Suppression of OsVPE3 Enhances Salt Tolerance by Attenuating Vacuole Rupture during Programmed Cell Death and Affects Stomata Development in Rice

Wenyun Lu†, Minjuan Deng†, Fu Guo, Mingqiang Wang, Zhanghui Zeng, Ning Han, Yinong Yang, Muyuan Zhu and Hongwu Bian*

Abstract

Background: Vacuolar processing enzymes (VPEs) are cysteine proteinases that act as crucial mediators of programmed cell death (PCD) in plants. In rice, however, the role of VPEs in abiotic stress-induced PCD remains largely unknown. In this study, we generated OsVPE3 overexpression and suppression transgenic lines to elucidate the function of this gene in rice.

Results: Survival rate and chlorophyll retention analyses showed that suppression of OsVPE3 clearly enhanced salt stress tolerance in transgenic rice compared with wild type. Furthermore, fragmentation of genomic DNA was inhibited in plants with down-regulated OsVPE3. Vital staining studies indicated that vacuole rupture occurred prior to plasma membrane collapse during salt-induced PCD. Notably, overexpression of OsVPE3 promoted vacuole rupture, whereas suppression of OsVPE3 attenuated or delayed the disintegration of vacuolar membranes. Moreover, we found that suppression of OsVPE3 caused decreased leaf width and guard cell length in rice.

Conclusions: Taken together, these results indicated that suppression of OsVPE3 enhances salt tolerance by attenuating vacuole rupture during PCD. Therefore, we concluded that OsVPE3 plays a crucial role in vacuole-mediated PCD and in stomatal development in rice.

Keywords: OsVPE3, Programmed cell death, Rice, Salt stress, Stomata, Vacuolar processing enzyme

Background

Rice (Oryza sativa L.) is one of the most important cereal crops for more than half of the world’s population. According to estimates, the world will need to produce 25% more rice by 2030 to meet the challenges of feeding increasing populations. However, the increasing soil salinization of limited farmland is becoming a serious global threat to sustained rice production (Khatun and Flowers 1995; Sahi et al. 2006; Gao et al. 2007). Therefore, enhancing salt-tolerance is a serious concern for crop breeding programs.

Programmed cell death (PCD) is a highly conserved and genetically controlled process in multicellular organisms. PCD is involved in maintaining cellular homeostasis, development and senescence(Azeez et al. 2007; Williams and Dickman 2008), and this process is triggered by a variety of abiotic and biotic stresses (Huh et al. 2002; Lam 2004; Gechev et al. 2006). Plant and animal cells share many hallmarks of PCD, including cytoplasm shrinkage, chromatin condensation, DNA cleavage, mitochondrial swelling, organelle disruption and plasma membrane collapse (Mittler et al. 1997; van Doorn 2011; De Pinto et al. 2012). However, plants also exhibit unique features of PCD due to the presence of chloroplasts and vacuoles (Samuilov et al. 2003; Hatsugai et al. 2006; Kim et al. 2012; Wituszynska et al. 2015). Vacuoles are storage organelles that function as reservoirs for both hydrolitic enzymes...
and defence proteins, and vacuoles play several roles in stress response, development and pathogen defence. Recent studies have suggested that vacuole-mediated cell death is a response to various stresses in plants (Hatsugai et al. 2006; Hatsugai et al. 2015). Under salt stress, early events include the production of reactive oxygen species (ROS) and increased cytoplasmic calcium concentrations (Dionisio-Sese and Tobita 1998; Menezes-Benavente et al. 2004; Kudla et al. 2010) followed by PCD. In addition, vacuole rupture is a trigger for nuclear degradation during PCD (Obara et al. 2001). However, the genes and regulatory networks involved in vacuole-mediated cell death remain unidentified.

Vacuolar processing enzymes (VPEs) are cysteine proteinases involved in the processing of vacuolar proteins and the maturation of seed storage proteins in plants (Haranishimura et al. 1991; Hiraiwa et al. 1993; Rojo et al. 2003; Wang et al. 2009). VPEs are expressed in senescent tissues, and their expression patterns have been linked to PCD (Hara-Nishimura et al. 1998; Kinoshita et al. 1999). For example, it has been shown that VPEs regulate systematic cell death induced by viral, aluminium and heat stress, which is mediated by caspase-1-like activity during PCD (Hatsugai et al. 2004b; Li et al. 2012; Kariya et al. 2013). VPEs and caspase-1 share conserved structural properties, particularly the Asp pocket of caspase-1, which includes three crucial amino acids (Arg-179, Arg-341 and Ser-347) (Hatsugai et al. 2006). Although VPEs and caspase-1 share many similarities, the subcellular localizations of these two proteases are completely different. Namely, VPEs localize to vacuoles, whereas animal caspases localize to the cytosol. As initiators of plant PCD, VPEs trigger cell death through vacuolar collapse (Hatsugai et al. 2004a). To date, several types of VPEs have been reported to be involved in PCD as follows: NbVPE1a and NbVPE1b in Nicotiana; and AtyVPE in Arabidopsis. In rice, however, the role of VPEs in the vacuole-mediated cell death remains unknown.

The rice genome contains four VPE homologous genes as follows: OsVPE1 (Os04g45470), OsVPE2 (Os01g37910), OsVPE3 (Os02g43010) and OsVPE4 (Os05g51570) (Deng et al. 2011). Phylogenetically, OsVPE1 and OsVPE3 are more similar to Arabidopsis AtβVPE, whereas OsVPE2 and OsVPE4 are more similar to AtyVPE and AtyVPE. OsVPE1, a homolog of AtβVPE, plays a crucial role in the maturation of glutelins in seeds (Wang et al. 2009). Previous work by our group has shown that the transcription of OsVPE2 and OsVPE3, but not of OsVPE1 and OsVPE4, can significantly enhance salt-induced PCD (Deng et al. 2011; Kim et al. 2014). In this study, we aimed to elucidate the role of OsVPE3 in the context of salt stress. To determine the function of OsVPE3 in rice under salt stress, we generated transgenic lines to either overexpress and suppress OsVPE3, and we found that OsVPE3 regulates the collapse of vacuolar membranes during PCD. In addition, we found that OsVPE3 plays a role in leaf and stomata development in rice.

Results
Expression pattern and localization of OsVPE3
A comparison between genomic DNA and cDNA revealed that OsVPE3 (Os02g43010) contains nine exons and eight introns (Fig. 1a), and OsVPE3 encodes a peptide of 496 amino acids. The red letters in Fig. 1 indicate essential amino acids in the catalytic site and substrate pocket that mediate caspase-like activity (Fig. 1b) (Nicholson 1999; Hara-Nishimura et al. 2005; Hatsugai et al. 2015).

To determine the subcellular localization of OsVPE3, the full-length cDNA was fused to green fluorescent protein (GFP) under control of the 35S promoter. An OsVPE1-GFP fusion gene driven by the 35S promoter was used as a control. OsVPE1, a seed-type vacuolar processing enzyme, has been shown to localize to vacuoles in onion cells (Wang et al. 2009). These constructs were transiently transformed into rice protoplasts. We searched for GFP signals in vacuoles for the 35S:OsVPE1-GFP and 35S:OsVPE3-GFP fusion constructs, and no GFP signal was observed in vacuoles for the 35S: GFP construct (Fig. 1c). It was reported that GFP was unstable in the acidic vacuoles of plants under the light condition, because light would cause the rapid degradation of 27-kDa GFP by pre-existing proteinase in vacuoles at pH5.5 (Tamura et al., 2003). In protoplast transient assay, isolated protoplasts were kept in the dark condition to protect from light damage, thus the GFP fluorescence can be observed in the vacuoles. Consequently, this result suggested that OsVPE3 is a vacuole-targeting protein similar to OsVPE1.

To investigate the expression pattern of OsVPE3 in rice, transcription levels were examined in various tissues by quantitative RT-PCR. Expression analysis revealed that OsVPE3 is expressed in tissues of the leaf, root, booting panicle and immature embryo (Fig. 1d). OsVPE3 expression increased rapidly in the leaves of 10-day-old seedlings compared with 5-day-old seedlings. The highest transcriptional expression level of OsVPE3 was detected in immature embryos. These results demonstrated that OsVPE3 is actively transcribed in the leaves and developing embryos of rice.

Generation of Transgenic Rice Lines
In our previous work, we found that the expression of OsVPE3 is dramatically induced by salt stress (Deng et al. 2011; Kim et al. 2014). However, the role of the OsVPE3 gene in rice remains largely unknown. To investigate if OsVPE3 is involved in the process of PCD, OsVPE3 was overexpressed and suppressed using transgenic lines (Fig. 2a, b). Homozygous T3 transgenic seeds
were used for further investigation. Quantitative RT-PCR analysis confirmed that the expression levels of OsVPE3 were strongly increased in the overexpression lines (OE-1 and OE-2) and decreased significantly in the RNA interference transgenic lines (RNAi-1) compared with WT (Fig. 2c).

Previous studies showed that OsVPE1 plays a crucial role in the maturation of glutelins in seeds (Wang et al. 2009). To examine the role of OsVPE3 on seed proteins, we performed SDS-PAGE analysis to compare the proteins in WT and transgenic lines. Our data showed that the major storage proteins yielded three bands of approximately 20, 40 and 57 kDa in rice (Fig. 2d). The 57-kDa protein is a glutelin precursor (Yamagata et al. 1982; Krishnan and Okita 1986). Compared with WT, 57 kDa protein levels was increased to 1.33 fold in the OsVPE3-RNAi line, whereas reduced to approximately 0.80-0.84 in the overexpression lines. Accordingly, the grain width and 1000-grain weight of the RNAi line decreased significantly compared with WT and overexpression lines (Additional file 1: Figure S1). These results indicated that OsVPE3 is involved in the processing of seed storage proteins in rice.

**Suppression of OsVPE3 Enhances Salt Stress Tolerance in Rice**

Based on our previous studies, we hypothesized that OsVPE3 might act as a trigger in salt-induced PCD. To determine if OsVPE3 is involved in salt stress tolerance in rice, 4-week-old WT and transgenic plants were treated with 150 mM NaCl for 3 days. Before NaCl treatment, the expression levels of OsVPE3 were measured in the fourth leaf of each line, confirming that the transcript levels of OsVPE3 were increased in the overexpression transgenic lines (OE-1 and OE-2) and significantly suppressed in the RNAi transgenic lines (Fig. 3a, b). Under salt stress, chlorosis is a common symptom in rice leaves. Chlorophyll contents were measured after 3 days of NaCl treatment, and then survival rates were calculated after 7 days of recovery culture under normal growth conditions. When the plants were exposed to 150 mM NaCl for 3 days, the leaves of WT and overexpression lines turned yellow. In contrast, the chlorosis phenotype was significantly weakened in the RNAi line (Fig. 3c, d). Approximately 56.1 ± 15.0% of RNAi plants remained alive after recovery culture, whereas the survival rates of WT and overexpression transgenic lines (OE-1, OE-2) were significantly lower than that of RNAi line.
Remarkably, the RNAi line had a significantly higher chlorophyll content and relative survival rate compared with the WT and overexpression lines (Fig. 3d, e). These results demonstrated that suppression of OsVPE3 inhibits the development of chlorosis and improves salt tolerance in rice.

Suppression of OsVPE3 Inhibits the Formation of DNA Ladders in Salt-Induced PCD

To further investigate the effect of OsVPE3 on rice tolerance to salt stress, 3-day-old WT and transgenic seedlings were treated with high concentration NaCl (100 mM, 150 mM) for 3 days. The results showed that RNAi seedlings had the highest root relative elongation rate and overexpression seedlings were the most sensitive to salt stress (Fig. 4a). At the same time, Evans Blue staining confirmed that the root tips of the RNAi line maintained highest cell viability after exposure to 150 mM NaCl for 3 days compared to other lines (Fig. 4b). The fragmentation of genomic DNA is a typical biochemical and morphological feature of PCD. To determine if the suppression of OsVPE3 can inhibit salt-induced PCD, DNA fragmentation was measured in transgenic lines and WT plants treated with 300 mM NaCl for 8 h. As shown in Fig. 4c, clear and visible DNA laddering was observed in the OsVPE3-overexpression lines, whereas DNA laddering was significantly inhibited in the RNAi line. This result suggested that suppression of OsVPE3 inhibits the formation of DNA ladders during salt-induced PCD.

Because OsVPE3 has high homology with OsVPE1, we performed qRT-PCR experiment to detect the expression levels of OsVPE homologous genes in the roots of transgenic lines (Fig. 4d). The results showed that the expression level of OsVPE3 was decreased dramatically in RNAi line; meanwhile, OsVPE1 expression was also reduced compared with the WT. The expression level of OsVPE3 was much more decreased than OsVPE1 in the RNAi line. Our previous work has shown that the transcription of OsVPE2 and OsVPE3, but not of OsVPE1 and OsVPE4, can significantly increase in the salt-induced PCD (Deng et al. 2011; Kim et al. 2014), suggesting that the transcription of OsVPE1 was not responsive to salt stress in rice roots (Deng et al. 2011). Therefore, we conclude that OsVPE3 plays a crucial role in salt stress-mediated PCD.

Suppression of OsVPE3 Helps Maintain the Integrity of Vacuolar Membranes During Salt-Induced PCD

Unlike in animal cells, the vacuole is a unique organelle used to store a variety of hydrolytic enzymes and defence proteins in plant cells (Neuhaus et al. 1991; Yamada et al. 2001). The rupture of vacuolar membranes releases these components and leads to cell death (Mino et al. 2006). To elucidate the role of OsVPE3 in the integrity of vacuolar membranes during PCD, rice protoplasts were stained with Trypan Blue and the BCECF-AM fluorescent probe. Trypan Blue staining is a reporter of cell death based on disintegrated cellular membranes, and BCECF-AM labels the vacuole lumen as green fluorescence to reveal intact vacuoles (Swanson and Jones 1996). BCECF-staining
revealed that protoplasts prior to NaCl treatment accumulated strong fluorescence signal in vacuoles in both WT and transgenic lines (Fig. 5a, the first horizontal low). Protoplasts subjected to 100 mM NaCl treatment for 3 h can be sorted into three types as follows (Fig. 5a, b): Type 1, BCECF-positive and Trypan Blue-negative, indicating living cells with intact cellular membranes and vacuoles; Type 2, BCECF-negative and Trypan Blue-negative, indicating living cells with disintegrated vacuolar membranes (as shown by distribution of BCECF fluorescence signal outside of the vacuoles); and Type 3, BCECF-negative and Trypan Blue-positive, indicating dead cells with collapsed vacuoles (as shown by no BCECF fluorescence signal). After 100 mM NaCl treatment, the
survival protoplasts (type 1) decreased to 54.6 ± 2.4 and 52.7 ± 2.4% of the levels in the WT and OE-1 lines, respectively. In contrast, the survival rate of protoplasts (Type 1) from the RNAi line was 70.3 ± 6.5%, which was significantly higher than that of the WT and OE-1 lines. The results showing that many of the protoplasts (Type 2) with degraded vacuolar membranes were alive suggested that cell death was preceded by vacuolar collapse. Only 8.4 ± 4.1% of the RNAi cells belonged to Type 2, which involved vacuole rupture before cell death. Interestingly, 37.5 ± 3.1% of OsVPE3-overexpressing cells belonged to Type 3, which was significantly higher than in the WT and RNAi lines. Notably, certain Type 3 protoplasts from the RNAi line still showed weak fluorescence in the vacuolar region, thereby suggesting that OsVPE3-RNAi attenuated or delayed the disintegration of vacuolar membranes even after cell death. Taken together, these results demonstrated that suppression of OsVPE3 prevents vacuole rupture during salt-induced PCD (Fig. 5a).

Suppression of OsVPE3 Results in Decreased Leaf Width and Stomatal Size

Compared with WT, there were no obviously morphological changes in the transgenic plants at the early seedling stage. However, after four weeks of growth, the RNAi transgenic line exhibited narrower leaves than WT (Fig. 6a). Blade width of the fourth leaf was approximately 6.00 ± 0.54 cm in the WT plants, whereas the blade width was approximately 5.50 ± 0.33 cm in the RNAi line (Fig. 6b). Further observation revealed stomata size in the RNAi line was smaller than in WT plants (Fig. 6c). Statistical analysis showed that only 25% of guard cells were longer than 20 μm in the RNAi line whereas 64% in the WT plants. The majority of guard cells were between 15
and 20 μm in the RNAi line (Fig. 6d). There were no significant differences in leaf width and guard cell length between the OE-1 line and WT plants. The expression pattern of OsVPE3 showed that OsVPE3 is actively transcribed in the leaves and developing embryos of rice (Fig. 1d). We speculated that the expression levels of OsVPE3 were abundant enough for the leaf development in WT, thus the OsVPE3-overexpression lines exhibited weak phenotype compared with the WT.

In addition, we tested the percentage of water loss of detached leaves from WT and transgenic lines. The data indicated that the percentage of water loss of RNAi leaves was significantly lower than other lines (Additional file 1: Fig. S2), suggesting suppression of OsVPE3 enhanced the dehydration tolerance. The above results demonstrated that suppression of OsVPE3 caused decreased leaf width and guard cell length, promoting a dehydration tolerance in rice leaves.

Furthermore, we analysed the expression of genes related to stomatal development by qRT-PCR. According to known reports, there are five critical genes related to the development of stomata in rice, namely OsTMM (TOO MANY MOUTHS), OsSPCH1 (SPEECHLESS), OsSPCH2, OsMUTE and OsFAMA (Liu et al. 2009). OsTMM is a receptor for extracellular ligand, characterized as a set of key regulators in stomatal production and patterning (Balcerowicz and Hoecker 2014). OsSPCH1, OsSPCH2, OsMUTE and OsFAMA are essential transcription factors in the stomata patterning and development (Liu et al. 2009). We monitored the expressions of these genes (the data of OsFAMA is not shown because of its low expression level in rice leaves). As shown in Fig. 7, the expression levels of OsTMM, OsSPCH1 and OsMUTE were significantly down-regulated in the RNAi line compared with WT. Consistently, these three genes showed up-regulated tendencies in overexpression lines, while only the expression of OsSPCH2 decreased significantly. It revealed that OsVPE3 might mainly affect the level expression of OsTMM, OsSPCH1 and OsMUTE in stomata developmental pathway to change the guard cell size.
Fig. 6 Fourth leaf width and guard cell size in WT and transgenic lines. 

a. Images of第四 leaves of WT and transgenic lines. Bars = 1 cm. 
b. Measurements of the maximum fourth leaf width in WT and transgenic lines. Values indicate the mean, and error bars represent the SD (n >10). An asterisk indicates a significant difference between the WT and transgenic lines (t-test; P < 0.05). 
c. Images of stomata in WT and transgenic lines. Bars = 5 μm. 
d. Percentage of stomata with various guard cell lengths in WT and transgenic lines (n > 500).

Fig. 7 Effect of OsVPE3 on the expression levels of genes related to stomatal development. Expression levels of OsTMM, OsSPCH1, OsSPCH2, and OsMUTE in WT and transgenic lines as determined by qRT-PCR. Total RNA was extracted from the shoots of 5-day-old plants. OsUBQ5 was used as an internal control. Values are means, and error bars represent the SD from three independent experiments. Asterisks indicate a significant difference between WT and transgenic lines (t-test; **, P < 0.01).
Discussion
The main purpose of this study was to understand the role of OsVPE3 in vacuole-mediated PCD following salt stress in rice. OsVPE3 overexpression and suppression transgenic lines were created to elucidate the function of OsVPE3 in rice. Our results demonstrated that OsVPE3 plays a crucial role in the salt stress response by regulating the collapse of vacuolar membranes during PCD. In addition, OsVPE3 affected leaf and stomata guard cell development in rice.

High salinity is one of the most important abiotic stresses during crop breeding. High salinity activates the salt-overly-sensitive (SOS) system in plants, which leads to sodium exclusion from the cytosol (Zhu 2003). Under high salt conditions, excess Na⁺ accumulates in the plant, thus increasing influx of Na⁺ and efflux of K⁺(Serrano and Rodriguez-Navarro 2001; Horie et al. 2012). It has been proposed that these changes can decrease the cytosolic K/Na ratio, thus elevating concentrations of the intracellular second messenger Ca²⁺ (Kudla et al. 2010) and causing ROS bursts (Zhu 2001). As a consequence of increased K/Na ratios and salinity-induced ROS, programmed cell death would be finally triggered (Huh et al. 2002; Lin et al. 2006; Shabala 2009). A recent study in rice by our group revealed that overexpression of BCL-2, an anti-apoptotic protein, significantly reduces NaCl-induced K⁺ efflux and represses the expression of VPEs, thereby alleviating PCD symptoms (Deng et al. 2011; Kim et al. 2014).

Salt-induced PCD in plants and animals shares many consequences, including DNA fragmentation, nuclear condensation, nuclear deformation, mitochondrial involvement and endonuclease activity (Li et al. 2007; Jiang et al. 2008). Analysis of DNA laddering indicated that PCD occurs in rice seedlings under salt treatment (Fig. 4c). Compared with WT, overexpression of OsVPE3 strongly enhanced genomic DNA fragmentation, whereas OsVPE3 interference strongly repressed DNA fragmentation during PCD. This finding suggested that OsVPE3 likely plays a crucial role in the salt-induced PCD in rice.

The VPE family was originally identified as a group of processing enzymes responsible for the maturation of seed storage proteins in protein storage vacuoles (Haranishimura et al. 1991; Haranishimura et al. 1993). It was originally reported that NtVPEs have caspase-1 activity and are essential for the virus-induced hypersensitive response involving PCD, which led to the proposition of a new cell death mechanism mediated by VPE and cellular vacuoles (Hatsugai et al. 2004b). Subsequently, a similar function was proposed for AtVPE in mycotoxin-induced cell death (Kuroyanagi et al. 2005). Recently, further studies have shown that VPEs are also involved in cell death under abiotic stresses (Zhang et al. 2013b). For example, AtyVPE, which is mediated by MPK6, affects heat-shock-induced PCD in Arabidopsis (Li et al. 2012). Our study found that OsVPE3 regulated salt-induced PCD in rice.

Vacuoles are essential organelles in plants, which have multiple functions including storage of a wide variety of ions, proteins and other metabolites, and maintain cytosolic ion homeostasis (Boller and Wiemken 1986; Rea and Sanders 1987). Plant vacuoles also play critical roles in stress responses, development and pathogen defence. There have been many reports concerning the relationship between vacuoles and cell death (Hara-Nishimura and Hatsugai 2011; Higaki et al. 2011). Vacuole rupture triggers nuclear degradation during PCD (Obara et al. 2001). The discovery of VPE functions in vacuoles could further explain the molecular mechanism of vacuole-mediated PCD. Similar to characterized OsVPE1, OsVPE3 localized to vacuoles (Fig. 1c). We found that vacuole integrity was impaired prior to cell death under salt treatment in WT plants. However, suppression of OsVPE3 markedly enhanced the maintenance of vacuolar membranes even in the context of cell death. In contrast, overexpression of OsVPE3 accelerated the rupture of vacuole membranes (Fig. 5). Our results strongly supported the hypothesis that OsVPE3 plays a crucial role in vacuole rupture during PCD. To date, the mechanism of VPE-mediated vacuole rupture remains unclear, due to a lack of evidence that the VPEs interact other vacuolar proteins. Based on the characteristics of VPEs, we propose that VPEs expression is upregulated by disturbances in ion homeostasis in response to abiotic stresses and that VPE precursors are self-activated to process vacuolar hydrolases and proteases, leading to vacuole rupture and cell death.

In addition to vacuole rupture, there is another interesting finding concerning the role of OsVPE3 in the development of stomata in rice. The stoma is known to be an important structure for controlling gaseous exchange and water release by transpiration (Assmann 1993), and function under stress conditions (Zhang et al. 2013a). At the same time, stomata movement may be affected by VPEs in Arabidopsis (Albertini et al. 2014). We found that leaves in the RNAi line were more curled than in wild type and that this phenomenon was more apparent under salt stress (Fig. 3). Further research showed that stomata size in the RNAi line was smaller than in WT plants (Fig. 6c, d). Small stomata may alter transpiration to improve resistance in plants. Transcriptome analysis has revealed strong expression of AtyVPE in Arabidopsis guard cells (Albertini et al. 2014). qRT-PCR analysis has confirmed that γVPE expression in guard cells is higher than in whole leaves, thus suggesting that this gene plays a critical role in guard cells (Albertini et al. 2014). Moreover, Arabidopsis γVPE knockout mutants reduced stomata opening and increased resistance to desiccation (Albertini et al. 2014). Our data showed that suppression of OsVPE3 down-regulated the expression levels of
OsTMM, OsSPCH1 and OsMUTE in the stomata developmental pathway, leading to affect guard cell size in the OsVPE3-RNAi line. This finding suggests that OsVPE3 might play a role in stomata development in rice.

Conclusions
Our results demonstrated that OsVPE3 plays a crucial role in the salt stress-induced PCD by regulating the collapse of vacuolar membranes. In addition, OsVPE3 affected leaf and stomata guard cell development in rice. These findings are relevant for enhancing salt-tolerance via genetic engineering in crop breeding programs.

Methods
Growth Conditions
Seeds were surface sterilized with 10% sodium hypochlorite (v/v) for 30 min, rinsed 5 times in deionized water and soaked in deionized water at 30 °C for 2 days in the dark. After germination, the seeds were transferred to a nutrient solution culture (Yoshida et al. 1976) at pH 5.0–6.0 in a greenhouse at 28 °C under a 16:8 h light: dark cycle. The 3-day-old seedlings were treated with 300 mM NaCl following seedling growth under these conditions. After 4 weeks of growth, the plants were treated with various concentrations of NaCl.

Constructs and Plant transformation
All wild-type and mutant transgenic lines were generated in the Oryza sativa L. ssp. japonica cv. Nipponbare rice background.

For the overexpression constructs, the cDNA sequence of OsVPE3 (approximately 1488 bp) was amplified from the cDNA library of Nipponbare using gene-specific primer pairs. The fragments were then cloned into the pENTR/D-TOPO vector (Invitrogen, Carlsbad, CA, USA) and then into the destination vector (pH7FWG2.0) by LR clonase reactions.

For the RNA interference (RNAi) constructs, a 745-bp fragment was amplified from OsVPE3, inserted into the pENTR/D-TOPO vector (Invitrogen), and then cloned into pH7GW1WG2 (I) by LR clonase reactions.

Rice transformation was performed using the Agrobacterium tumefaciens-mediated co-cultivation method. Transformed calli were selected on hygromycin medium. T0 plants were self-pollinated over two generations to obtain homozygous T2 transgenic seeds. Homozygous T3 seeds were used in this study.

Primers used in this work are listed in Additional file 1: Table S1. The gene constructs used for rice transformation were verified by sequencing.

Total RNA extraction and qRT-PCR Assay For OsVpe3 Expression
Total RNA was isolated using the TRIzol RNA extraction kit (Invitrogen), and first-strand cDNA was synthesized using the ReverTra Ace qPCR RT Master Mix with gDNA Remover for qRT-PCR (TOYOBO). Quantitative RT-PCR was performed using the Master cycler ep realplex system (Eppendorf, Hamburg, Germany) and the SYBR PrimerScript RT-PCR kit (Perfect Real Time; TaKaRa). OsUBQ5 was amplified as a control for the template. All primers used in this work are listed in Additional file 1: Table S1.

Protein extraction from rice grains and SDS-PAGE assays
Mature seeds harvested from T3 homozygous plants were used for protein extraction. The protein extraction was performed on the described methods (Takemoto et al. 2002), and the proteins were analysed by SDS-PAGE. The density of the protein bands was measured by using the Image J software.

Subcellular Localization
The OsVPE1:GFP and OsVPE3:GFP fusions were created using Gateway cloning (Invitrogen). The full-length cDNAs of OsVPE1 and OsVPE3 (lacking stop codons) were amplified and inserted into the pENTR/D-TOPO vector (Invitrogen), and they were subsequently cloned into pUGW5 using LR clonase reactions. The fusion constructs were transformed into rice protoplasts using PEG-mediated transfection according to our previous study (Bai et al. 2014). Following transformation, rice protoplasts were incubated at 25 ± 2 °C in the dark for 12–16 h prior to observation with a Zeiss LSM710 NLO two-photon microscopy (Germany).

Chlorophyll Content Assay
Four-week-old plants were treated with 150 mM NaCl for 3 days prior to measuring chlorophyll content. Chlorophyll was extracted from the tip of the fourth leaf. Briefly, each sample (0.1 g) was placed into a 10-mL tube containing 4 mL of extraction solution (1: 1 ethanol: acetone) and incubated in the dark for 24 h at 25 °C. Absorbance of the extracts at 663 and 645 nm was measured using a Spekol spectrophotometer (Carl Zeiss GmbH, Jena, Germany). Total chlorophyll content was calculated using the following formulas (Yu et al. 2006):

\[ \text{Chl a} = 12.7 \times A663 - 2.69 \times A645 \times V/(1000 \times W) \]

\[ \text{Chl b} = 22.9 \times A645 - 4.68 \times A663 \times V/(1000 \times W) \]

\[ \text{Chl a + b} = 20.2 \times A645 + 8.02 \times A663 \times V/(1000 \times W) \]

All experiments were repeated three times.
Root Elongation Assay
After germination, sterilized seeds were sown on filter paper moistened with 0.1 mM CaCl₂. Before treatment with NaCl, the primary root lengths of 3-day-old seedlings were measured and recorded as $L_{C0}$ and $L_{T0}$. After treatment with NaCl (0, 100, 150 mM) for 3 days, the primary root lengths were recorded as $L_{C3}$ and $L_{T3}$. Root relative elongation rates were calculated using the formula previously reported by Pan et al. (2004):

$$\text{RER} (%) = \left( \frac{L_{T3} - L_{T0}}{L_{C3} - L_{C0}} \right) \times 100\%$$

Evans Blue Staining
The cell viability of primary root was evaluated using Evans Blue staining. After treatment with 0 or 150 mM NaCl for 3 days, 6-day-old seedlings were stained with 2.5% Evans Blue for 10 min and then washed twice with deionized water. Prior to observation, the seedlings were soaked in transparent agent for 12 h.

DNA Ladder Analysis
After 8 h of treatment with 300 mM NaCl, the roots tips of the rice seedlings were collected and ground in liquid nitrogen. DNA was isolated using the CTAB method and then digested with 100 g/mL DNase-free RNase for 1 h at 37 °C to eliminate RNA contamination. For each sample, an aliquot of DNA (20 μg) was separated using a 2% (w/v) agarose gel, stained with 0.1 μg/mL ethidium bromide in TE buffer (10 mM Tris-HCl, pH 8.0; 0.5 mM EDTA) and washed once with TE buffer. The fragmented DNA was observed under UV light using a photostation (UVI, Cambridge, UK).

BCECF and Trypan Blue Staining
Protoplasts were incubated with 10 μM BCECF-AM (Molecular Probes, USA) for 2 h at 25 °C in the dark and then treated with 100 mM NaCl for 3 h. Prior to observation, protoplasts were washed twice with W5 solution and stained with 0.04% Trypan Blue for 3 min. BCECF signal was visualized with excitation at 465–495 nm and emission at 515–555 nm using a band pass filter based on a previous method (Tang et al. 2012).

Leaf Water Loss Assay
Plants germinated under normal growth conditions for 4 weeks. The leaves were detached from various lines with same age and position, and weighed immediately as the initial fresh weight. They were then placed in clean filter papers, and incubated at 25 °C. The decreases in fresh weight were recorded at every 20 min for 5 h. Water loss was presented as percentage of fresh weight loss versus the initial fresh weight (Zhang et al. 2012).

Stomata Observation
The dental resin impression method was used with nail polish as an impression material (Kagan et al. 1992; Geisler et al. 2000). Impressions were observed on glass slides using a Zeiss LSM710 NLO two-photon microscope (Mannheim, Germany).

Additional files

**Additional file 1:** Supporting information. Table S1. List of primers used in this study (F, forward primer; R, reverse primer; q, quantitative real-time PCR). Figure S1. The grain width and 1000-grian weight of the WT, over-expression lines and RNAi line.Values are means, and error bars represent the SD from three independent experiments. Asterisks indicate a significant difference between WT and transgenic lines (t-test, ***, P < 0.01). Figure S2. The percentages of water loss of detached leaves from WT and transgenic lines. Values are means, and error bars represent the SD from three independent experiments. (DOCX 825 kb)

Abbreviations
BCECF-AM: 2,7’-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein-acetoxyethyl; BCL-2: B-cell lymphoma-2; GFP: Green fluorescent protein; MPK6: Mitogen-activated protein kinase 6; PCD: Programmed cell death; qRT-PCR: Real time quantitative polymerase chain reaction; ROS: Reactive oxygen species; SOS: Salt-overly-sensitive; VPE: Vacuolar processing enzymes

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

Authors’ contributions
LW, DM, ZM and BH contributed to the experimental design. LW, DM, GF, WM, ZZ and BH contributed to experiment performance, data analysis and drafted the manuscript. HN and YY contributed to good advice of designing experiment. All authors read and approved the final manuscript.

Author details
1Institute of Genetics and Regenerative Biology, Key Laboratory for Cell and Gene Engineering of Zhejiang Province, College of Life Sciences, Zhejiang University, Hangzhou, China. 2State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control, Institute of Virology and Biotechnology, Zhejiang Academy of Agricultural Sciences, Hangzhou, China. 3Department of Plant Pathology and Huck Institute of Life Sciences, Pennsylvania State University, University Park, PA 16802, USA.

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