The Membrane-Associated Transcription Factor NAC089 Controls ER-Stress-Induced Programmed Cell Death in Plants

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Abstract

The unfolded protein response (UPR) is activated to sustain cell survival by reducing misfolded protein accumulation in the endoplasmic reticulum (ER). The UPR also promotes programmed cell death (PCD) when the ER stress is severe; however, the underlying molecular mechanisms are less understood, especially in plants. Previously, two membrane-associated transcription factors (MTFs), bZIP28 and bZIP60, were identified as the key regulators for cell survival in the plant ER stress response. Here, we report the identification of another MTF, NAC089, as an important PCD regulator in Arabidopsis (Arabidopsis thaliana) plants. NAC089 relocates from the ER membrane to the nucleus under ER stress conditions. Inducible expression of a truncated form of NAC089, in which the transmembrane domain is deleted, induces PCD with increased caspase 3/7-like activity and DNA fragmentation. Knock-down expression of a truncated form of NAC089, in which the transmembrane domain is deleted, induces PCD with increased caspase 3/7-like activity and DNA fragmentation. Knock-down NAC089 in Arabidopsis confers ER stress tolerance and impairs ER-stress-induced caspase-like activity. Transcriptional regulation analysis and ChIP-qPCR reveal that NAC089 plays important regulatory roles in regulating downstream genes involved in PCD, such as NAC094, MC5 and BAG6. Furthermore, NAC089 is up-regulated by ER stress, which is directly controlled by bZIP28 and bZIP60. These results show that nuclear relocation of NAC089 promotes ER-stress-induced PCD, and both pro-survival and pro-death signals are elicited by bZIP28 and bZIP60 during plant ER stress response.

Introduction

In eukaryotic cells, ER is a major site for the production of secreted, plasma membrane and organelle proteins. Cells have evolved a sophisticated quality control system to ensure the accuracy of protein folding through optimizing the protein-folding machinery and ER-associated degradation (ERAD) [1,2,3]. To coordinate protein-folding capacity with protein-folding demand, a collection of phylogenetically conserved signaling pathways, termed the UPR, senses the accumulation of misfolded proteins in the ER and sustains homeostatic balance according to the protein folding needs which change constantly depending on different developmental programs and/or environmental conditions [1,4,5].

Three arms of UPR signaling pathways, namely inositol requiring enzyme 1 (IRE1), double-stranded RNA-activated protein kinase (PKR) like ER kinase (PERK), and activating transcription factor 6 (ATF6), were identified in mammalian cells that have the ability to promote cell survival by reducing misfolded protein accumulation in the ER. IRE1 is a key component in the most conserved branch, which acts by splicing messenger RNA encoding transcription factor Hac1p in yeast or XBP1 in mammalian cell, respectively [6,7,8]. Recently, the equivalent pathways were discovered in plants (e.g. the IRE1-bZIP60 pathway in Arabidopsis), which also play important roles in heat stress response, as well as in plant immune response [9,10,11,12,13,14]. PERK is an ER-localized kinase and its activation upon ER stress leads to the attenuation of bulk protein translation in metazoan cells [15]. ATF6 is an ER membrane-associated bZIP transcription factor; its activation requires ER-to-Golgi translocation and regulated intramembrane proteolysis (RIP) [16]. Although the plant PERK ortholog has not yet been reported, the ER membrane-associated Arabidopsis bZIP28 was found to be the functional homolog of mammalian ATF6, which is activated in a manner similar to ATF6 [17,18,19,20,21].

Severe or chronic ER stress can also lead to PCD, a process that kills unwanted cells under ER stress conditions to protect other cells [22]. In contrast to what is known about how UPR protects cells, less is known about the mechanisms that link UPR to PCD, especially in plants [23]. In mammalian cells, IRE1 can trigger PCD by activating the Jun amino-terminal kinase (JNK) pathway [24]. Phosphorylation of JNK leads to the activation of pro-death...
protein BIM and inhibition of anti-death protein BCL-2 [25]. Mammalian IRE1 also binds to BAX and BAK, two cell-death-inducing proteins involved in the mitochondrial cell death pathway [26]. The activation of mammalian IRE1 is able to cause rapid decay of selected microRNAs (miRs -17, -34a, -96, and -125b) that normally repress translation of caspase-2 mRNA, and thus sharply elevates protein levels of this initiator protein in the mitochondrial cell death pathway [27]. The ER stress-induced mammalian bZIP transcription factor CHOP is one of the major players that induces PCD, most probably through suppression of the pro-survival protein BCL-2 and up-regulation of ERO1α to further perturb the cellular redox state [28]. CHOP is a downstream target of all three aforementioned UPR signaling pathways in mammals [4]. Recently, transcriptional induction through ATF4 and CHOP was shown to increase protein synthesis leading to oxidative stress and PCD [29]. Orthologs of mammalian JNK, BAX, BAK, CHOP and ATF4 are not found in the Arabidopsis genome [30]. However, ER stress-induced PCD is reported in plants with the hallmark of DNA segmentation, and the conserved BAX inhibitor-1 (BI-1) plays important roles in suppression of ER stress-induced PCD in Arabidopsis plant [31,32,33,34,35,36,37,38]. When animal cells are subjected to severe ER stress, IRE1 loses its specificity and begins to degrade mRNAs in a process called regulated IRE1-dependent decay (RIDD) [39]. IRE1 in Arabidopsis also has similar function in the RIDD process in the UPR for degradation of mRNA encoding proteins in the secretory pathway to decrease the amount of proteins entering the ER [40]. Different from the animal system, knock-outs of both IRE1s in Arabidopsis impairs UPR and enhances PCD upon ER stress, indicating that RIDD may play a negative role in PCD in plants [40]. Despite the emerging evidence on ER stress-mediated PCD in plants, the underlying molecular mechanisms of PCD in plant UPR is still largely unknown. In soybean plants, prolonged ER stress and osmotic stress synergistically activate N-rich proteins (NRPs) to induce the expression of NAC6/NAC30 to regulate PCD together with NAC081 [33,41,42], however, the link from ER stress-sensing machinery to these NRPs is still missing.

Here we show that NAC089 plays important roles in regulating ER-stress-induced PCD in the model plant Arabidopsis. NAC089 relocates from the ER membrane to the nucleus during ER stress response. Inducible expression of a truncated form of NAC089 promotes PCD with increased caspase 3/7-like activity and DNA fragmentation. Down-regulation of NAC089 confers ER stress tolerance and impairs ER-stress-induced caspase 3/7-like activity. Several UPR downstream genes including the PCD regulators MC5, BIK6 and NAC094 are shown to be regulated by NAC089 under ER stress condition. NAC089 itself is also up-regulated by ER stress, which is directly controlled by bZIP28 and bZIP60. Therefore, NAC089 is an important PCD regulator in plant UPR, linking ER stress-sensing to downstream PCD regulators during ER stress response in plants.

Results

Up-regulation of NAC089 by ER stress is controlled by both bZIP28 and bZIP60

An ER-stress-related NAC (for NAM, ATAF, and CUC) transcription factor NAC089 (also known as ANAC089) was identified from our previous microarray analysis [43]. Its expression was up-regulated rapidly by ER stress inducers tunicamycin (TM) and dithiothreitol (DTT) (Figure 1A and Figure S1). Knock-out of either bZIP28 or bZIP60 partially suppressed, while knock-outs of both bZIP28 and bZIP60 in zip28zip60 double mutant completely abolished the up-regulation of NAC089 under ER stress condition (Figure 1B). Previously two ER stress responsive cis-elements UPRE and ERSE-I were identified as the binding sites of bZIP28 and bZIP60 [21,43,44]. We searched the NAC089 promoter region and found that one copy of UPRE and one copy of ERSE-I like (one mismatch) cis-elements are present over the segment [−95, −49] relative to the TSS site of NAC089. To assess the activation of NAC089 promoter by bZIP28 and bZIP60, an effector-reporter dual-luciferase transient assay was set up. The NAC089 promoter fragment containing the aforementioned cis-elements was fused to the firefly luciferase reporter and tested in Arabidopsis leaf protoplasts. As expected, the reporter was activated by either TM or DTT treatment (Figure 1C). Using this assay system, co-expression of either bZIP28D or bZIP60S dramatically enhanced the firefly luciferase reporter activity (Figure 1D). To demonstrate the direct binding of bZIP28 or bZIP60 to the NAC089 promoter, electrophoretic mobility shift assays (EMSAs) were performed with the biotin-labeled NAC089 promoter DNA. When the truncated form of either bZIP28 or bZIP60 was incubated with the biotin-labeled DNA probe, a band shift was observed reflecting the formation of the respective complex. To show the binding specificity, excess un-labeled probe was added and shown to be an effective competitor for the formation of each complex. On the contrary, the un-labeled mutated UPRE probe could not compete with the binding (Figure 1E–F). Through further mutation analysis, it was found that neither bZIP28 nor bZIP60 binds to the ERSE-I like cis-element presented in the probe (Figure S2). Thus, the expression of NAC089 is up-regulated by ER stress, which is directly controlled by both bZIP28 and bZIP60 through the UPRE cis-element.

NAC089 relocates from the ER membrane to the nucleus under ER stress condition

NAC089 is predicted to be a membrane-associated transcription factor [45] with the N-terminal DNA-binding domain facing the cytoplasm (Figure 2A). It has transcriptional activation activity and forms homodimers [46] (Figure S3). To confirm the membrane association of NAC089 and also to investigate the possible membrane-to-nucleus translocation of NAC089 in response to ER stress, 4X MYC tag was fused to NAC089 at the N-terminus and the fusion protein was expressed in Arabidopsis plants. Total proteins were extracted from transgenic
seedlings and MYC-NAC089 was detected with the western blotting analysis. Without TM or DTT treatment, one prominent band reacted with the anti-MYC antibody. After TM or DTT treatment for 6 hr, one excess band with smaller molecular weight was induced, with a similar migration rate to the truncated form NAC089D, in which the C-terminal 24 amino acids of NAC089 were replaced with the 4X MYC tag (Figure 2B). To track the movement of NAC089 in response to ER stress, mGFP-NAC089 was expressed in Arabidopsis and observed under confocal laser scanning microscopy. In the mock (H2O) treatment, most of the mGFP-NAC089 signals were observed in the ER (Figure 2C–D); after either TM or DTT treatment for 6 hr, the fluorescence

Figure 1. Up-regulation of NAC089 by ER stress is directly controlled by bZIP28 and bZIP60. (A–B) Up-regulation of NAC089 in the wild-type (wt) plants by ER-stress inducers tunicamycin (TM, 5 μg/ml) and dithiothreitol (DTT, 2 mM) (A) and in the wt, bZIP28 single mutant (zip28), bZIP60 single mutant (zip60) or double mutant (zip28zip60) by TM (5 μg/ml) treatment (B). The expression of NAC089 is normalized to the expression of the internal control actin. (C–D) Transactivation of NAC089 promoter in the dual-luciferase leaf protoplast assays. Activations of the NAC089 promoter by ER stress treatments (C) and by co-expression of either activated bZIP28 (bZIP28D) or activated bZIP60 (bZIP60S) (D). Relative reporter activity is the firefly luciferase activity normalized to the renilla luciferase activity. Bars depict SE (n = 3) in A–D. The empty vector (EV) was used as a negative control. ** P<0.01, * P<0.05. (E–F) EMSA experiments to detect the protein-DNA binding. Either the purified His-bZIP28D (E) or Trx-His-bZIP60T (F) was incubated with the biotin-labeled pNAC089 DNA. Lane 3, 50× un-labeled pNAC089; lane 4, 200× un-labeled pNAC089; lane 5, 200× un-labeled mutated form pNAC089M1. Arrows and arrow heads point to the positions of shifted bands and free probes, respectively.
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signals were largely found in the nuclei of the Arabidopsis leaf protoplasts and root cells (Figure 2C–D). The nuclear relocation of mGFP-NAC089 was not observed in the root cells when the transgenic plants were treated with TM (5 μg/ml) for short period of time (e.g. 2 hr). The ER-to-nucleus movement was also confirmed in the protein fractionation studies (Figure S4). Taken together, NAC089 is an ER membrane-associated protein and it relocates from the ER membrane to the nucleus in response to ER stress.

Down-regulation of NAC089 confers ER stress tolerance in plants

To investigate the biological function of NAC089 in the ER stress response, we created partial loss-of-function mutants by RNA interference (RNAi) and chimeric repressor silencing technology (CRES-T). NAC089 knock-down plants (RNAi089) grew as well as the wild-type (wt) control under normal growth condition, but they were more tolerant to ER stress than the wt (Figure 3A-B, Figure S5). More greenish big (G-B) plants and less yellowish small (Y-S) plants were observed in the RNAi089 plants than that in the wt under ER stress conditions (Figure 3B). In CRES-T system, fusion of an EAR-motif repression domain to a transcription factor converts an activator into a repressor, which results in partial loss function of the transcription factor [47]. We replaced the C-terminal hydrophobic tail of NAC089 with the EAR-motif, and expressed the chimerical fusion protein NAC089D-EAR in Arabidopsis with NAC089’s native promoter. NAC089D-EAR expression did not affect seedling development under normal growth condition, but also conferred ER stress tolerance in plants (Figure S6A-D). ER stress should be built-up in plants which had been grown on solid growth medium with low concentrations of tunicamycin for a long period of time, as ascertained by the up-regulation of UPR marker genes in the wild-type plants (Figure S6E). All together, we concluded that partial loss-of-function of NAC089 in Arabidopsis increases chronic ER stress tolerance.

NAC089 promotes programmed cell death in plants

To gain insight into mechanisms by which NAC089 operates, we conditionally expressed a MYC-tagged truncated form of NAC089 (NAC089D-MYC) with the beta-estradiol (BE) inducible system [48] (Figure S7). This truncated form co-migrates with the ER stress-induced nuclear form of MYC-NAC089, has the transcriptional activation activity and localizes in the nucleus as mentioned above. The wt control and NAC089D-MYC expressing plants (XVE089D) were transferred to growth medium supplemented with or without BE. There was no obvious difference between the wt and XVE089D plants on the growth medium without BE (Figure 4A). However, when BE was included in the growth medium, root growth of the XVE089D plants was inhibited, and chlorotic leaves were observed, representing a typical PCD phenotype (Figure 4B-C). Cysteine-dependent aspartate-directed proteases (caspases) are the key regulators of PCD in animals, of which caspase-3 is the crucial executor of PCD and recognize tetra-peptide motif DEVD [49]. Although the ortholog of animal caspase-3 is absent in plants, caspase 3/7-like activity has been reported in many examples involved in plant development and adaptation to environmental stresses [50]. To investigate whether the NAC089D-MYC-induced PCD was associated with the caspase-like activity, we performed caspase-3/7 activity assays using the same tetra-peptide substrate as previously reported [32,33,41]. It was found that the expression of NAC089D-MYC considerably induced caspase 3/7-like activity in the XVE089D plants (Figure 4D). The caspase 3/7 activity was also checked in the wt control and NAC089 RNAi plants (line RNAi089-25). ER stress gradually induced caspase 3/7-like activity in both the wt and RNAi089-25 plants in response to chronic ER stress. However, the caspase 3/7-like activity in the RNAi089-25 plants was about half of that in the wt plants after 3 days of TM treatment (Figure 4E), suggesting that NAC089-regulated caspase 3/7-like activity is stress severity-dependent. It is possible that other pathways also regulate such caspase 3/7-like activity. Loss of cell viability, accumulation of H2O2 and rupture of plasma membrane are often associated with PCD [30]. To assess cell viability, roots of XVE089D plants were stained with fluorescein diacetate (FDA), which is a substrate for many endogenous esterases. NAC089D-MYC expression dramatically reduced the endogenous esterase activities (Figure 4F-G). Further 3′-diaminobenzidine (DAB) staining demonstrated that H2O2 was accumulated in the roots when NAC089D-MYC was induced (Figure 4H-I). Propidium iodide (PI) binds to DNA, but it is often used to stain plant cell wall or plasma membrane (Figure 4J) because it is membrane impermeant. When NAC089D-MYC was induced, PI signals were observed in both the cytoplasm and nucleus (Figure 4K), indicating that expression of NAC089D-MYC reduced the rigidity of cell membrane. Another characteristic of PCD is the morphological changes in the nucleus which could be revealed by 4, 6-diamidino-2-phenylindole (DAPI) staining. Intact and round nuclei were found in most of the XVE089D root cells without BE treatment (Figure 4L). In contrast, nuclei with stretches and speckles were observed in the XVE089D root cells when the plants were treated with BE. (Figure 4M-N). Cleavage of genomic DNA at internucleosomal sites by endogenous nuclease is always associated with PCD and terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) assay is frequently used to label the fragmentation of nuclear DNA in situ [31]. Compared to the low level of background green fluorescence in normal-grown roots (Figure 4O), strong TUNEL-positive signals were observed in the XVE089D root cells when the plants were treated with BE. (Figure 4P-Q). BE treatment had no obvious effect on the aforementioned histochemical staining in the wt control plants (Figure S8). These results suggest that NAC089 has the ability to promote PCD in plants.

NAC089 regulates many downstream genes including PCD regulators under ER stress condition

PCD is a genetically controlled process that plays important roles in plant development and responses to abiotic stress or pathogens [51,52]. Many PCD regulators that have been well characterized in humans, worms and flies are absent from the Arabidopsis genome, indicating that plants may use different regulators to execute PCD [52,53]. To understand how NAC089 regulates plant PCD, we performed microarray (Agilent 4X44K) experiments with the BE inducible gene expression system [48]. We conditionally expressed a MYC-tagged truncated form of NAC089 (NAC089D-MYC) with the beta-estradiol (BE) inducible system [48] (Figure S7). This truncated form co-migrates with the ER stress-induced nuclear form of MYC-NAC089, has the transcriptional activation activity and localizes in the nucleus as mentioned above. The wt control and NAC089D-MYC expressing plants (XVE089D) were transferred to growth medium supplemented with or without BE. There was no obvious difference between the wt and XVE089D plants on the growth medium without BE (Figure 4A). However, when BE was included in the growth medium, root growth of the XVE089D plants was inhibited, and chlorotic leaves were observed, representing a typical PCD phenotype (Figure 4B-C). Cysteine-dependent aspartate-directed proteases (caspases) are the key regulators of PCD in animals, of which caspase-3 is the crucial executor of PCD and recognize tetra-peptide motif DEVD [49]. Although the ortholog of animal caspase-3 is absent in plants, caspase 3/7-like activity has been reported in many examples involved in plant development and adaptation to environmental stresses [50]. To investigate whether the NAC089D-MYC-induced PCD was associated with the caspase-like activity, we performed caspase-3/7 activity assays using the same tetra-peptide substrate as previously reported [32,33,41]. It was found that the expression of NAC089D-MYC considerably induced caspase 3/7-like activity in the XVE089D plants (Figure 4D). The caspase 3/7 activity was also checked in the wt control and NAC089 RNAi plants (line RNAi089-25). ER stress gradually induced caspase 3/7-like activity in both the wt and RNAi089-25 plants in response to chronic ER stress. However, the caspase 3/7-like activity in the RNAi089-25 plants was about half of that in the wt plants after 3 days of TM treatment (Figure 4E), suggesting that NAC089-regulated caspase 3/7-like activity is stress severity-dependent. It is possible that other pathways also regulate such caspase 3/7-like activity. Loss of cell viability, accumulation of H2O2 and rupture of plasma membrane are often associated with PCD [30]. To assess cell viability, roots of XVE089D plants were stained with fluorescein diacetate (FDA), which is a substrate for many endogenous esterases. NAC089D-MYC expression dramatically reduced the endogenous esterase activities (Figure 4F-G). Further 3′-diaminobenzidine (DAB) staining demonstrated that H2O2 was accumulated in the roots when NAC089D-MYC was induced (Figure 4H-I). Propidium iodide (PI) binds to DNA, but it is often used to stain plant cell wall or plasma membrane (Figure 4J) because it is membrane impermeant. When NAC089D-MYC was induced, PI signals were observed in both the cytoplasm and nucleus (Figure 4K), indicating that expression of NAC089D-MYC reduced the rigidity of cell membrane. Another characteristic of PCD is the morphological changes in the nucleus which could be revealed by 4, 6-diamidino-2-phenylindole (DAPI) staining. Intact and round nuclei were found in most of the XVE089D root cells without BE treatment (Figure 4L). In contrast, nuclei with stretches and speckles were observed in the XVE089D root cells when the plants were treated with BE. (Figure 4M-N). Cleavage of genomic DNA at internucleosomal sites by endogenous nuclease is always associated with PCD and terminal deoxynucleotidyl transferase-mediated dUTP nick and labeling (TUNEL) assay is frequently used to label the fragmentation of nuclear DNA in situ [31]. Compared to the low level of background green fluorescence in normal-grown roots (Figure 4O), strong TUNEL-positive signals were observed in the XVE089D root cells when the plants were treated with BE. (Figure 4P-Q). BE treatment had no obvious effect on the aforementioned histochemical staining in the wt control plants (Figure S8). These results suggest that NAC089 has the ability to promote PCD in plants.
especially after 12 hr TM treatment (Figure S11). The up-regulation of these 13 genes by ER stress was also checked in the wt and NAC089 knock-down plants (line RNAi089-25). Previously, the BAX inhibitor 1 (BI-1) was reported to be an important modulator of ER stress-mediated PCD in Arabidopsis [31]. The transcription factor WRKY33, which is required for resistance to necrotrophic pathogens, plays critical roles in autophagy [55]. These two PCD markers along with other cell survival UPR markers were also included in the expression study. It was found that the up-regulation of 6 NAC089D-MYC-regulated genes (i.e. AT1G69325, encoding remorin-like protein; AT1G79330, encoding metacaspase MC5; AT2G46240, encoding

**Figure 2. NAC089 relocates from the ER membrane to the nucleus during ER stress responses.**

(A) Topology of NAC089 protein. (B) Activation of MYC-NAC089 in response to ER stress. Plant seedlings were treated with H2O (control), tunicamycin (TM, 5 μg/ml) or DTT (2 mM) for 6 hr. Star and asterisk represent the precursor and the processed form, respectively. The beta-estradiol (BE) induced truncated form NAC089D-MYC was used as the migration marker. Coomassie blue staining of RbcS serves as the loading control. (C–D) Nuclear relocation of mGFP-NAC089 in response to ER stresses in Arabidopsis leaf protoplast (C) and root cells (D). TM = 5 μg/ml in C and TM = 10 μg/ml in D. Dashed lines highlight the cell boundary. Bar = 50 μm.

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BCL-2-associated athanogene BAG6; AT3G52350, encoding unknown protein; AT4G30880, encoding lipid transfer protein; and AT5G39820, encoding transcription factor NAC094) and autophagy-related gene WRKY33 was impaired in the RNAi089-25 plants under ER stress condition comparing to the wt control (Figure 6A–B). These results indicate that NAC089 plays critical roles in regulating these ER-stress-induced genes including several PCD-related genes. ER stress also up-regulates several cell survival UPR marker genes and BI-1 in both the wt and RNAi089-25 plants (Figure 6B), suggesting that NAC089 plays minor role in regulating the expression of these genes. In order to know whether NAC089 directly regulates these downstream genes, chromatin immunoprecipitation (ChIP) experiments were carried out with NAC089D-MYC plants (line XVE089D-13) using anti-MYC antibody. It was found that NAC089D-MYC was enriched significantly with fold change greater than 2 at the promoter regions of 7 genes (i.e. AT1G65240, AT1G71390, AT1G79330, AT2G46240, AT1G39880, AT3G39820 and AT5G40010) (Figure 7), indicating that these genes might be the direct targets of NAC089. Among the 7 NAC089 possible targets, the up-regulation of 3 genes (i.e. AT1G65240, encoding aspartyl protease; AT1G71390, encoding receptor-like protein RLP11 and AT5G40010, encoding AAA ATPase 1) by ER stress was not suppressed in the NAC089 knock-down mutants (Figure 6A), suggesting that other factors may also up-regulate these genes under ER stress condition. We concluded that NAC089 has the ability to regulate some of the UPR downstream genes, including the PCD regulatory genes MG5, BAG6 and NAC094, and the autophagy regulatory gene WRKY33. The function of other NAC089 downstream genes in ER stress-induced PCD needs to be investigated in the future.

Discussion

The unmitigated ER stress is believed to induce PCD in animals [3], as well as in plants [31,56]. Given that PCD components are not highly conserved between animals and plants [30,57], our knowledge on ER stress-induced PCD in plants is very limited [58]. Previously, BI-1 and IRE1 were reported to be the negative regulators of PCD in plants [31,40]. In the current study, a membrane-associated transcription factor NAC089 was identified as an important transcriptional regulator of plant PCD under ER stress condition based on the following evidences: 1) NAC089 is up-regulated by UPR regulators bZIP28 and bZIP60 under ER stress condition; 2) NAC089 relocates from the ER membrane to the nucleus in response to ER stress; 3) Inducible expression of the truncated form of NAC089 induces PCD; 4) Partial loss-of-function of NAC089 confers resistance to chronic ER stress with reduced caspase 3/7-like activity; 5) NAC089 has transcriptional activity and binds to the promoter of many downstream targets; 6) Knock-down NAC089 suppresses the ER-stress-induced expression of several PCD regulators.

As in animals, plant development and adaptations to environmental stresses are intimately connected to PCD [59,60]. In
mammals, PCD is controlled predominately through functionally conserved proteins such as CED9/BCL-2 and BAX, but such genes have not been identified in plants [52]. Interestingly, the heterotrimeric G protein signaling was reported to be involved in ER stress-associated PCD. Null mutants of G beta subunit (AGB1) were more resistant to ER stress than either the wt plants or null mutants of G alpha subunit, but the underlying molecular mechanism was not known yet [61]. On the contrary, Chen and Brandizzi recently reported that the null AGB1 mutants were more sensitive to ER stress [62]. The function of AGB1 in ER stresses response needs to be further clarified. Caspases are cysteinyl-aspartic proteases that play essential roles in PCD in animals [4]. Plant caspase homologs are not found so far, and the metacaspases were demonstrated to have similar function in plant PCD [53, 63, 64]. Caspase-like activity has been detected in plant PCD associated with xylem formation and adaptations to heavy metal

Figure 4. NAC089 promotes programmed cell death in plants. (A–C) Photos of Arabidopsis NAC089D-MYC transgenic lines (XVE089D) and wild-type (wt) control plants transferred to 1/2 MS medium supplied without (A) or with (B) beta-estradiol (BE) for additional 5 days with the quantitative data in (C). (D–E) Caspase 3/7-like activity in protein extracts from NAC089D-MYC overexpression plants treated with BE (D) or in the wild and NAC089 RNAi plants treated with TM (0.5 µg/ml) (E). The caspase-like activity was normalized to the activity of the wt control under normal condition. Bars depict SE (n = 3) in C–E. ** P < 0.01, * P < 0.05. (F–I) Esterase activities (F–G) and H2O2 accumulation (H–I) in the XVE089D-13 roots as revealed by FDA and DAB staining without (F, H) or with (G, I) BE treatment for 3 days. (J–N) Membrane rigidity and nucleus morphology in the XVE089D-13 roots as reflected by PI (J–K) and DAPI (L–N) staining without (J, L) or with (K, M–N) BE treatment for 5 days. (O–Q) DNA breakage in the XVE089D-13 roots as detected by TUNEL assay without (O) or with (P–Q) BE treatment for 5 days. Bar = 10 mm in A–B and bar = 10 μm in F–Q. doi:10.1371/journal.pgen.1004243.g004
**Figure 5. NAC089 regulates many downstream genes.** Expression of downstream genes identified through microarray analysis was examined with qRT-PCR in two lines of NAC089D-MYC plants. Fold change is the gene expression level of plants treated with beta-estradiol (BE) for 16 hr divided by that of plants treated with DMSO (solvent control), both of which were normalized to the expression of actin. Bars depict SE (n = 3). The fold change for each gene is significant (P < 0.01) in line XVE089D-13. doi:10.1371/journal.pgen.1004243.g005

**Figure 6. Up-regulation of NAC089D-MYC-regulated genes by ER stress is impaired in the NAC089 RNAi plants.** The expressions of NAC089D-MYC downstream genes (A) and other UPR markers (B) were quantified with qRT-PCR in the wild-type control (wt) and NAC089 knock-down plants (line RNAi089-25). Fold change is the gene expression value in TM (5 μg/ml) -treated plants divided by the value in non-treated plants, both of which were normalized to the expression of actin. Bars depict SE (n = 3). Values for the gene locus with # are log2 values. Fold change of BiP1 (AT5g28540, highlighted with 1) also includes that of BiP2 (AT5g42020). ** P < 0.01, * P < 0.05. doi:10.1371/journal.pgen.1004243.g006
stress, pathogen infection, as well as exposure to ultraviolet-C [50,65,66,67,68]. In the current study, chronic ER stress induced caspase 3/7-like activity, and such induction was impaired in the NAC089 knock-down plants. Several NAC089 downstream targets including some known PCD regulators were also indentified in the current study. Among them, one metacaspase (M5) and several other proteases were induced by ER stress, which was suppressed in the NAC089 knock-down plants. BAG (BCL2-associated athanogene) family proteins were originally identified as the anti-cell-death protein in mammals [69]. Among the seven animal BAG homologs found in Arabidopsis [70], overexpression of BAG6 induced PCD in yeast and plants [71], indicating that BAG6 is a pro-death protein in plants. In the current study, BAG6 was induced by ER stress in the Arabidopsis wt plants, which was impaired in the NAC089 knock-down plants. Previously, the soybean NAC transcription factor NAC6/NAC30 was shown to induce caspase 3-like activity and promote extensive DNA fragmentation when it was overexpressed in soybean protoplasts [33]. Here in the current study, we found that one of the direct targets of NAC089, NAC094, is the close-related homolog of soybean NAC6/NAC30 in Arabidopsis. ER stress-induced expression of NAC094 was greatly suppressed in the NAC089 knock-down plants. These results support that NAC089 controls the expression of several PCD-related downstream genes in Arabidopsis under ER stress condition. Interestingly, the autophagy-related gene WRKY33 [55] was also up-regulated by ER stress, which was dependent on NAC089. Other NAC089 downstream genes such as genes encoding protease and nuclease were also identified in the current study. The identification of NAC089 as a PCD regulator provides more opportunities for further understanding new molecular components involved in plant PCD, especially under ER stress condition.

NAC089 is regulated by ER stress at both transcriptional and post-translational levels. At the transcriptional level, NAC089 is up-regulated by ER stress, which is directly controlled by bZIP28 and bZIP60, two important regulators in plant UPR [9,18,44,72]. At the protein level, NAC089 is an ER membrane-associated transcription factor (MTF) and it relocates from the ER to the nucleus under ER stress condition. Interestingly, bZIP28 and bZIP60 are also ER MTFs. bZIP28 is activated through regulated proteolysis. In response to ER stress, bZIP28 relocates from the ER to the Golgi where it is cleaved by two Golgi-resident proteases S1P and S2P, and the C-terminal lumen-facing domain is thought to be responsible for the sensing of ER stress [17,18,19,72,73]. The activation mechanism for bZIP60 is unconventional, and the activation of bZIP60 is dependent on the ER membrane-localized IRE1 proteins. Under ER stress conditions, bZIP60 mRNA is spliced by IRE1, which results in an open reading frame (ORF) shift and elimination of the transmembrane domain [9,11,12]. The N-terminal part of yeast IRE1 is inserted into the ER lumen and plays important role in direct sensing the unfolded proteins in the ER in yeast [74]. Recently, at least 13 MTFs in NAC family are found in Arabidopsis, of which some are activated during development and adaptations to environmental stresses [45,75,76,77,78]. However, the activation mechanisms of these NAC MTFs are still largely unknown. The NAC089 mRNA does not have the predicted double stem-loop structure that has been shown to be very important for IRE1 splicing [9]. Furthermore, there is no alternative spliced transcript of NAC089 observed in the ER stressed wt seedlings (Figure S12), suggesting that NAC089 might be activated in a manner different from bZIP60. The C-terminal ER lumen facing tail of NAC089 is very short and does not have the canonical S1P cutting site, which implicates that NAC089 might not be proteolytically processed in the same way as bZIP28. We did not include protease inhibitors in the NAC089 activation experiments because most of the protease inhibitors are not permeable to live plant cells. Further investigation of the activation mechanism of NAC089 will improve our understanding of MTFs in plants. Surprisingly, one rare nucleotide polymorphism caused by natural variation in the Arabidopsis Cvi ecotype results in premature stop constitutive nuclear localization of NAC089, in which the C-terminus (114 AA) including the hydrophobic tail is not translated. Although Cvi ecotype is much more sensitive to fructose than Ler ecotype, expressing the Cvi NAC089 suppresses fructose sensitivity in Ler seedlings [79]. The truncated form (114 AA deletions) of NAC089 was found in the nucleus, however, the activation or nuclear relocation of NAC089 in response to fructose treatment is not reported, and how the truncated form of NAC089 represses...
fructose signaling is not clear. Since the deletion occurred in Cvi was found neither in over 100 Arabidopsis accessions nor in the Arabidopsis Genome 1001 sequence collections, the biological function of NAC089 in fructose signaling other than in the Cvi ecotype is elusive [79]. Recently, it was reported that fructose feeding induced ER stress in mice [90]. It would be interesting to determine whether the high concentration of fructose could also induce ER stress in Arabidopsis plant. Recently, NAC089 was reported to be involved in redox regulation [81]. Besides its effect on redox status, DTT also inhibits disulfide bond formation and therefore promotes protein misfolding. However, TM is a more specific ER stress inducer because of its specific effect on blocking protein N-glycosylation in the ER. In the current study, both TM and DTT treatments were employed to demonstrate the specific role of NAC089 in ER stress response. Our current study has also advanced the understanding of the function of NAC089 in ER-stress-induced plant PCD and the underlying molecular mechanisms.

How cells make the cell fate selection between life and death remains enigmatic. In human cells, ER stress activates all the three arms of UPR pathways; each branch has different effect on cell survival or cell death, but attenuation of each branch is different. Switch between cell survival and cell death outputs lies in part in the duration of individual branch activity, which guides the cell toward survival or demise [82]. In plants, except the PERK pathway, the bZIP28 and bZIP60 branches of signaling pathway have been previously discovered to regulate downstream genes involved in promoting cell survival [83]. Knock-outs of both branches in the zip28zip60 double mutant causes high sensitivity to ER stresses and accelerated PCD under prolonged ER stress condition [72]. IRE1A and IRE1B redundantly control the activation of bZIP60 and RIDD; knock-outs of both IRE1s in Arabidopsis also promotes PCD while knock-out of single bZIP60 gene has no PCD phenotype [40]. The ER stress-induced up-regulation of NAC089 is dependent on both bZIP28 and bZIP60. Different from the zip28zip60 mutant, knock-down NAC089 confers ER stress tolerance and over-expression of the truncated form of NAC089 promotes PCD. These results may not necessarily be controversial. Firstly, bZIP28 and bZIP60 regulate many survival genes whose expressions are almost completely abolished in the zip28zip60 mutant [72]. Lacking the expression of survival genes in the zip28zip60 mutant may lead to the accelerated PCD. Secondly, NAC089 has substantial constitutive expression and the NAC089 pathway may still operate for PCD in the zip28zip60 mutant even without further NAC089 up-regulation. Thirdly, it is possible to have other PCD pathways turned on to execute PCD in the zip28zip60 mutant. Recently, heterotrimeric G protein signaling [61], vacuolar processing enzyme (VPE)-triggered cell death [42,84] and IRE1-mediated autophagy [85] pathways were reported to be involved in the ER stress-induced PCD in plants.

A hypothetical working model has been emerged from the current study (Figure 8). When Arabidopsis cells are confronted with ER stress, both bZIP28 and bZIP60 pathways are activated to mitigate the stress by up-regulation of genes involved in protein folding or ERAD to improve survival [1,83]. The activated bZIP60 also induces its own transcription [44] and another transcription factor NAC103 [86] to amplify the cell survival signal. The ER-localized IRE1 protects cell through a process called RIDD to reduce the protein folding demand [40]. In the meantime, besides the constitutive high expression of NAC089 under normal condition, both bZIP28 and bZIP60 up-regulate the expression of NAC089 under ER stress condition. Up-regulation of NAC089 mRNA may increase the protein level of the membrane-associated NAC089 precursor. However, nuclear relocation of NAC089 is tightly controlled, in which bZIP28 and bZIP60 may play negative roles. When the ER stress is severe, NAC089 is activated and relocates from the ER to the nucleus, inducing the expression of PCD regulators to promote cell death. It is possible that the stress intensity and/or duration of ER stress might determine the signaling output and the final cell fate. Further understanding on how cells balance the cell survival and cell death effects in UPR is of great fascination.

Materials and Methods

Plant materials, growth conditions and phenotypic analysis

All Arabidopsis (Arabidopsis thaliana) wild-type, T-DNA mutants and transgenic plants in the current study were in the Columbia (Col-0) ecotype background. The double mutant zip28zip60 was made as previously reported [72]. Methods for plant growth were described previously [43]. For short time treatment, different concentrations of TM (5 μg/ml), DTT (2 mM) or BE (10 μM) were added in the half-strength MS medium unless mentioned in the text. For long time treatment, 10 μM BE or various low concentrations of TM were supplied in the solid growth medium. Root length was measured and emergence rate of true leaves was calculated. Total chlorophylls were extracted from seedlings with 80% (v/v) aceton at 4°C overnight and measured from A663 and A646 readings for each sample [87]. All the data in the paper were subjected to Student’s t-test or two-way ANOVA (analysis of variance) analysis.

Plasmid construction and generation of transgenic plants

The coding sequence of NAC089 was amplified with PCR and inserted into pSKM36 after digestion with AclI and SpeI restriction enzymes to produce the vector Pro35S:NAC089. Modified green fluorescence protein (mGFP) tag and 4X MYC tag were amplified and inserted into Pro35S:NAC089 at AclI site to generate the Pro35S:mGFP-NAC089 and Pro35S:MYC-NAC089 constructs, respectively. For RNAi construct preparation, part of the NAC089 gene sequence covering cDNA 651–1150 was inserted into pHANNIBAL in both sense and antisense orientations separated by an intron sequence. The entire RNAi cassette was cut with NdeI and inserted into pART27 to make the RNAi expression vector. To express the dominant negative fusion protein ProNAC089- NAC089D-EAR, the sequence encoding EAR motif (QDDLDLIRLGF) was synthesized and firstly inserted into pCAMBIA1300; about 1 kb upstream sequence of NAC089 and sequence encoding the truncated form of NAC089 (NAC089D, aa 1–316) were amplified and subsequently inserted. To generate the conditionally overexpression construct [48], nucleotides encoding NAC089D was amplified and inserted into pER101M. For dual luciferase activity assay, fragment of the NAC089 promoter (−98 bp to −46 bp relative to the TSS site) was synthesized and inserted into pGreen0800-II after the 35S minimal promoter was introduced. Constructs expressing bZIP28D and bZIP60S were made as described [9,10]. For protein expression in E. coli, the respective sequence of bZIP18D (aa 1–321) or bZIP60T (aa 87–217) was amplified and inserted into pET28 and pET32, respectively. All the primers were listed in Table S1 and error-free clones were introduced into plants by either transient expression or stable transformation.

Protein extraction and immunoblotting analysis

Total protein was extracted from plants with extraction buffer described previously [17]. Membrane fraction and nuclear fraction were prepared with sucrose gradient centrifuge according to the
standard protocol [88]. Proteins were resolved on 8–10% SDS-PAGE gels and visualized by western blotting using antibodies against c-MYC (Santa Cruz Biotechnology), nuclear protein marker histone H3 (Abcam) and ER protein marker BiP (Santa Cruz Biotechnology).

Caspase-like activity and luciferase reporter assays

Caspase-like activity was measured with luminescent assays based on DEVD short peptides with Caspase-Glo 3/7 Assay Kit (Promega). Both caspase 3 and caspase 7 recognize the same DEVD substrate. Briefly, seedlings were harvested after various treatments and total proteins were extracted with liquid nitrogen in a buffer containing 100 mM sodium acetate, pH 5.5, 100 mM NaCl, 1 mM EDTA, and 5 mM DTT. To measure caspase-3/7 activity, 30 μl caspase-3/7 luminescent substrate (Z-DEVD-aminoluciferin) was added to 50 μg protein extracts and incubated at 22°C for 1 hr protected from light. The luminescence of each sample was measured with the Synergy 2 Multi-Mode Microplate Reader (BioTek). For dual-luciferase activity assays [89], Arabidopsis leaf protoplasts were isolated from 4-week-old soil-grown seedlings and transfected according to a standard protocol [43] with various reporter constructs or cotransfected with different effectors. Firefly and renilla luciferase were quantified with Dual-Luciferase Reporter Assay Kit (Promega) according to the manufacturer’s instructions in the Synergy 2 Multi-Mode Microplate Reader (BioTek).

Histochemistry and microscopy

For FDA and DAB staining, seedlings were stained with 2.5 mg/ml FDA (Sigma-Aldrich) in phosphate-buffered saline for 10 min or 1 mg/ml DAB (pH 5.5, Sigma-Aldrich) for 2 hr at room
temperature, immersed into boiled ethanol for 10 min according to the standard protocol [31]. For PI and DAPI staining, samples were stained using DAPI (Sigma-Aldrich) at 1 μg/ml in 0.1% (v/v) Triton X-100 for 10 min or PI (Sigma-Aldrich) at 10 μg/ml for 1 min, and washed twice with water. For in situ TUNEL staining, roots were stained in microcentrifuge tubes (1.5 ml) using the in situ cell death detection kit (Takara) according to the manufacturer’s instructions. Except DAB staining, which was observed under differential interference contrast (DIC) microscopy, other staining, BiFC and subcellular localization of mGFP-NAC089 were visualize with laser confocal fluorescence microscopy (Zeiss LSM A710).

Chromatin immunoprecipitation (ChIP) and gene expression analysis

ChIP was performed according to the standard protocols. Briefly about 3 g of 2-week-old XTEE099D transgenic seedlings were treated with either 10 μM BE or DMSO (solvent control) for 16 hr and fixed with 1.0% formaldehyde for 10 min subsequently. Antibodies against c-MYC (Santa Cruz Biotechnology) and GST (AgG control, Abmart) were used for immunoprecipitation. Protein-A-agarose beads were blocked with salmon sperm DNA and used to pull down the protein-DNA complex. Equal amounts of starting plant material and the ChIP products were used for quantitative PCR. Primers were selected in the promoter regions of each selected gene. DNA levels were calculated relative to TAJ (AT1G37110) using a comparative threshold cycle method. The ChIP experiments were performed 3 times with biological replications and similar results were obtained. For microarray analysis or qRT-PCR, the wt were obtained. For microarray analysis or qRT-PCR, the wt was grown vertically on agar plates for one week and then transferred to 1/2 MS liquid supplied with 10 μM BE or DMSO or TM for a period of time as noted. Total RNA was extracted and purified according to the manufacturer’s instructions [43]. Agilent Arabidopsis gene chips were used for microarray analysis. The predicted ER stress responsive cis-elements were selected in the promoter regions of each selected gene. DNA levels were calculated relative to TAJ (AT1G37110) using a comparative threshold cycle method. The ChIP experiments were performed 3 times with biological replications and similar results were obtained. For microarray analysis or qRT-PCR, the wt control, XTEE099D and NAC089 RNAi plants were grown vertically on agar plates for one week and then transferred to 1/2 MS liquid supplied with 10 μM BE or DMSO or TM for a period of time as noted. Total RNA was extracted and purified according to the manufacturer’s instructions [43]. 

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a LightShift Chemiluminescent EMSA Kit (Pierce), according to the manufacturer’s protocols [43]. Briefly, each 20 μl binding reaction contained 2 μl binding buffer, 0.3 μl Poly (di-dC), 4 μg purified protein, 0.83 μmol biotin-labeled probe or certain amount of unlabeled probe as the competitor. The pNAC089 wt or pNAC089M1-M3 probes were created by annealing together complementary oligonucleotides and biotinylated with a labeling kit (Pierce). His-tagged bZIP28D or Trx-His-tagged bZIP60T proteins were expressed in E. coli strain BL21 and purified with Ni-NTA agarose beads (Qiagen). The binding reactions were allowed to incubate on ice for 1 hr and run on a 5% polyacrylamide mini-gel (37.5:1 acrylamide-bisacrylamide in 0.5x Tris-Borate-EDTA (TBE) containing 3% glycerol). The complex was transferred to a membrane and developed according to a standard protocol.

Supporting Information

Dataset S1 List of genes regulated by conditional overexpression of NAC089D-MYC.
(XLS)

Figure S1 NAC089 is up-regulated by ER stress. (A–B) Up-regulation of NAC089 by tunicamycin (TM, A) and dithiothreitol (DTT, B) was examined in the dose-response experiments. The expression of NAC089 is normalized to the expression of the internal control actin. The wild-type Arabidopsis seedlings were stressed for 4 hr. Bars depict SE (n = 3).

Figure S2 bZIP28 and bZIP60 bind to UPRE but not to ERSE-I-like cis-element. (A) DNA sequences of biotin-labeled probes. The predicted ER stress responsive cis-elements UPRE and ERSE-I-like were highlighted in bold. The mutated sites were underlined. (B–C) EMSA for protein and DNA interactions. Either the purified His-bZIP28D (B) or Trx-His-bZIP60T (C) was incubated with various biotin-labeled DNA. Arrows and arrow heads point to the positions of shifted bands and free probes, respectively.

Figure S3 NAC089 has transcriptional activation activity and forms homodimers. (A) Schematic structure of NAC089 protein. DBD: DNA binding domain; TMD: transmembrane domain. (B) Transcriptional activation activity of NAC089. Segment of NAC089 lacking the TMD (NAC089D) was fused to yeast GAL4 DNA binding domain and the activation of HIS and LacZ reporters were evaluated. (C–F) Evaluation of dimmer formation in yeast 2-hybrid assays (C–D) and BiFC assays (E–F). Bar = 50 μm.

Figure S4 NAC089 is enriched in the membrane fraction or nuclear fraction depending on different conditions. Plant seedlings were treated with H2O (control), TM or DTT for 6 hr and the used for protein fractionation studies. Anti-Histone and anti-BiP antibodies were employed to detect the nuclear protein marker Histone H3 and ER protein marker BiP, respectively. The beta-estradiol (BE) induced truncated form NAC089D-MYC was used as the migration marker.

Figure S5 NAC089 is specifically knocked-down in the RNAi plants. (A–B) The expression of NAC089 (A) and its close-related homologs NAC060 and NAC040 (B) in the NAC089 knock-down plants. The relative gene expression in the transgenic plants is the value normalized to the expression in the wild-type control (wt), both of which are normalized to the internal control actin. Bars depict SE (n = 3).

Figure S6 Partial loss-of-function of NAC089D enhances ER stress tolerance. (A–D) The C-terminal hydrophobic region of NAC089 was replaced by the transcriptional repressor domain EAR and the fusion protein was validated by RT-PCR in Arabidopsis plants (A). Photos of 12-day-old Arabidopsis grown on 1/2 MS medium supplied without or with different concentration of TM were taken (B). Emergence rate of true leaves was counted (C) and total chlorophyll content of seedlings was measured (D). The wild-type (wt) and zip28zip60 double mutant were used as the controls. Bars depict SE (n = 3). (E) Expression of UPR marker genes in the wild-type plants after growing on 1/2 MS medium supplied without or with different concentration of TM for 12 days. UBQ5 was used as an internal control.

Figure S7 NAC089D-MYC is expressed in the overexpression plants. Validation of transgenic expression in two lines of NAC089D-MYC overexpression plants by western blotting. Coomassie blue

PLOS Genetics | www.plosgenetics.org 12 March 2014 | Volume 10 | Issue 3 | e1004243
staining of RbS serves as a loading control for western blotting. Plant seedlings were treated with beta-estradiol (BE) for 16 hr. (TIF)

**Figure S8** Beta estradiol has little effect on histochemical staining in the wild-type control plants. (A-D) Esterase activities (A-B) and H$_2$O$_2$ accumulation (C-D) in the wild-type control roots as revealed by FDA and DAB staining without (A, C) or with (B, D) beta-estradiol (BE) treatment for 3 days. (E-H) Membrane rigidity and nucleus diffusion in the wild-type control roots as reflected by PI (E-F) and DAPI (G-H) staining without (E, G) or with (F, H) BE treatment for 5 days. (I-J) DNA breakage in the wild-type control roots as detected by TUNEL assay without (I) or with (J) BE treatment for 5 days. Bar = 10 µm. (TIF)

**Figure S9** Beta estradiol does not induce NAC089 downstream genes in the wild-type control plants. Wild-type control (wt) and XVE089D-MYC plants were treated with DMSO (control) or beta-estradiol (BE) for 16 hr and gene expressions were checked with RT-PCR. *UBQ5* was used as an internal control. (TIF)

**Figure S10** Transcription factor activity is enriched in the NAC089 downstream genes. Gene Ontology (GO) analysis was performed with NAC089D-MYC induced genes in *agriGO*. (TIF)

**Figure S11** Some of the NAC089D-MYC-regulated genes are up-regulated by ER stress. Totally 23 genes were selected from the microarray experiment and their expressions were examined with qRT-PCR. The wild-type plants were treated with tunicamycin for 4 and 12 hr and the expression of NAC089 target genes was also quantified with qRT-PCR. The relative gene expression is the value in the treated sample normalized to the untreated control, both of which are normalized to the expression of *actin*. Bars depict SE (n = 3). (TIF)

**Figure S12** NAC089 is not alternatively spliced under ER stress condition. (A) Gene model of *NAC089*. White rectangles represent UTRs and black rectangles denote exons. The region encoding the transmembrane domain of NAC089 is boxed. (B) Detection of *NAC089* transcript with RT-PCR. The wild-type plants were treated with H$_2$O (control), 5 µg/ml tunicamycin (TM) or 2 mM DTT for 4 hr and the expression of *NAC089* was examined by RT-PCR with different primer pairs. *UBQ5* was used as a loading control. (TIF)

**Table S1** Primers used in the study. (DOC)

**Author Contributions**
Conceived and designed the experiments: ZTY MJW LiS SJL JXL. Performed the experiments: ZTY MJW LiS DLB LeS ZTS SSZ SFZ. Analyzed the data: ZTY MJW LiS SJL JXL. Wrote the paper: JXL.

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