AtOZF1 Positively Regulates Defense Against Bacterial Pathogens and NPR1-Independent Salicylic Acid Signaling

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Plant hormone salicylic acid (SA) plays critical roles in defense signaling against biotrophic pathogens. Pathogen inoculation leads to SA accumulation in plants. SA activates a transactivator protein NPR1, which, in turn, transcriptionally activates many defense response genes. Reports also suggest the presence of NPR1-independent pathways for SA signaling in *Arabidopsis*. Here, we report the characterization of a zinc-finger protein-coding gene *AtOZF1* that positively influences NPR1-independent SA signaling. Mutants of *AtOZF1* are compromised, whereas *AtOZF1*-overexpressing plants are hyperactive for defense against virulent and avirulent pathogens. *AtOZF1* expression is SA-inducible. *AtOZF1* function is not required for pathogenesis-associated biosynthesis and accumulation of SA. However, it is required for SA responsiveness. By generating *atozf1npr1* double mutant, we show that contributions of these two genes are additive in terms of defense. We identified *AtOZF1*-interacting proteins by a yeast-two-hybrid screening of an *Arabidopsis* cDNA library. VDAC2 and NHL3 are two *AtOZF1*-interacting proteins, which are positive regulators of basal defense. *AtOZF1* interacts with NHL3 and VDAC2 in plasma membrane and mitochondria, respectively. Our results demonstrate that *AtOZF1* coordinates multiple steps of plant-pathogen interaction.

Plants possess a highly complex and well-elaborated immune system to combat diverse groups of pathogens they encounter. Recognition of microbe- or pathogen-associated molecular pattern genes through plant pattern recognition receptors (PRR) triggers a basal level defense (pattern-triggered immunity [PTI]) in plants (Li et al. 2016; Macho and Zipfel 2014). Successful pathogens release effector molecules to subdue PTI (Boller and He 2009; da Cunha et al. 2006; Jones and Dangl 2006). Recognition of these effectors by host cells activates effector-triggered immunity (ETI) (Boller and He 2009; Godiard et al. 1994; Jones and Dangl 2006; Nimchuk et al. 2003). Defense responses induced by PTI include activation of mitogen-activated protein kinases and transcription factors, production of reactive oxygen species (ROS),

enhanced accumulation of plant hormones like salicylic acid (SA), ethylene, and jasmonic acid, and induced expression of pathogenesis-related (*PR*) genes (Bigeard et al. 2015; Cohn et al. 2001; Nürnberger and Scheel 2001; Tsuda and Katagiri 2010). Activation of ETI induces similar but much higher-level responses than PTI. In addition, ETI is often associated with rapid programmed cell death surrounding the infection site, known as hypersensitive response (HR) (Morel and Dangl 1997). HR restricts the spread of the pathogen and serves as a signal for defense in the distal tissues to activate systemic acquired resistance (SAR) (Alvarez et al. 1998; da Cunha et al. 2006). However, HR is not a prerequisite for SAR activation, as virulent pathogen inoculation also activates SAR (Liu et al. 2011).

Plant hormone SA plays the most significant role in defense against biotrophic pathogens such as bacteria and oomycetes. SA and its glycoside (SAG) accumulate at an elevated level in the inoculated tissue as well as in pathogen-free distal tissue. The mutants of *Arabidopsis* that are defective in one or both SA biosynthesis and accumulation show impaired resistance against biotrophic pathogens (Giri et al. 2014, 2017; Glazebrook et al. 1996; Nandi et al. 2004; Roetschi et al. 2001; Singh et al. 2013; Wildermuth et al. 2001; Zhou et al. 1998). NONEXPRESSOR OF PR 1 (NPR1) protein is the master regulator of SA signaling (Dong 2004; Yan and Dong 2014). The mutants of NPR1 are defective both in local resistance and SAR. NPR1, which predominantly localizes in the cytoplasm of the resting cells, moves to the nucleus upon pathogen infection or exogenous SA application (Mou et al. 2003). In the nucleus, NPR1 increases binding of the TGA class of transcription factors to induce expression of *PR* genes (Kesarwani et al. 2007; Saleh et al. 2015; Zhang et al. 1999). Though the NPR1 pathway remained as the primary SA signal transduction pathway in *Arabidopsis*, experiments suggest the existence of NPR1-independent pathways. Several *npr1* suppressor mutants such as *suppressor of npr1-1*, *constitutive 1* (*snc1*), *suppressor of SA insensitivity* (*ssi1*, *ssi2*), and *constitutive defense without defect in growth and development1* (*cddl*) constitutively activate SA accumulation and signaling in *npr1* mutant background (Li et al. 2001; Nandi et al. 2005; Shah et al. 1999, 2001; Swain et al. 2011). These mutants developmentally accumulate high levels of SA and are constitutive for SA signaling. A report indicated the role of TGA2 in NPR1-independent transcription of glutaredoxin GRXC9 in *Arabidopsis* (Herrera-Vásquez et al. 2015). However, a NPR1-independent pathway for SA signaling in plants mostly remained elusive.

We reported earlier that mutant of *At2g19810* show enhanced susceptibility to *Pseudomonas syringae* pv. *tomato* DC3000 (Swain et al. 2015). *At2g19810* codes for the *Arabidopsis thaliana* OXIDATION-RELATED ZINC FINGER 1 (*AtOZF1*)

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protein, which is required for oxidative stress tolerance (Huang et al. 2011). AtOZF1 is a member of the conserved tandem CCCH zinc finger (TZF) family that contains three CCCH-type zinc-finger domains (Bogamuwa and Jang 2014; Jang 2016; Wang et al. 2008). AtOZF1 expression increases upon H₂O₂ treatment (Dong 2004). Mutants of AtOZF1 show a severe reduction in primary root elongation and seedling growth, upon exogenous H₂O₂ application, compared with wild-type (WT) plants (Huang et al. 2011). In addition, AtOZF1 (also known as *Arabidopsis thaliana* ZINC-FINGER PROTEIN AtC3H20/AtTZF2) expression is increased by multiple abiotic stresses, such as abscisic acid (ABA), salt, mannitol, and cold (Lee et al. 2012). Overexpression of AtOZF1/AtTZF2 enhances resistance to exogenous H₂O₂ application and drought in *Arabidopsis* (Lee et al. 2012). We report here that AtOZF1 is a

positive regulator for defense against both virulent and avirulent pathogens. Our results also show that AtOZF1 takes part in NPR1-independent mechanisms of SA signaling.

RESULTS

AtOZF1 positively modulates defense against virulent and avirulent bacterial pathogens.

A mutant of AtOZF1 showed reduced resistance against *P. syringae* pv. *tomato* DC3000 (Swain et al. 2015). For further support, we used additional allelic T-DNA mutants and AtOZF1-overexpressing plants and tested against virulent and avirulent pathogens. We obtained two T-DNA insertion mutant alleles of AtOZF1 (Salk_151571, *atozf1-1*; CS810235, *atozf1-2*) and confirmed T-DNA insertions by polymerase chain reaction (PCR) in

Fig. 1. Influence of AtOZF1 in defense against virulent pathogens. **A**, *Pseudomonas syringae* pv. *maculicola* (*Psm*) counts in wild type (WT) and *atozf1-1* (*ozf1-1*) plants. **B**, *P. syringae* pv. *tomato* (*Pst*) counts and disease symptoms in WT, *atozf1-1*, and *npr1-2* plants at 3 days postinoculation (dpi). **C**, *P. syringae* pv. *maculicola* counts and disease symptoms in WT, *atozf1-2* (*ozf1-2*), and *npr1-2* plants at 3 dpi. **D**, *P. syringae* pv. *maculicola* counts and disease symptoms in WT, *atozf1-2*, and two different AtOZF1 overexpressor (OE) lines at 3 dpi. In A to D, each bar represents the mean $\pm$ standard deviation (SD) of four leaf samples, each carrying five leaf discs of 7 mm in diameter. Different letters above the bars indicate values that are significantly different ($P \leq 0.05$) from each other, as analyzed by one-way analysis of variance (Holm-Sidak method). **E**, Relative abundance of *PR1* transcript 48 h after *P. syringae* pv. *maculicola* inoculation. Each bar indicates mean $\pm$ SD ($n = 3$). **F**, Callose deposition after 10 h of flg22 treatment. Each bar represents mean $\pm$ SD ($n = 10$). Representative microscope pictures are shown on the right. **G**, Callose deposition after 9 h of mock or *P. syringae* pv. *maculicola* inoculation. Each bar represents mean $\pm$ SD of 10 samples. In all except F, overnight-grown bacterial suspension at 5×10^5 CFU/ml in 10 mM MgCl₂ was pressure infiltrated. In E, F, and G, one or two asterisks (*) indicate the values that are significantly different from the corresponding samples of WT plants as determined by *t* test ($P \leq 0.05$ and $P \leq 0.001$, respectively). Each experiment was repeated at least two times with similar results.

these plants (Supplementary Fig. S1A). We monitored growth of two virulent strains of bacteria, *P. syringae* pv. *tomato* and *P. syringae* pv. *maculicola* ES4326 on WT and *atozf1* mutant plants. In several experiments, we also included the *npr1-2* mutant as control. We observed that mutants of *AtOZF1* supported more bacterial load than WT plants (Fig. 1A to C). Susceptibility of *atozf1* mutants were almost similar to that of the *npr1-2* mutant (Fig. 1B and C). To further ascertain the influence of *AtOZF1* in defense, we generated *AtOZF1*-overexpressing plants. Plants overexpressing *AtOZF1* were significantly more resistant than WT plants against *P. syringae* pv. *maculicola* (Fig. 1D). In agreement with the defense against bacterial pathogens, *atozf1* expressed the *PR1* gene at a lower level, whereas *AtOZF1*-overexpressing lines were expressed at higher levels than WT plants (Fig. 1E). The *atozf1-1* mutants also expressed other SA-inducible genes, such as *PR2* and *PR5*, at a lower level than WT plants (Supplementary Fig. S2). Resistance against bacterial pathogens is associated with callose, a mixture of β -1,3-glucans, deposition in the infected tissue (Dong et al. 2008). In agreement with the positive regulatory role of *AtOZF1* in basal immunity, the *atozf1-1* plants showed reduced callose deposition than WT plants upon treatment with flg22, an elicitor of PTI response in *Arabidopsis* (Fig. 1F). The *atozf1* mutants also showed reduced callose deposition after *P. syringae* pv. *maculicola* inoculation, whereas it was enhanced in the *AtOZF1* overexpressing plants (Fig. 1G). The above results suggest that *AtOZF1* is essential for defense against virulent pathogens in *Arabidopsis*.

To get more insight about the role of *AtOZF1* in plant defense, we examined the defense response against avirulent

bacterial pathogens such as *P. syringae* pv. *tomato* carrying *AvrRpt2* (*Pst-AvrRpt2*) or *AvrB* (*Pst-AvrB*) genes. As observed for virulent pathogens, against avirulent pathogens, also, *atozf1* mutants showed higher susceptibility than WT plants. Bacterial titers in *atozf1-1* plants were much higher than WT plants and were similar to *npr1-2* plants (Fig. 2A and B). Moreover, plants overexpressing *AtOZF1* showed a significant reduction in bacterial load compared with WT and *atozf1-1* plants (Fig. 2C), suggesting *AtOZF1* is necessary to mount a resistance against avirulent pathogens. High-level infection with an avirulent pathogen results in an apoplastic oxidative burst, invoking production of higher amounts of ROS in the leaves. The ROS inhibit future growth of the pathogen by creating a highly oxidizing environment, which also activates death of surrounding plant cells, called the hypersensitive response (HR). To determine the role of *AtOZF1* in ROS accumulation, we performed diaminobenzidine (DAB) staining in WT and *atozf1-1* after *Pst-AvrRpt2* infection. The *atozf1-1* leaves showed significantly reduced DAB staining compared with the WT leaves (Fig. 2D), suggesting that *AtOZF1* is essential for normal oxidative burst upon avirulent pathogen infection. The cells undergoing HR release ionic contents to the apoplast. Such tissues, when floated in distilled water, release ions and thereby increase the electrical conductivity of water, which quantitatively reflects the level of cell death. The *atozf1-1* mutant shows a significantly lower level of ion leakage compared with WT plants upon *Pst-AvrRpt2* inoculation (Fig. 2E), suggesting a role of *AtOZF1* that is important for HR induction. Altogether, these results indicate that *AtOZF1* positively influences defense against virulent and avirulent bacterial pathogens.

Fig. 2. Influence of *AtOZF1* in defense against avirulent pathogens. **A**, *Pst-AvrRpt2* counts in wild type (WT), *atozf1-1*, and *npr1-2* plants. **B**, *Pst-AvrB* counts in WT, *atozf1-1*, and *npr1-2* plants. **C**, *Pst-AvrRpt2* counts in WT and *AtOZF1* overexpressor (OE) plants. Bacterial culture suspended at 1×10^6 CFU/ml in 10 mM MgCl₂ was pressure-infiltrated, and bacterial load was counted at 3 days postinoculation. Each bar represents the mean $\pm$ standard deviation (SD) of four leaf samples, each carrying five leaf discs of 7 mm in diameter. Different letters above the bars indicate values that are significantly different ($P \leq 0.05$) from each other, as analyzed by one-way analysis of variance (Holm-Sidak method). **D**, Diaminobenzidine staining for H₂O₂ detection after 3 h of floating in water containing *Pst-AvrRpt2* at 10^8 CFU/ml. **E**, Electrolyte leakage from *Pst-AvrRpt2*-inoculated leaves of WT and *atozf1-1*. Each point represents mean $\pm$ SD ($n = 3$). Two asterisks (**) indicate values from *atozf1-1* that are significantly different ($P \leq 0.001$) from WT, as obtained through *t* test for that specific timepoint. Each experiment was repeated at least two times with similar results.

AtOZF1 is not required for pathogen-induced SA accumulation.

Immune response against hemibiotrophic pathogens like bacteria is mediated by defense hormone SA (Delaney et al. 1994). Pathogen infection activates SA signaling by enhancing accumulation of endogenous SA and SAG. Since AtOZF1 positively modulates defense response and pathogen-induced *PR1*-transcript accumulation (Figs. 1 and 2), we speculated its influence in pathogen-induced SA accumulation. To investigate this possibility, we measured basal and pathogen-induced accumulation of SA in WT, *atozf1-1*, and *AtOZF1*-overexpressing plants. High-pressure liquid chromatography (HPLC) analysis for SA accumulation in *P. syringae* pv. *maculicola* or mock-inoculated leaves suggested that AtOZF1 had not much influence on pathogenesis-associated SA biosynthesis pathway or accumulation in *Arabidopsis* (Fig. 3A and B). Disruption of *AtOZF1* expression did not affect basal SA accumulation in mock-treated plants. There was only a small reduction in total SA level in *P. syringae* pv. *maculicola*-inoculated leaves of *atozf1-1* compared with the WT plants (Fig. 3A). We did not observe any increase of pathogen-induced total SA level in the *AtOZF1*-overexpressing plants; however, we observed a slight increase in free SA content in some of our experiments (Fig. 3B). From these results, we conclude that AtOZF1 function is not significantly required for pathogen-induced SA accumulation but may, however, positively influence free-SA levels when overexpressed.

Fig. 3. Salicylic acid (SA) content in wild type (WT), *atozf1-1*, and *AtOZF1* overexpressing plants. **A**, Total SA (SA+SAG) content in mock or *Pseudomonas syringae* pv. *maculicola* (Psm)-inoculated WT, *atozf1-1*, and *AtOZF1* overexpressor (OE) plants. **B**, Free SA content in mock or *P. syringae* pv. *maculicola*-inoculated WT, *atozf1-1*, and *AtOZF1*-OE plants. Plants were inoculated with either *P. syringae* pv. *maculicola* at 5×10^5 CFU/ml suspended in 10 mM MgCl₂ or only 10 mM MgCl₂ as mock control. Samples were harvested 2 days after inoculation. Each bar represents mean $\pm$ standard deviation of five samples. Different letters above the bars indicate values that are significantly different ($P \leq 0.05$) from each other, as analyzed by one-way analysis of variance (Holm-Sidak method). Experiments were repeated two times with similar results.

AtOZF1 is essential for SA sensitivity and positively regulates NPR1-independent defense responses.

AtOZF1 positively regulates defense and *PR1* expression without impacting SA accumulation. It is most likely possible that AtOZF1 functions as a positive regulator of SA signaling downstream of SA accumulation. To support such a hypothesis, we examined the effect of exogenous SA application on disease resistance and *PR1* transcript accumulation in WT, *atozf1-1*, and *npr1-2* plants. Five-week-old soil-grown plants were sprayed with water (0 μM SA) or SA (50, 100 or 150 μM) and, 24 h later, all plants were inoculated with *P. syringae* pv. *maculicola*. Bacterial counts at 3 days postinoculation (dpi) showed that SA application in WT plants significantly reduced bacterial load, whereas it had little effect on *npr1-2* mutant plants (Fig. 4A). Interestingly, compared with WT plants, the *atozf1-1* plants were less-responsive to exogenous SA when applied at a low concentration (50 μM). To compare, we calculated fold protection by SA treatment as a ratio of mean CFU in water-treated plants to that of SA-treated plants. The results showed that in the low concentration of SA (50 μM), fold protection in WT plants is much higher than *atozf1-1* plants (Fig. 4B). A similar reduced SA sensitivity in *atozf1-1* plants was also observed in terms of *PR1* expression. Exogenous SA application significantly enhanced *PR1* transcript accumulation in WT plants but not in *npr1-2* plants (Fig. 4C). The *atozf1-1* plants were not defective in *PR1* expression but were less responsive than WT plants toward exogenous SA treatment at higher concentrations (100 and 150 μM) (Fig. 4C). Together, these results suggest that *AtOZF1* is required for the normal level of SA sensitivity of *Arabidopsis*.

To see the interrelationship of AtOZF1 and NPR1 in defense signaling, we generated double mutant *atozf1-1 npr1-2*. We observed that the double mutant showed additive susceptibility of each of the single mutants toward both virulent pathogen *P. syringae* pv. *maculicola* (Fig. 4D) and avirulent pathogen *Pst-AvrRpt2* (Fig. 4E). This additive property was also observed in terms of pathogen-induced *PR1* expression. Pathogen-induced *PR1* expression was lower in *atozf1-1* and *npr1-2* than WT plants. Moreover, the *atozf1-1 npr1-2* double mutant expressed *PR1* at a lower level than each single mutant (Fig. 4F). Together these results demonstrated that AtOZF1 and NPR1 function additively in defense signaling, suggesting a NPR1-independent role for AtOZF1 in SA signaling.

AtOZF1 expression is SA-inducible.

The results described above demonstrated that AtOZF1 influences SA response and SA-mediated signaling. To investigate whether *AtOZF1* expression is SA-regulated, we monitored *AtOZF1* mRNA accumulation after spraying SA on WT plants. Northern blot hybridization showed enhanced accumulation of *AtOZF1* mRNA in plants treated with 500 μM of SA (Fig. 5A). Spraying plants with different concentrations of SA showed that *AtOZF1* mRNA accumulation was enhanced with increasing concentrations of SA (Fig. 5B). Results suggest that *AtOZF1* expression is transcriptionally controlled by SA.

AtOZF1 interacts with VDACC2 and NHL3.

To reveal the possible mechanism of AtOZF1-mediated plant defense, we looked for its interacting partners. Using AtOZF1 as bait in a yeast-two-hybrid assay, we screened an *Arabidopsis* cDNA library and obtained a total of 25 interacting clones. Sequencing of the plasmids identified 24 different cDNA-containing clones (Supplementary Table S1, At5g10360 appeared in two clones). Among the interacting proteins, NDR1/HIN1-LIKE 3 (NHL3, coded by *At5g06320*) was the only previously known modulator of plant defense (Varet et al. 2002, 2003). Besides

NHL3, we also selected VOLTAGE-DEPENDENT ANION CHANNEL 2 (VDAC2, coded by *At5g67500*) for further analysis. *Nicotiana benthamiana* VDAC2 (NbVDAC2) promotes nonhost resistance and pathogenesis-induced H₂O₂ production (Tateda et al. 2009). Expression of *Arabidopsis* VDAC2 increases upon virulent and avirulent pathogen inoculation (Tateda et al. 2011). Members of the *Arabidopsis* VDAC family, such as AtVDAC1, influence resistance against avirulent pathogen *Pst-AvrRpt2* (Tateda et al. 2011). To determine the part of AtOZF1 protein that is required for interaction with NHL3 and VDAC2, we performed yeast-two-hybrid interaction with a series of truncated AtOZF1 protein (Fig. 6A). We used AtOZF1, VDAC2, and NHL3, individually, along with corresponding empty vectors as negative controls (Supplementary Fig. S3). We found *d6*, having only the C-terminal 142 amino acids without any zinc-finger, was the smallest fragment that strongly interacts with VDAC2 (Fig. 6B). The other fragments, like *d5*, *d2*, and full-length AtOZF1, interact with VDAC2 but in a lower efficiency than the *d6* fragment. It is possible that the other domains function as autoinhibitory for the interaction. Alternatively, certain yeast proteins may interact and cause a steric hindrance in the larger AtOZF1 proteins. Unlike VDAC2, we did not find the interaction of *d6* with NHL3. The smallest fragment that interacted with NHL3 was *d5*, which contains the C-terminal 180 amino acids along with the third zinc-finger (Fig. 6B). However,

similar to VDAC2, the interaction weakened in the larger AtOZF1 fragments (Fig. 6B).

In planta interaction of AtOZF1 with VDAC2 and NHL3 was confirmed by a bimolecular fluorescence complementation (BiFC) assay. AtOZF1 and NHL3 coding sequences were transcriptionally fused with N- and C-terminal halves of yellow fluorescent protein (YFP) present in pSPYNE and pSPYCE, respectively (Waadt et al. 2008). Fusion proteins were transiently expressed in *N. benthamiana*. As a positive control, we used pGMAS-CIPK24 and pSPYNE-CBL1, which interact in the plasma membrane (Waadt et al. 2008) (Supplementary Fig. S4A). NHL3 was known to localize in the plasma membrane (Varet et al. 2003). We observed that NHL3 and AtOZF1 interact to generate a functional YFP and the fluorescence merges with FM64-red, a plasma membrane-binding dye (Fig. 6C). The results suggest the plasma membrane as the place of interaction for AtOZF1 and NHL3. *Arabidopsis* VDAC2-GFP (green fluorescent protein) localizes in the mitochondria and the cytoplasm (Tateda et al. 2011). Our BiFC analysis suggests that interaction of VDAC2 and AtOZF1 takes place predominantly in the mitochondria (Fig. 6C). Coexpression of only the N-terminal half of YFP-NHL3 or VDAC2 and empty vector pSPYCE failed to produce functional YFP, suggesting that interactions were required for YFP reconstitution. Further, to confirm the physical interaction of AtOZF1 with VDAC2 and

Fig. 4. Role of AtOZF1 in salicylic acid (SA) sensitivity and signaling. **A**, *Pseudomonas syringae* pv. *maculicola* (*Psm*) counts at 3 days postinoculation (dpi) in wild type (WT), *atozf1-1*, and *npr1-2* plants after spraying with 0, 50, 100, or 150 µM of SA. *P. syringae* pv. *maculicola* inoculation was done after 24 h of SA spray. **B**, Fold reduction in *P. syringae* pv. *maculicola* counts after SA spray, with respect to water-treated plants. The fold indicates the ratio of mean water-treated CFU counts to individual CFU counts for each sample. **C**, *PR1* transcript accumulation after 24 h of SA treatment in WT, *atozf1-1*, and *npr1-2* plants. Each point in the line plot indicates mean $\pm$ standard deviation (SD) ($n = 3$). One or two asterisks indicate the values in mutants that are significantly different from the corresponding samples of WT plants ($P \leq 0.05$ and $P \leq 0.001$, respectively). **D**, *P. syringae* pv. *maculicola* counts at 3 dpi in the indicated genotypic plants. **E**, *Pst-AvrRpt2* counts at 3 dpi in the indicated genotypic plants. **F**, *P. syringae* pv. *maculicola*-induced *PR1* transcript accumulation. Plants of the indicated genotypes were inoculated with *P. syringae* pv. *maculicola* and samples were harvested at 24 and 48 h postinoculation. Northern blot hybridization was done using *PR1* cDNA as probe. Ethidium bromide (EtBr)-stained gel picture indicates relative amount of RNA loaded in the gel. In all experiments, pathogens were inoculated at 5×10^5 CFU/ml. In A, D, and E, each bar or point in the line plot indicates mean $\pm$ SD of four samples carrying five leaf-discs of 7 mm in diameter. Different letters above the bars indicate values that are significantly different ($P \leq 0.05$) from each other, as analyzed by one-way analysis of variance (Holm-Sidak method). Experiments were repeated at least two times with similar results.

NHL3, we performed coimmunoprecipitation (CoIP) followed by Western blot analysis. Transiently expressed AtOZF1-Myc protein in *N. benthamiana* coimmunoprecipitated with VDAC2-HA (hemagglutinin) or NHL3-HA and vice versa (Fig. 6D; Supplementary Fig. S5A). Expression of AtOZF1 or its interacting factor alone served as required negative control. As a positive control of the experiment, we used CIPK24 and CBL1.

VDAC2 and NHL3 are positive regulators of basal defense.

Expression of *NHL3* is induced by virulent and avirulent strains of *P. syringae* pv. *tomato* (Varet et al. 2002). Overexpression of *NHL3* confers resistance against *P. syringae* pv. *tomato* DC3000 (Varet et al. 2003). The reports suggested NHL3 as a positive regulator of basal defense. To validate further, we used a *T-DNA* insertion mutant line of *NHL3* (salk_150318) (Supplementary Fig. S6A and B) and challenged it with the virulent pathogen *P. syringae* pv. *maculicola*. As anticipated, we observed higher bacterial growth in the *nhl3* mutant compared with WT plants (Fig. 7A). To determine the role of VDAC2 in defense, we studied disease resistance in a *T-DNA* insertion mutant line of *VDAC2* (CS818558). Similar to *nhl3*, the *vdac2* mutant also supported a higher bacterial proliferation than WT plants (Fig. 7A). However, the influence of AtOZF1 in defense was higher than both VDAC2 and NHL3. The mutants, along with WT and *atozf1-1*, were also challenged with the avirulent pathogen *Pst-AvrRpt2*. We did not find much difference regarding resistance against avirulent pathogen (Fig. 7B). The results suggest that VDAC2 and NHL3 are positive regulators of PTI-mediated basal defense.

Fig. 5. *AtOZF1* mRNA accumulation after salicylic acid (SA) treatment. **A**, SA-induced expression of *AtOZF1* mRNA in wild-type (WT) plants, as determined by Northern blot hybridization. Plants were sprayed with 500 μM SA and samples were harvested after 0, 12, and 18 h of treatment. **B**, *AtOZF1* expression after spraying SA at different concentrations. WT plants were sprayed with SA solution and samples were harvested 18 h after spraying. *AtOZF1* transcript accumulation was determined by quantitative polymerase chain reaction. Each bar represents mean $\pm$ standard deviation ($n = 3$). One or two asterisks (* or **) indicate values of $P \leq 0.05$ or $P \leq 0.001$, respectively, in the treatment compared with control (0 h sample). Experiments were repeated two times with similar results.

AtOZF1 is required for pathogen-induced VDAC2 mRNA accumulation.

To study the pathogenesis-associated expression of *VDAC2* and *NHL3*, we monitored their mRNA accumulation by quantitative (q)PCR after *P. syringae* pv. *maculicola* inoculation in WT plants. We also included *atozf1-1* mutant in the study, to examine whether AtOZF1 influences their expression. As expected, we observed enhanced expression of both *VDAC2* and *NHL3* in WT plants after *P. syringae* pv. *maculicola* inoculation (Fig. 7C and D). We did not observe much difference between WT and *atozf1-1* in terms of *NHL3* mRNA accumulation, suggesting that AtOZF1 function is not required for pathogen-induced expression of *NHL3* (Fig. 7D). In contrast, accumulation of *VDAC2* mRNA was significantly lower in *atozf1-1* than in WT plants (Fig. 7C). Results suggest that AtOZF1 is essential for *VDAC2* expression under pathogen stress and the inability to induce *VDAC2* may partly be responsible for enhanced bacterial growth in *AtOZF1* mutant plants.

DISCUSSION

This work and the previously published literature clearly demonstrated the significance of AtOZF1 as a positive regulator of defense responses against multiple abiotic and biotic stresses. *AtOZF1* overexpression provides enhanced resistance to abiotic stresses and exogenous H_2O_2 application through yet unidentified mechanisms (Huang et al. 2011; Lee et al. 2012). Our results show that, on defense against pathogens, AtOZF1 coordinates multiple steps, including ROS accumulation, ETI-induced ion leakage, and SA signaling (Fig. 8A). Exogenous SA application induces *AtOZF1* expression. Thus, AtOZF1 may be considered as an integral component of disease defense mechanism of *Arabidopsis*.

Role of AtOZF1 in plant defense.

AtOZF1 positively regulates defense against bacterial pathogens. Mutants of *AtOZF1* are susceptible to both compatible and incompatible pathogens and express reduced level of *PR1* transcript compared with WT plants. In contrast, plants overexpressing *AtOZF1* are hyper-resistant compared with WT plants. Interestingly, the *AtOZF1* overexpression does not make plants constitutive for defense. Most of the mutants or overexpression plants that constitutively activate defense are compromised for growth, developmentally accumulate SA, and develop spontaneous necrosis (Bolton 2009; Heidel et al. 2004; Huot et al. 2014; Jirage et al. 2001). In contrast, *AtOZF1* overexpression plants do not accumulate a high level of SA without pathogen inoculation and do not express *PR* genes constitutively. Morphology of overexpression plants is also comparable to that of the WT plants. Thus, AtOZF1 may function as an enhancer of inducible plant defense in *Arabidopsis*.

AtOZF1 influences several aspects of plant defense. It promotes ROS accumulation, HR-mediated ion-leakage, SA signaling and bacterial growth. These events of defense response are interlinked but not necessarily follow synchronized regulation (Torres et al. 2005; Galletti et al. 2008; Yun et al. 2011; Chaouch et al. 2012). *Arabidopsis* respiratory burst oxidase homologs (RBO; NADPH oxidase), AtRbohD and AtRbohF contribute to ROS accumulation induced by both virulent and avirulent pathogens, but influence defense only against virulent pathogens (Torres et al. 2002; Chaouch et al. 2012). Since, AtOZF1 influences ROS accumulation, ion-leakage, defense against both virulent and avirulent pathogens, but does not influence SA accumulation, we presume two distinct nodes at which AtOZF1 interacts with defense signaling. In the first node, it influences ROS accumulation, ion-leakage and general defense against virulent pathogens (Fig. 8A). Functions at this

node are similar to the earlier reported roles of AtOZF1, which include oxidative stress and ABA signaling (Huang et al. 2011; Lee et al. 2012). The role of AtOZF1 in ABA signaling may also be associated with its function in oxidative stress. Double mutant *atrbohD/F* is impaired in ABA-induced ROS production, stomatal closure and seed germination inhibition (Kwak et al. 2003). At the second node, AtOZF1 influences SA signaling downstream to SA accumulation (Fig. 8B). AtOZF1 function at this node contributes to resistance against both virulent and avirulent pathogens and expression of *PR* genes.

Contribution of AtOZF1 in SA signaling.

NPR1 is the most important regulator of SA signaling. Though the existence of NPR1-independent pathway is established, the machinery remained elusive. Many *Arabidopsis* mutants, for example, *snc1*, *ssi1* and *ssi2*, and *cdd1*, constitutively activate SA accumulation and signaling in the *npr1* mutant

background (Li et al. 2001; Nandi et al. 2005; Shah et al. 1999, 2001; Swain et al. 2011). These genes are negative regulators of NPR1-independent signaling. Our results identified AtOZF1 as a positive regulator of defense that functions in a pathway that is parallel to NPR1. Effects of individual mutations in NPR1 or AtOZF1, in terms of restricting bacterial growth, are similar. The double mutants show an additive effect on bacterial growth and suppression of pathogen-induced *PR* gene expression, which suggest a function parallel to NPR1 (Fig. 8B). However, exogenous SA application prior to pathogen inoculation, suggest the involvement of AtOZF1 in both NPR1-dependent and -independent pathways. At lower concentration of SA, *AtOZF1* mutant fails to provide WT-level protection in the presence of the NPR1 gene. Thus, AtOZF1 has a NPR1-independent effect in restricting the growth of pathogens in response to exogenous SA application. Similarly, SA-induced expression of *PR1* is also influenced by AtOZF1, even in the presence of NPR1.

Fig. 6. Interaction of AtOZF1 with VDAC2 and NHL3. **A**, Schematic representation of full-length AtOZF1 protein and its deleted derivatives. Boxes indicate zinc-finger domains. Numbers indicate amino-acid positions from the N-terminal end. **B**, Growth of yeast cells in Y2H selection medium. +Ve control cells contain pGBKT7-P53 and pGADT7-T antigen. VDAC2 or NHL3 was cloned in pGADT7. AtOZF1 and its deleted fragments were cloned in pGBKT7. Overnight-grown yeast cells were suspended at 10⁸ cells/ml and serially diluted before spotting in the selection medium. All photographs were taken at the same magnification after two days of growth at 28°C. **C**, BiFC assay to detect interaction of AtOZF1 with VDAC2 or NHL3 in onion cells. pSPYNE and pSPYCE vectors provide N and C terminals of YFP respectively. YFP and FM64-red or mitotracker images were taken from the same observation field at the same magnification using a confocal microscope. **D**, Coimmunoprecipitation of AtOZF1-Myc and NHL3-HA or VDAC2-HA after transient expression in *Nicotiana benthamiana* leaves.

However, since exogenous SA-induced *PR1* expression is dependent on NPR1 (Fig. 4C), we believe that AtOZF1 functions as an enhancer of NPR1 in that pathway (Fig. 8C).

Mechanism of AtOZF1-mediated defense.

The biochemical function of AtOZF1 is not elucidated in detail. The protein contains three CCCH-type zinc fingers. Several tandem-arrayed CCCH-type zinc-finger proteins are implicated in mRNA turnover (Lee et al. 2012; Kim et al. 2014; Qu et al. 2014). In an *in vitro* RNase activity assay, AtOZF1 has been shown to degrade several *Arabidopsis* RNA molecules (Lee et al. 2012). However, it is not known whether it is a general RNase activity or specific against certain RNA species. Besides RNase activity, tandem-CCCH zinc-finger domains also take part in protein-protein interaction (Guo et al. 2009). Cotton (*Gossypium hirsutum*) zinc-finger protein 1 (GhZFP1) contains two tandem-CCCH zinc-fingers. Screening of a cotton cDNA library with GhZFP1 identified two proteins, GZIRD21A (GhZFP1 interacting and responsive to dehydration protein 21A) and GZIPR5 (GhZFP1 interacting and pathogenesis-related protein 5), which interact with GhZFP1. Yeast-two-hybrid analysis with deleted domains of GhZFP1 suggests the part of the protein carrying zinc-finger domains assists in the interaction (Guo et al. 2009). We identified two AtOZF1 interactors, VDAC2 and NHL3, that positively influence defense against bacterial pathogens. VDACs are mitochondrial outer membrane-localized proteins that play roles in plant defense, cell death, and H₂O₂ production (Kusano et al. 2009). Our results show that VDAC2 function is required for resistance to virulent pathogens. VDAC2 and NHL3 are localized in the mitochondria and the plasma membrane, respectively, and interact with AtOZF1 in their respective subcellular organelles. Since

AtOZF1 protein does not contain any specific subcellular localization signal, we believe that the interacting proteins influence its localization. Thus, it may be possible that VDAC2 and NHL3 by interacting with AtOZF1 in its C-terminal domain help in moving to mitochondria and plasma membrane, respectively (Fig. 8A). Our results show that AtOZF1 function is also required for pathogen-induced transcription of *VDAC2*. Earlier, another tandem CCCH zinc-finger protein, AtC3H4, is reported to bind single- and double-stranded DNA and RNA and to function as a transcriptional activator (Kim et al. 2014). Thus, we cannot rule out the possibility of AtOZF1 functioning as a transcriptional activator for plant defense.

In summary, our report illustrated the functional significance of the zinc-finger protein AtOZF1 in plant immune responses. The results revealed two crucial roles of AtOZF1. First, AtOZF1 functions downstream of SA accumulation and positively modulates NPR1-independent SA signaling. Second, it interacts with VDAC2 and NHL3, which were shown to promote basal defense against bacterial pathogens. Further investigations will uncover how these interactions help plants to fight against pathogens.

MATERIALS AND METHODS

Plant materials and growth conditions.

All *Arabidopsis* plants were of Col-0 ecotype. The *T-DNA* insertion mutants *atozf1-1* (Salk_151571), *atozf1-2* (CS810235), *vdac2* (CS818558), and *nhl3* (Salk_150318) were obtained from the Arabidopsis Biological Resource Center, Ohio State University, Columbus, OH, U.S.A. Allelic compositions were determined by standard PCR-based techniques. Primer sequences are mentioned in Supplementary Table S2. The double mutant *atozf1-1npr1-2* was generated by crossing homozygous parental lines. The *atozf1-1*

Fig. 7. Role of VDAC2 and NHL3 in defense against bacterial pathogens. **A**, *Pseudomonas syringae* pv. *maculicola* counts at 3 days postinoculation (dpi). **B**, *Pst-AvrRpt2* counts at 3 dpi. **C**, VDAC2 mRNA accumulation during pathogenesis. **D**, NHL3 mRNA accumulation during pathogenesis. Pathogens were inoculated at 5×10^5 CFU/ml and bacterial loads were determined at 3 dpi. Each bar represents the mean $\pm$ standard deviation (SD) of four leaf samples, each carrying five leaf discs of 7 mm in diameter. Different letters above the bars indicate values that are significantly different ($P \leq 0.05$) from each other, as analyzed by one-way analysis of variance (Holm-Sidak method). In C and D, samples were harvested at the indicated period postinoculation with *P. syringae* pv. *maculicola*. Transcript accumulation was determined by qPCR. Each point in line plot indicates mean $\pm$ SD ($n = 3$). Asterisks (*) indicate the values that are significantly different ($P \leq 0.05$) from wild-type (WT) plants for that specific timepoint. Experiments were repeated two times with similar results.

mutation was identified by PCR amplification. For *npr1-2* identification, we developed a pair of primers for which the PCR amplicon from WT plant gets digested at two positions by *BsmI* enzyme but at only one in the *npr1-2* plant. AtOZF1 overexpressing plants were generated by an *Agrobacterium tumefaciens* (C58 strain)-mediated floral-dip transformation method. Coding sequences of AtOZF1 was cloned into the binary vector pCXS-NHA (Chen et al. 2009) as N-terminal fusion with the HA tag. Transgenic plants were confirmed by reverse transcription (RT)-PCR and Western blot. *Arabidopsis* plants were grown in an environmentally controlled growth chamber at 22°C and 60% relative humidity, with a 12-h light and 12-h dark cycle (Singh et al. 2013). *N. benthamiana* plants were grown on garden soil at 25°C temperature in the glass house.

Pathogen inoculation, SA, and flg22 treatment.

Pathogen inoculation and determination of bacterial growth were carried out as described previously (Nandi et al. 2003a; Singh et al. 2014). Overnight-grown bacteria were resuspended in 10 mM MgCl₂ and the suspension was infiltrated into the abaxial sides of leaves with a needleless syringe. Only 10 mM MgCl₂ was used as control, whenever required. For SA treatment, 4-week-old plants were sprayed with a solution of SA (Sigma-Aldrich, St. Louis) made in distilled water or only water as the control, and plants were covered overnight with a plastic dome. For flg22 treatment, leaves were infiltrated with either 1 µM solution flg22 (Genscript) made in water or only water as control, and plants were covered with a plastic dome for 10 h before collecting samples for callose deposition study.

Ion-leakage experiment.

Ion-leakage experiments were performed as described earlier (Bhattacharjee et al. 2017; Roy and Nandi 2017). A suspension of *Avr-Pst* (at 1×10^7 CFU/ml in 10 mM MgCl₂) was pressure infiltrated in the abaxial side of fully grown leaves. The control set received only 10 mM MgCl₂. Leaf discs of 5 mm in diameter were taken out immediately after inoculation, were briefly washed with sterile water, and were floated on distilled water in a 6-well plate. We used seven leaf discs per 5 ml of water. The electrical conductivity (microsiemens per centimeter) measured immediately after floating leaf discs was taken as the 0-h reading. The floated leaves were incubated in the dark inside the growth chamber for 6 h and were then transferred to light, after which electrical conductivity readings were taken at 2-h intervals. After completion of the experiment, the liquid, along with the leaf discs, was transferred to 50-ml falcon tubes and was autoclaved, to liberate the entire cellular contents. The electrical conductivity after autoclaving was taken as 100% ion leakage, and the readings at other timepoints were expressed as the percentage of total ion leakage.

SA estimation.

Total SA and SAG levels were determined by HPLC (Agilent 1220 LC), as described previously (Singh et al. 2013), with minor modifications. In brief, total SA and SAG was extracted from 200 mg of leaf samples and was dissolved separately in 200 µl HPLC running buffer (0.2 M NaOAc, pH 5.2, and 10% methanol). Using an autosampler, 100 µl was loaded into the column (5 µm C-18 reverse-phase 4.6 mm × 150 mm). HPLC was run at 30°C with a flow rate of 0.8 ml/min.

RNA extraction and gene expression analyses.

Isolation of total RNA and cDNA synthesis was done as described earlier (Giri et al. 2017). For Northern blot, total RNA (approximately 6 µg) was separated on agarose gel, was transferred to nylon membrane, and was hybridized with radio-

labeled probes for Northern blot analysis (Nandi et al. 2003b, 2004). For qPCR analysis, cDNA was prepared from 1 µg of total RNA, using a kit (Bio-Rad) per the manufacturer's protocol. RT-qPCR was done, using 50 ng of cDNA and power SYBR green master mix (Applied Biosystem) in an ABI-PRISM 7500 FAST sequence detector, and was analyzed by the system-given software. Typically, each set contained three biological replicates and qPCR was carried out by taking two technical replicates of each cDNA samples. Average of two technical replicates was taken as the value of that biological replicate. Normalization with *ACTIN2* (*At3g18780*) and *TUB2* (*At5g62690*) and primer efficiency calculation was done as described earlier (Giri et al. 2017). Statistical analysis was carried out using log₁₀-transformed values of expression level.

DAB staining and callose deposition.

DAB staining for ROS detection was carried out as described previously (Roy and Nandi 2017). In brief, leaves were detached along with the petiole and were floated in distilled water overnight. The next day, water was replaced by *Pst-AvrRpt2* (a concentration of 10⁸ cells per milliliter suspended in 10 mM MgCl₂) or 10 mM MgCl₂. After 3 h, the liquid was replaced with DAB solution (1 mg/ml in 10 mM Na₂HPO₄) and vacuum (–5 mm Hg pressure) was applied for 5 min. The color was developed by slow shaking for 4 to 5 h at room temperature and was washed with destaining solution (ethanol/acetic acid/glycerol, 3:1:1). For callose deposition studies, flg22 or pathogen-treated leaves were detached and were incubated with 95% ethanol overnight for removing chlorophyll. The leaves were washed three times with water and were stained with 0.01% aniline blue (in 1.5 mM Na₂HPO₄ buffer, pH 9.0) for 10 h, before observing

Fig. 8. Models depicting the role of AtOZF1 in plant defense. **A**, Role of AtOZF1 in pathogen-associated molecular pattern- and effector-triggered immunity (PTI and ETI, respectively). AtOZF1 interacts with VDAC2 and NHL3. Both proteins influence PTI. AtOZF1 also may interact with some other factor (?) for influencing ETI. **B**, AtOZF1 and NPR1 function in parallel pathways for mounting defense against pathogens and for pathogen-induced *PR1* expression. **C**, Exogenous salicylic acid (SA) treatment shows both NPR1-dependent and -independent mechanisms of defense and *PR1* expression.

under a microscope. The amount of callose per observation field (0.04 mm²) was calculated by the imageJ software.

Screening of *Arabidopsis* cDNA library through a yeast-two-hybrid assay.

The full-length coding sequence of AtOZF1 was cloned in bait expression vector pGBKT7 (Clontech). An *Arabidopsis* normalized cDNA library prepared in pGADT7 vector (Clontech) was used for screening AtOZF1-interacting factors, following manufacturer's protocol and as described earlier (Banday and Nandi 2017). The yeast clones or transformants were selected on synthetic defined medium lacking Trp, Ura, His, and Ade. Plasmids were isolated from yeast cells and were transformed into *Escherichia coli* DH5 α . For each interacting clone, the bacterial plasmid was isolated and a yeast-two-hybrid assay was repeated. During this step, pGADT7-T and pGBKT7-P53 expressing plasmids DNAs were used as positive control and individual vectors were used as negative control. The yeast transformants were serially diluted before plating in quadruple dropout medium. We selected only the clones that showed interaction strength equal to or more than the positive control. The plasmid DNA was sequenced to get the gene identity.

BiFC and transient expression studies.

BiFC assay was performed after cloning AtOZF1 and its interacting partners in pSPYNE(R)173 and pSPYCE(M) vectors for generating N- and C-terminal parts of YFP fused proteins respectively (Waadt et al. 2008). Overnight-grown *A. tumefaciens* (strain C58) culture having BiFC constructs were expressed in onion cells as described previously (Waadt et al. 2008). For transient expression in *N. benthamiana*, fully grown leaves of 5 to 6 week-old were coinfiltrated with *A. tumefaciens* carrying the BiFC construct and HcPro for RNAi suppression constructs (del Toro et al. 2014) both at 0.5 OD₆₀₀ suspended in MES (10 mM, pH 5.7), acetosyringone (100 μ M) and MS (0.4%, Sigma Cat # M5519).

CoIP.

CoIP was done as described by Chinchilla et al. (2007), with some modifications. About 1 g of leaf tissue was frozen in liquid N₂ and total protein was extracted by mortar pestle in 2.5 ml of extraction buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 10% [wt/vol] glycerol, 1% [vol/vol] NonidetP-40, 0.5% [wt/vol] sodium deoxycholate, and protease inhibitor [Sigma]) The extracted protein was incubated overnight at 4°C, with protein A-agarose beads (Invitrogen) and anti-HA (Abcam) or anti-Myc (AbCam) antibodies. The beads were collected and were washed three times with ice-cold extraction buffer and once with 50 mM Tris-HCl, pH 7.5. Finally, elution was done by heating for 10 min at 50°C with and without 100 mM dithiothreitol. Proteins bounded with beads were loaded on sodium dodecyl sulfate-polyacrylamide gels (12% [wt/vol]) and were analyzed by immunoblot using anti-Myc or anti-HA antibodies.

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