. A degenerate W-box (TTGACN) sequence, which is used as a lenient W-box definition (Ciolkowski et al., 2008), can be found in six of the seven promoters without a canonical W-box in the upregulated candidate list. ChIP–qPCR was then used to validate these in vivo protein–DNA interactions. Fourteen candidates that were randomly selected from the upregulated candidate list were tested using ChIP–qPCR. Twelve candidates (~86%) passed the validation. Enrichment of many promoter regions was observed, including the promoters of *At5g44910*, *1-AMINO-CYCLOPROPANE-1-CARBOXYLATE SYNTHASE7 (ACS7)*, *ENDOPLASMIC RETICULUM OXIDOREDUCTINS2 (ERO2)*, *PLANT U-BOX 24 (PUB24)*, and *TREHALASE1 (TRE1)* (see Supplemental Table 3 online). This result suggests that WRKY22 directly targets the promoter regions of these submergence-responsive genes.

Innate Immunity Conferred by Submergence Is Mediated by WRKY22

Plant innate immunity is usually triggered through PRRs in response to pathogen-produced MAMPs. For instance, exogenous applications of MAMPs triggered coinduction patterns of WRKYs and innate immunity marker genes (see Supplemental Figure 2B online). Surprisingly, MAMP-free submergence conditions were also able to induce coexpression of WRKYs and innate immunity marker genes (Figures 1A and 1C). Thus, we speculated that submergence may activate *Arabidopsis* endogenous elicitors that trigger defense mechanisms similar to those elicited by MAMPs. The WRKY22 target *FRK1* is known to be triggered through receptor-like kinase (RLK) signaling of FLAGELLIN-SENSITIVE2 (FLS2) in response to flagellin or flg22 (a peptide fragment of flagellin) from microbes (Gómez-Gómez and Boller, 2000). A recent report showed that FLS2 not only recognizes flg22 peptide originally from microbes, but also interacts with a plant endogenous peptide hormone, CLAVATA3 peptide (Lee et al., 2011), suggesting that PRRs can recognize both MAMPs and other molecular patterns to trigger similar signaling pathways.

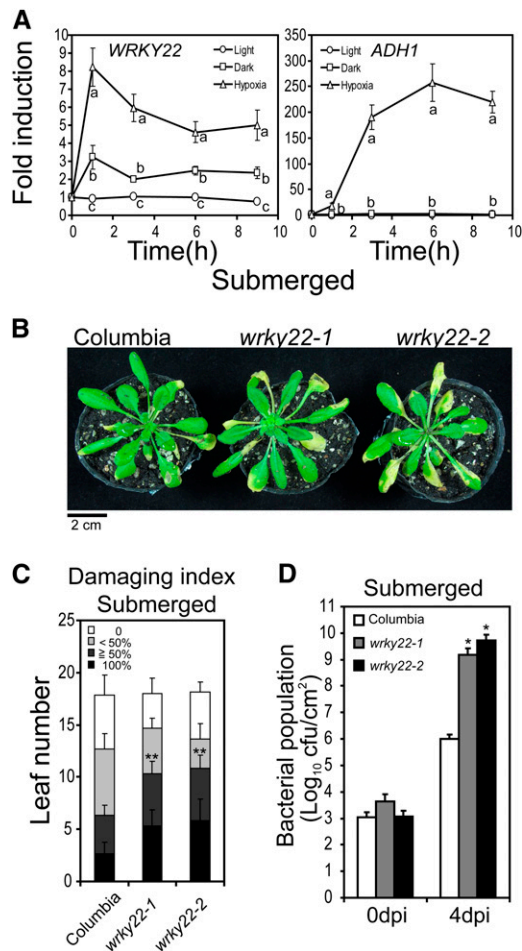


Figure 4. *WRKY22* Mediates Submergence-Triggered Immunity.

(A) Transcript levels of *WRKY22* and *ADH1* were detected by qRT-PCR. Nine-day-old Columbia seedlings were in light, dark, or hypoxia (0.5% oxygen gas balanced with nitrogen in dark) for up to 9 h and were collected at specific time points (0, 1, 3, 6, and 9 h). *TUBULIN* mRNA was used as an internal control. The data represent means \pm SD from seven independent biological replicates and were subjected to a one-way analysis of variance and Tukey's honestly significant difference tests ($P < 0.05$). Data from the same time point with different lowercase letters were significantly different from each other.

(B) Disease symptoms assessed 4 d postinoculation in submerged Columbia, *wrky22-1*, and *wrky22-2* plants. Bar = 2 cm.

(C) Levels of resistance in submerged Columbia, *wrky22-1*, and *wrky22-2* plants. The levels of resistance were defined using a damage index based on the necrotic and chlorotic area of leaves, as described in Figure 3. The data represent means \pm SD from eight independent repeats. Pairwise statistical differences between Columbia and *WRKY22* mutants were determined by Student's *t* test for the sum of 100% and $\geq 50\%$ indexes. ** $P < 0.01$.

(D) Bacterial populations at 0 and 4 d postinoculation in submerged Columbia, *wrky22-1*, and *wrky22-2* plants. The data represent means \pm SD from five to six independent repeats. Statistical differences between Columbia and *WRKY22* mutants were identified using Student's *t* test. * $P < 0.05$.

By integrating our results of *WRKY22* targets derived from expression array and ChIP with validation by qRT-PCR (Figure 6; see Supplemental Figure 7 online), we found that *WRKY22* mediates PRR-related and stress-related signals in response to submergence. Notably, a toll/interleukin-1 receptor domain-containing protein, encoded by a *WRKY22* direct target At5g44910 (Figure 6A), is also a RLK, which is a potential peptide receptor. An indirect target *PSK1* (At1g13590; Figure 6A), which encodes a plant peptide hormone, could generate or amplify signals. *OLIGO PEPTIDE TRANSPORTER1* (*OPT1*; At5g55930) and several *OPT* genes, which were found to be *WRKY22* targets (Figure 6A; see Supplemental Table 2 online), could transport peptide- or amino acid-related signals. Another type of RLK, wall-associated kinases, can recognize oligogalacturonides that are released from plant cell wall fragments as DAMPs (Brutus et al., 2010; De Lorenzo et al., 2011). Several members of the WAK-like (WAKL) family (i.e., *WAKL2*, *WAKL6*, and *WAKL10*) were identified as putative targets of *WRKY22* upon submergence (see Supplemental Table 2 online). Moreover, several biotic stress-related or MAMP-induced genes were also identified as targets of *WRKY22* in response to submergence (Figure 6B). In addition to *WRKY22*-mediated induction of *FRK1* under submergence (Figure 5; see Supplemental Table 2 online), these characteristics of *WRKY22* targets strengthen the idea that submergence may lead to PRR-mediated signaling through the mediation of *WRKYs* to trigger innate immunity.

***WRKY22*-Regulated Networks upon Submergence Are Associated with Pathogen Resistance**

To uncover *WRKY22*-regulated transcriptional networks upon submergence, we integrated our experimentally derived lists of *WRKY22* targets from qRT-PCR results (Figures 5 and 6; see Supplemental Figure 7 online), microarray analysis (asterisk-labeled genes in Supplemental Table 2 online), and ChIP-qPCR

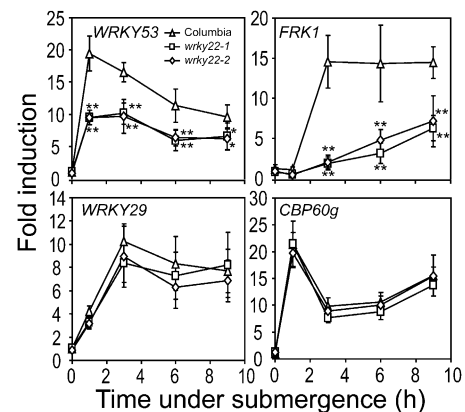


Figure 5. Innate Immunity Markers *FRK1* and *WRKY53* Are Transcriptionally Regulated by *WRKY22*.

Transcript levels were detected by qRT-PCR using specific primers. *TUBULIN* mRNA was used as an internal control. The data represent means \pm SD from four to eight independent biological replicates. * $P < 0.05$ and ** $P < 0.01$ in Student's *t* test.

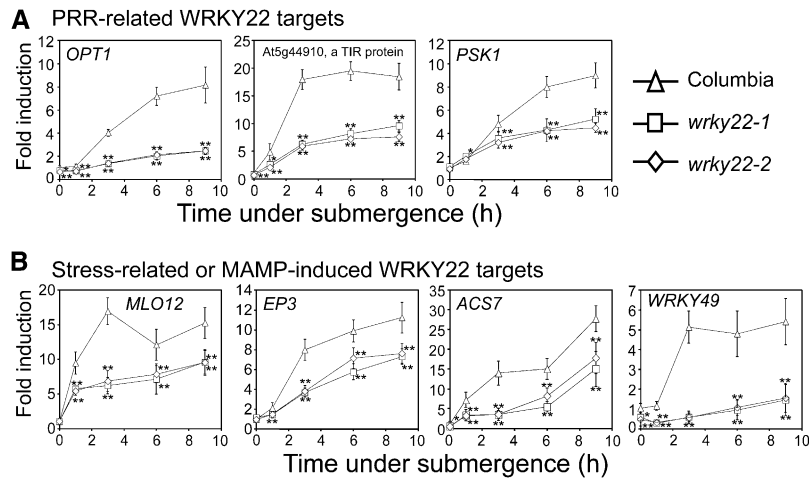


Figure 6. Several PRR-Related and MAMP-Induced Genes Are WRKY22 Targets under Submergence.

Transcript levels of PRR-related (**A**) and MAMP-induced (**B**) genes were detected by qRT-PCR using specific primers. *TUBULIN* mRNA was used as an internal control. The data represent means \pm SD from four to eight independent biological replicates. * $P < 0.05$ and ** $P < 0.01$ in Student's *t* test.

results (see Supplemental Table 3 online) and then used the STRING Web-based database (Szklarczyk et al., 2011) to build functional interaction networks based on available experimental evidence (Figure 7). In addition to the previously mentioned PRR-related genes affected by WRKY22, many pathogen-associated genes are highly connected as subnetworks. One subnetwork includes two WRKY22-regulated innate immunity markers, *FRK1* and *WRKY53*, and an RLK gene (*At4g11890*) that is upregulated during downy mildew disease infection (Hok et al., 2011). Another subnetwork contains *MILDEW RESISTANCE LOCUS O 12* (*MLO12*), which is a member of a conserved protein family in plants required for powdery mildew fungi pathogenesis (Panstruga, 2005; Consonni et al., 2006). Another WRKY22 target, *EP3* (*At3g54420*), encoding a class IV chitinase, is rapidly induced by MAMPs (e.g., flg22) as well as bacterial and viral pathogens (de A. Gerhardt et al., 1997; Whitham et al., 2003; Navarro et al., 2004). In qRT-PCR analysis, both *MLO12* and *EP3* were expressed at a lower level in *wrky22* mutants (Figure 6B). In a WRKY22-regulated subnetwork, *MYB15* (*At3g23250*) and *PUB24* (*At3g11840*), encoding a TF and a U-box E3 ubiquitin ligase, respectively, have been shown to respond to the plant defense elicitor chitin (Libault et al., 2007). Collectively, the existence of WRKY22-regulated networks associated with pathogen responses suggests that WRKY22 plays a role in regulating submergence-triggered pathogen resistance.

WRKY22-regulated networks are also associated with abiotic stress. Notably, ethylene biosynthesis is known to be regulated through transcriptional control of a key enzyme, 1-aminocyclopropane-1-carboxylate synthase, during flooding (Van Der Straeten et al., 2001; Peng et al., 2005; Rieu et al., 2005). We found that *ACS7* was transcriptionally regulated by WRKY22 via directly binding to the *ACS7* promoter under submergence (Figures 6B and 7; see Supplemental Table 3 online). A recent article shows activation of the *ACS7* promoter by abiotic stresses and further suggests *ACS7* is involved in the crosstalk

between ethylene and abscisic acid for the abiotic stress adaptation of plants (Dong et al., 2011). Submergence induction of another WRKY22 direct target, *TRE1*, was negatively regulated by WRKY22 (Figure 7; see Supplemental Figure 7B and Supplemental Table 3 online). Expression of *TRE1* is involved in stomatal function and leads to increased drought tolerance (Van Houtte et al., 2013). Responsiveness of *WOUND-RESPONSIVE3/NITRATE TRANSPORTER3.1* (*WR3/NRT3.1*), a wound-induced marker gene (León et al., 1998), to submergence was reduced in *wrky22* mutants (Figure 7; see Supplemental Figure 7B and Supplemental Table 2 online). Interestingly, other than the involvement of *EP3* in defense responses, the WRKY22 target *EP3* has also been shown to be induced by an abiotic stress, phosphate starvation (Hammond et al., 2003). Taken together, these WRKY22-regulated networks associated with abiotic stresses suggest that WRKY22 could act as a universal node in the mechanisms commonly responsive to a spectrum of abiotic stresses, including wounding, drought, and submergence.

DISCUSSION

Here, we report previously unknown regulatory pathways that mediate responses in submergence and pathogen resistance in *Arabidopsis*. We demonstrated that submergence can activate innate immunity markers and WRKY TFs to confer higher disease resistance to plants. Using mutant analysis, we were able to demonstrate that a submergence-inducible WRKY22 acts as a regulator to mediate the activation of innate immunity. This example lends genetic support to the hypothesis that other members of submergence-inducible WRKYs in the family could also play roles in submergence-triggered disease resistance. Via identifying downstream targets of WRKY22, we further demonstrated potential genes and their molecular functions involved in the underlying signaling pathways.

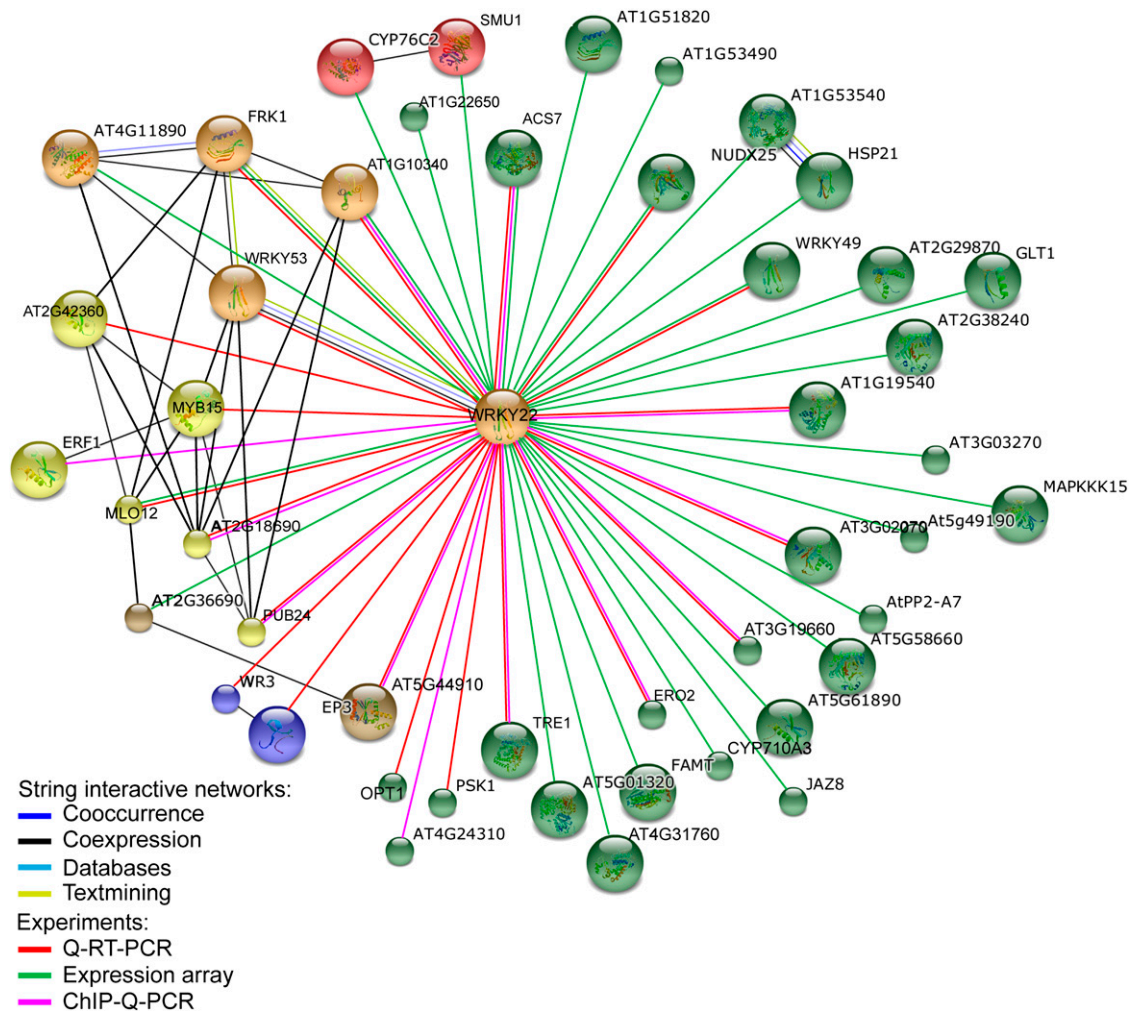


Figure 7. Integrated Networks of WRKY22 Downstream Targets upon Submergence.

Networks were constructed with a Web-based analysis tool STRING (Szklarczyk et al., 2011). Gene lists of WRKY22 downstream targets are integrated from qRT-PCR results (Figures 5 and 6; see Supplemental Figure 7 online), microarray analysis (asterisk-labeled genes in Supplemental Table 2 online), and ChIP-qPCR results (see Supplemental Table 3 online). These three types of experimental evidence are represented by three different line colors. The STRING database assembles information about co-occurrence, coexpression, databases, and text mining, which are also marked with different line colors. Nodes of genes were clustered to six groups by KMEANS in STRING and marked by a different color code.

Interconnection between Biotic and Abiotic Stresses

Stress research is typically focused on a single environmental challenge. However, in natural environments, plants are simultaneously or sequentially exposed to multiple stresses. To survive stresses, plants have evolved sophisticated defense mechanisms against both biotic and abiotic stresses. Submergence is a complex stress that consists of multiple environmental changes, including light intensity, temperature, pH, and dissolved oxygen concentration. In fields, water logging may increase the incidence of root rot diseases caused by plant pathogens (Davison and Tay, 1987; Walker, 1991; Yanar et al., 1997). The increased incidence of disease could be due to higher probability of pathogen infection after the flood and/or faster development of the pathogen under higher humidity (Huber and Gillespie, 1992). Although a current report shows

two rice chromosomal regions associated with biotic stress and submergence tolerance based on *in silico* data (Kottapalli et al., 2006), defense mechanisms against biotic and submergence stresses are largely unknown.

Defense responses in abiotic and biotic stresses are regulated by groups of cross-communicating signal transduction pathways. Hormones, such as abscisic acid, jasmonic acid, salicylic acid, and gibberellic acid, mediate signaling pathways required for both pathogen resistance and tolerance of abiotic stresses, including wounding, drought, salt, and cold (Ding et al., 2002; Xiong and Yang, 2003; Chini et al., 2004; Tanaka et al., 2006). A rice mitogen-activated protein kinase gene, *MAPK5*, negatively regulates pathogen defenses and positively mediates abiotic stresses (Xiong and Yang, 2003). An *Arabidopsis* disease resistance protein, *ACTIVATED DISEASE RESISTANCE1*, which processes

N-terminal kinase subdomains, enhances drought tolerance (Chini et al., 2004). In addition, many TFs mediate responses for both disease defense and abiotic stresses (Guo et al., 2004; Cao et al., 2006; Sohn et al., 2006; Zhang et al., 2007; Liu et al., 2008; Seo et al., 2010; Zhang et al., 2009). A recent review discussed the central roles of some WRKY TFs in mediating both abiotic and biotic stresses from the point of view of systems biology (Friedel et al., 2012). Together, these data suggest that common signaling pathways interconnect biotic stress and some abiotic stresses. Our study describes such a signaling pathway that specifically connects biotic stress and submergence.

Stomatal Immunity Could Contribute to Part of Submergence-Triggered Immunity

Stomatal closure is a part of innate immunity responses that mediated by the FLS2 receptor against bacterial invasion

(Melotto et al., 2006; Zeng and He, 2010). Recent studies demonstrate the involvement of L-type lectin receptor kinases (LecRK) in stomatal immunity. LecRK-VI.2 mediates PAMP-triggered immunity response and acts as a positive mediator of stomatal immunity (Singh et al., 2012). Mechanisms reversing such bacteria-mediated stomatal closure involve negative regulation of stomatal immunity by LecRK-V.5 (Desclos-Theveniau et al., 2012). Our data showed that the bacterial population was lower in submerged plants than in nonsubmerged control plants (Figure 3C). Interestingly, this bacterial titer in submerged plants was also statistically lower at 0 days after inoculation, suggesting that stomatal immunity could be involved in submergence-triggered immunity. Notably, transcripts of both positive and negative mediators of stomatal immunity, *LecRK-VI.2* and *LecRK-V.5*, were highly accumulated under submergence (see Supplemental Figure 8 online), suggesting that stomatal immunity is transcriptionally regulated under

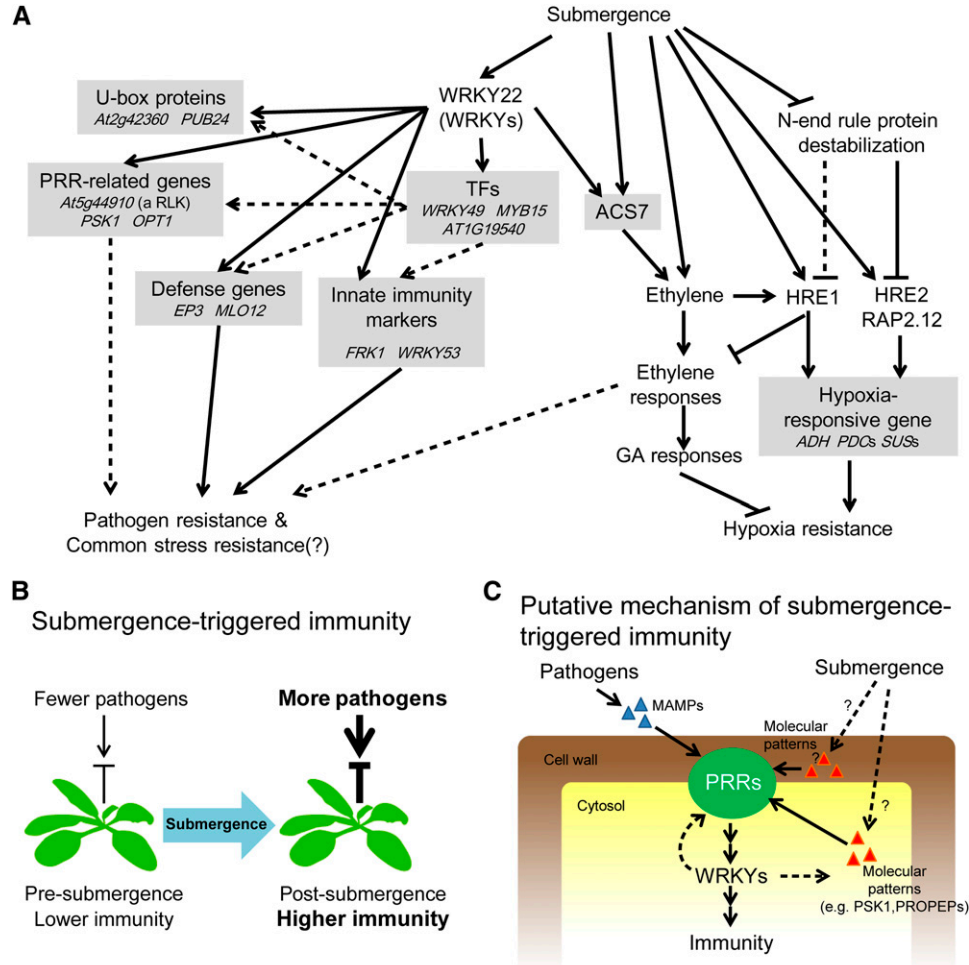


Figure 8. Models of WRKY22-Mediated Submergence-Triggered Responses.

(A) Summary of WRKY22-mediated and -independent pathways under submergence.

(B) Model of submergence-triggered immunity.

(C) Putative molecular mechanism of submergence-triggered immunity. Solid lines indicate validated events. Dotted lines and question marks indicate predicted events.

submergence. Additionally, a chloroplastic enzyme, ASPARATE OXIDASE (AO), is required for stomatal immunity (Macho et al., 2012). Our data showed transcripts of AO and several other *LecRK* genes were regulated under submergence (see Supplemental Figure 8 online). Collectively, stomatal immunity could represent a part of the mechanism for submergence-triggered immunity against bacterial invasion. However, further evidence associated with stomatal immunity, such as oxidative burst, stomatal aperture, and regulatory mechanisms of LecRKs, is required from future studies. Understanding of such submergence-triggered stomatal immunity is important to elucidate defense mechanisms of submergence-triggered immunity.

WRKY TFs May Modulate Regulatory Networks in Response to Submergence

A number of WRKY TFs were highly induced by submergence (Figure 1), suggesting the existence of WRKY-mediated transcriptional regulation in submergence signaling. Some of these submergence-responsive WRKYs were induced during early stages of submergence treatments, while others were induced later. Due to the different temporal expression patterns among induced WRKYs, it is likely that WRKYs could modulate early and late submergence signals and might be positioned upstream and downstream within submergence signaling pathways. Some WRKYs are regulated by mitogen-activated protein kinase cascades in plant defense signaling through protein phosphorylation and/or transcriptional regulation (Asai et al., 2002; Miao et al., 2007; Qiu et al., 2008). Several WRKYs are known to regulate their own expression or expression of other WRKY genes, indicating the occurrence of WRKY auto/cross-regulation (Miao et al., 2007; Qiu et al., 2008; Skibbe et al., 2008). Given the auto- and cross-regulation observed for some WRKY TFs, one may further speculate that WRKY proteins regulate themselves or with other TFs to form regulatory networks in submergence signaling. Through identification of WRKY22 targets, we indeed found that many WRKYs and other families of TFs are targets of WRKY22 to form regulatory networks (Figures 5 to 8; see Supplemental Tables 2 and 3, Supplemental Figure 7, and Supplemental Data Set 1 online).

Submergence Triggers Hypoxia-Specific Pathways and Pathways Commonly Induced by Stresses

Oxygen deficiency induces a wide spectrum of TFs in *Arabidopsis* (Licausi et al., 2010b; Hsu et al., 2011). Among these TFs, some are specifically induced by oxygen deficiency and some are commonly induced by stresses. For example, we previously studied *HRE1* (At1g72360), an AP2/ERF that is specifically induced by hypoxic treatments (Yang et al., 2011). In this study, we here show that a group of WRKY TFs were strongly induced by submergence (Figure 1) and other stresses, including drought and salt (see Supplemental Figure 2 online). We not only found that these WRKY genes were coexpressed with innate immunity marker genes (Figure 1; see Supplemental Figure 2 online), but also determined that a representative WRKY TF, WRKY22, regulates innate immunity genes, defense genes, and PRR-related genes (summarized in Figure 8A). Submergence-triggered responses of several signaling components, such as TFs, U-box

proteins, and ACS7, were also mediated by WRKY22. Regarding pathways specifically responsive to hypoxia, *HRE1* and *HRE2* are specifically and strongly induced by hypoxia and mediate induction of hypoxic core genes, including *ADH*, *PDCs*, and *SUSs* (Licausi et al., 2010a; Yang et al., 2011). The induction of *HRE1* can be triggered by ethylene and then plays a negative regulatory role in modulating ethylene responses (Yang et al., 2011). In addition to regulation on the transcript level, HRE2 protein stability is enhanced under hypoxia through an N-end rule protein destabilization mechanism (Gibbs et al., 2011). Through identification of WRKY22-regulated networks, we here provide an example of regulatory pathways upon submergence for TFs that are commonly induced by stresses.

Activation of Innate Immunity Could Have Coevolved with Submergence

A recent report demonstrated that plant immune responses are under the control of a circadian regulator, CIRCADIAN CLOCK-ASSOCIATED1, and suggested that this temporal control of plant defense allows plants to anticipate infection at the time of day when a pathogen normally disperses the spores and time immune responses accordingly (Wang et al., 2011). This example supports the notion that plants have evolved mechanisms that are intimately intertwined with environmental conditions. Our study showed that submergence equips *Arabidopsis* with higher innate immunity through WRKY22. In natural conditions, submergence commonly leads to a higher probability of pathogen infection and faster development of pathogens (Davison and Tay, 1987; Huber and Gillespie, 1992; Kottapalli et al., 2006). Therefore, we suggest that plants have evolved disease defense mechanisms in response to submergence in anticipation of a higher risk of pathogen attack (Figure 8B).

Summary

The establishment of a link between submergence stress and plant defense highlights a hitherto unrevealed aspect of the arsenal plants evolved against environmental variation. Although the underlying signaling networks have yet to be fully elucidated, this study gives a glimpse into the possible signaling pathways involved in submergence-triggered disease resistance (Figures 8B and 8C). Understanding the perception and upstream signaling that primes immunity in response to submergence will be the key to revealing the signaling networks. The next challenge is to elucidate the specific cues, particularly the roles of related PRRs and molecular patterns that trigger plant immunity under submergence.

METHODS

Plant Materials

Experiments were performed on *Arabidopsis thaliana* ecotype Columbia-0. Two T-DNA insertion mutant lines of *WRKY22*, *wrky22-1* (SALK_047120) and *wrky22-2* (SALK_098205), were obtained from the ABRC, Ohio State University. For ChIP, transgenic lines expressing *c-myc*-tagged *WRKY22* in *wrky22-2* background plants were generated using a T-DNA insertion containing 1000 bp of the upstream promoter

sequence and *WRKY22* full-length genomic DNA in frame with 18 copies of *c-myc* sequence at 3' end in a pMDC110-derived binary vector. The fragment of *WRKY22* promoter sequence and full-length genomic DNA were amplified from Columbia-0 genomic DNA with a set of primers fused with attB sites, AtWRKY22p-1attB1(5'-GGGGACAAGTTTGTAC-AAAAAAGCAGGCTTTTCCAAGTGTTCATACT-3') and AtWRKY22-2 w/o-stop-attB2 (5'-GGGGACCACATTTGTACAAGAAAGCTGGGTTATTC-CTCCGGTGGTAGTGG-3'), for Gateway cloning.

Growth Conditions

All seeds were surface sterilized with 0.5% sodium hypochlorite for 20 min and washed at least five times with sterilized water. Seeds were sown on plates with 0.55% Phytigel (Sigma-Aldrich) in half-strength Murashige and Skoog (MS) medium (Duchefa Biochemie) containing 0.5% Suc at pH 5.7 and kept at 4°C in the dark for 3 d to achieve uniform germination. The plates were then transferred to a growth chamber and placed vertically. Plants were grown at 22°C under a 16-h-light (81 $\mu\text{mol s}^{-1} \text{m}^{-2}$)/8-h-dark cycle until the indicated ages. To obtain 5-week-old plants for inoculation assays, the plates with 5-d-old seedlings were transferred to a growth chamber and grown under a 9-h-light (81 $\mu\text{mol s}^{-1} \text{m}^{-2}$, 09:00 to 18:00 h; at 22°C)/15-h-dark (at 18°C) cycle for 2 d. These 7-d-old seedlings were then transplanted onto a 6:2:1 mixture of peat moss, vermiculite, and perlite in pots until they were 5 weeks old. To obtain 14-d-old seedlings for ChIP assay, 5-d-old seedlings were transplanted onto fresh plates, and the plates were placed vertically to prevent roots from growing into medium. The transplanted seedlings were grown in the growth chamber until they were 14 d old.

Submergence and Low Oxygen Treatments

For presubmergence treatment of 5-week-old plants for inoculation, pots with plants were placed into distilled water that was bubbled with 3% oxygen balanced with nitrogen at a depth of at least 3 cm from the water surface. To prevent the pot and medium floating during submergence, four glass beads (1.6 cm diameter) were loaded into the bottom of pots before the seedlings were transplanted. For submergence treatments of 9-d-old seedlings, plates with plants on the surface of the medium were placed into half-strength MS liquid medium that was bubbled with 3% oxygen balanced with nitrogen for the indicated times. The liquid MS medium was pretreated with 3% oxygen for 1 h before use. For gas treatments, plates with plants on the surface of the medium were placed into air-tight jars. For hypoxic treatments, the gas in the jars was replenished with at least 10 \times volume of premixed 0.5% oxygen balanced with nitrogen for the time indicated. For anoxic treatments, the gas in the jars was replenished with at least 10 \times volume of pure nitrogen and supplemented with a pack of oxygen absorbent (GasPak EZ Anaerobe Container System; Becton, Dickinson and Company) for the indicated times. All treatments were performed in the dark.

Pseudomonas syringae Inoculation

Strain DC 3000 of *P. syringae* pv *tomato* (*Pst* DC3000) was cultivated at 28°C, 200 rpm in King's medium B (King et al., 1954) containing rifampicin (50 mg/L). For bacterial inoculation, *Pst* DC3000 was collected by centrifugation and resuspended in 10 mM MgCl₂ at $A_{600} = 0.2$, corresponding to a titer of 10⁸ colony-forming units/mL. Bacteria were then diluted to 10⁷ colony-forming units/mL in 10 mM MgCl₂ and 0.02% Silwet L-77 for dip inoculation. After inoculation, plants were kept in 100% relative humidity.

Quantification of Bacterial Population

For quantification of bacterial populations, three leaf discs (0.38-cm diameter) from one individual plant were pooled as a single data point. Leaf discs were collected from leaves washed twice with sterile water and

homogenized in King's B containing rifampicin, followed by plating appropriate dilutions on solid medium.

Microarray Analysis

Total RNA isolation, DNase treatment, and RNA integrity assays were conducted as previously described (Hsu et al., 2011). Preparation of fluorescence-labeled cDNA and microarray experiments were performed at the DNA Microarray Core Facility, Institute of Plant and Microbial Biology, Academia Sinica, Taiwan, as described at <http://ipmb.sinica.edu.tw/microarray/protocol.htm>. Arrays in this study were performed with the Agilent *Arabidopsis* (V4) Gene Expression Microarray, 4 \times 44k, based on the manufacturer's two-color microarray protocols. Four independent biological replicates were performed, of which two included a dye-swap. Array signals were detected and analyzed using the Agilent DNA Microarray Scanner G2565CA and Agilent Feature Extraction 10.7.1.1 software, respectively. The acquired results were then imported into GeneSpring 11.5 (Agilent Technologies) using lowess normalization. The data discussed in this publication have been deposited in the National Center for Biotechnology Information (NCBI)'s Gene Expression Omnibus (GEO) (Edgar et al., 2002) and are accessible through GEO Series accession number GSE40139 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40139>).

qRT-PCR

Reverse Transcription and qRT-PCR were conducted as previously described (Hsu et al., 2011). Briefly, qRT-PCR was performed using 1 μL cDNA (from 2 μg total RNA to 100 μL cDNA), 0.2 μM each primer, and SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7500 real-time PCR machine (Applied Biosystems) using the default settings. Sequences of primers used can be found in Supplemental Table 4 online. *TUB3* (AT5G62700) was used as an internal control for normalization. Relative expression levels were compared by calculating the expression of the gene at a certain time point to a common reference sample from the same tissues obtained at time zero from Columbia.

ChIP

To immunoprecipitate the WRKY22 protein, transgenic plants expressing *c-myc* epitope-tagged WRKY22 were used. To minimize the competition of protein-DNA interaction with endogenous WRKY22, this tagged WRKY22 was expressed in *wrky22-2* plants. The antibody for the ChIP experiments was a monoclonal antibody against *c-myc* (9E10), which was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. ChIP protocols were as previously described by Kaufmann et al. (2010). Briefly, 1.5 g of 14-d-old seedlings was chemically cross-linked by the addition of 1% formaldehyde for 15 min at room temperature. The fixed seedlings were rinsed five times with MC buffer (10 mM sodium phosphate, pH 7, 50 mM NaCl, and 0.1 M Suc) and frozen in liquid nitrogen. The frozen samples were homogenized, filtered, centrifuged, and washed to isolate nuclei. The nuclear pellets were resuspended, lysed, and sonicated to shear cross-linked DNA. The sonication was performed with a Bioruptor UCD-200 (Diagenode) until the average size of sheared DNA was ~500 bp. The sonicated chromatin was precleared with protein-A agarose beads for 1 h and immunoprecipitated with 2 μg anti-*c-myc* antibody for 2 h followed by addition of protein-A agarose beads for 3 h. The beads were washed five times with IP buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 10 μM ZnSO₄, 1% Triton X-100, and 0.05% SDS). The bound IP complexes were eluted with elution buffer (0.1 M Gly, 0.5 M NaCl, and 0.05% Tween 20, pH 2.8) and neutralized with 1 M Tris, pH 9.0. The eluates were digested with proteinase K overnight at 37°C to remove proteins followed by incubation at 65°C for at least 6 h to reverse cross-linking. The reverse cross-linked DNA was then purified by

extraction with phenol:chloroform:isoamyl alcohol and ethanol precipitation in the presence of glycogen. The DNA pellets were washed with 70% ethanol, air dried, and resuspended in TE 8.0.

ChIP-Chip and ChIP-qPCR

One hundred picograms of DNA were amplified using the GenomePlex Whole Genome Amplification Kit (Sigma-Aldrich) according to the manufacturer's instructions. The amplified DNA was then purified using the purification kit (Qiagen). The purified DNA was quantified using a Nano-drop spectrophotometer and labeled with Cy3 (for Columbia) and Cy5 (for *WRKY22*-18×c-myc in *wrky22-2*) fluorescent dyes. The labeled DNA was hybridized to NimbleGen *A. thaliana* ChIP-chip 385K Minimal Promoter Arrays (NimbleGen). The hybridization and washing of arrays were performed following the NimbleGen standard operating protocols (www.nimblegen.com). Array images were acquired with Axon GenePix 4000B and GenePix 6.0 software (Axon Instruments). The images were then converted to scaled log₂ ratio of the probes for peak finding analysis according to the genomic position in NimbleScan software (NimbleGen). Peaks of enriched DNA fragments were identified and mapped to promoters in the *Arabidopsis* genome using the default setting in the NimbleScan software (NimbleGen) according to the manufacturer's description. The data discussed in this publication have been deposited in NCBI's GEO (Edgar et al., 2002) and are accessible through GEO Series accession number GSE40138 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40138>). For ChIP-qPCR, Cy dye labeling was omitted. The DNA was diluted with TE 8.0 to 2 ng/μL. ChIP-qPCR was performed using 1 μL of the diluted DNA using the methods described above for qRT-PCR. The primer sequences used can be found in Supplemental Table 4 online.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At1g10340, At1g19540, At1g22650, At1g51820, At1g53490, At1g53540, At2g18690, At2g29870, At2g36690, At2g38240, At2g42360, At3g03270, At3g19660, At3g20270, At4g11890, At4g24310, At4g31760, At5g01320, At5g44910, At5g49190, At5g58660, At5g61890, ACS7 (At4g26200), *ADH1* (At1g77120), *AO* (At5g14760), *At-PP2-A7* (At5g45090), *CBP60g* (At5g26920), *CYP710A3* (At2g28850), *CYP76C2* (At2g45570), *CYP81F2* (At5g57220), *EP3* (At3g54420), *ERF1* (At3g23240), *ERO2* (At2g38960), *FAMT* (At3g44860), *FRK1* (At2g19190), *GLT1* (At5g53460), *HSP21* (At4g27670), *JAZ8* (At1g30135), *LecRK-V.5* (At3g59700), *LecRK-VI.2* (At5g01540), *MAPKKK15* (At5g55090), *MLO12* (At2g39200), *MYB15* (At3g23250), *NUDX25* (At1g30110), *OPT1* (At5g55930), *PDF1.2* (At5g44420), *PER62* (At5g39580), *PHI-1* (At1g35140), *PR-3* (At3g12500), *PR-4* (At3g04720), *PROPEP2* (At5g64890), *PROPEP3* (At5g64905), *PSK1* (At1g13590), *PUB24* (At3g11840), *RALF1* (At1g02900), *SMU1* (At1g73720), *TRE1* (At4g24040), *WAKL10* (At1g79680), *WAKL2* (At1g16130), *WAKL6* (At1g16110), *WR3/NRT3.1* (At5g50200), *WRKY22* (At4g01250), *WRKY29* (At4g23550), *WRKY30* (At5g24110), *WRKY49* (At5g43290), and *WRKY53* (At4g23810). Microarray data from this article can be found in NCBI's GEO (Edgar et al., 2002) under GEO series accession numbers GSE40139 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40139>) and GSE40138 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE40138>).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Induction of DAMP Markers, *PROPEP2* and *PROPEP3*, in Response to Submergence.

Supplemental Figure 2. Expression of Innate Immunity Marker Genes, *PR* Genes, Submergence Early-Induced *WRKYs*, and Submergence Late-Induced *WRKYs* upon Abiotic and Biotic Stresses.

Supplemental Figure 3. Plant Immunity Is Triggered by Short Submergence.

Supplemental Figure 4. Isolation of *WRKY22* T-DNA Insertion Mutants.

Supplemental Figure 5. *WRKY22* Mediates Basal Immunity and Submergence-Triggered Immunity to *Pseudomonas syringae*.

Supplemental Figure 6. Transgenic c-myc-Tagged *WRKY22* Are Induced by Submergence in Transcript and Protein Levels.

Supplemental Figure 7. Effects of *WRKY22* Knockout on Transcript Levels of *WRKY22* Target Genes.

Supplemental Figure 8. Regulation of *LecRKs* and Stomatal Immunity-Associated Genes under Submergence.

Supplemental Table 1. List of Innate Immunity and Stress-Regulated Marker Genes.

Supplemental Table 2. Differential Expression of Selected Submergence-Responsive Genes in *WRKY22* Knockout Plants.

Supplemental Table 3. Potential Direct Targets of *WRKY22* by ChIP-Chip and ChIP-Q-PCR Experiments.

Supplemental Table 4. Primer Lists.

Supplemental Data Set 1. Submergence-Responsive Genes That Are Differentially Expressed in *WRKY22* Knockout Plants.

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AUTHOR CONTRIBUTIONS

F.-C.H. designed the research, performed research, analyzed data, and wrote the article. M.-Y.C. performed qRT-PCR. S.-J.C. performed the expression microarray and ChIP-chip microarray hybridization. Y.-R.L. contributed to the construction of transgenic plants. H.-P.P. performed ChIP. M.-C.S. designed the research and wrote the article.

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