Calcium pumps and interacting BON1 protein modulate calcium signature, stomatal closure, and plant immunity

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JH and DLY conceived the project; DLY, ZS, YB, JY and JH designed the experiments, analyzed the data and made the figures; DLY, ZS, YB, JY, ZY, HY, YL, MG, SW, BZ, DX, ZM and JK conducted experiments; JH and DLY wrote the paper.
One-sentence summary:

Calcium pumps ACA10 and ACA8 and their interacting protein BON1 regulate calcium signatures and impact stomatal movement and plant immunity in Arabidopsis.
Abstract

Calcium signaling is essential for environmental responses including immune responses. Here we provide evidence that the evolutionarily conserved protein BONZAI1 (BON1) functions together with autoinhibited calcium ATPase 10 (ACA10) and ACA8 to regulate calcium signals in Arabidopsis. BON1 is a plasma membrane localized protein that negatively regulates the expression of immune receptor genes and positively regulates stomatal closure. We found that BON1 interacts with the autoinhibitory domains of ACA10 and ACA8, and the aca10 loss of function (LOF) mutants have an autoimmune phenotype similar to that of the bon1 LOF mutants. Genetic evidences indicate that BON1 positively regulates the activities of ACA10 and ACA8. Consistent with this idea, the steady level of calcium concentration is increased in both aca10 and bon1 mutants. Most strikingly, cytosolic calcium oscillation imposed by external calcium treatment was altered in aca10, aca8, and bon1 mutants in guard cells. In addition, calcium and pathogen induced stomatal closure was compromised in the aca10 and bon1 mutants. Taken together, this study indicates that ACA10/8 and BON1 physically interact on plasma membrane and function in the generation of cytosol calcium signatures that are critical for stomatal movement and impact plant immunity.
Introduction

Calcium ion (Ca\(^{2+}\)) is an important cellular second messenger for diverse developmental processes and environmental responses in both plants and animals (Lewis, 2001; Dodd et al., 2010; Kudla et al., 2010). The extreme low calcium concentration in cytosol (100-200 nM) creates a unique environment where calcium concentration can be regulated dynamically (Bush, 1995). Increases of calcium transient in cytosol are activated in various environmental and developmental processes including root growth, stomatal movement, pollen growth, abiotic stress responses and plant-microbe interaction. Calcium spiking with unique magnitude, frequency, shape and duration in response to environmental and endogenous cues is referred as “calcium signature” and is thought to encode stimulus-specific information. It is shown that, in the guard cell where calcium is a key signal for stomatal movement control (Murata et al., 2015), calcium oscillation is indeed essential for stomatal closure (Allen et al., 2000).

The stimulus-specific calcium signature is thought to be generated by coordinated action of various Ca\(^{2+}\) influx and efflux systems including channels, pumps, and exchangers located at different cellular membranes. However, the molecular identities of calcium specific influx channels remain controversial (Steinhorst and Kudla, 2013). Plasma membrane (PM) localized cyclic nucleotide-gated channels (CNGCs), glutamate receptor like (GLR) and TWO-PORE CHANNEL (TPC) are considered as main channels that release Ca\(^{2+}\) in plant (Dodd et al., 2010; Kudla et al., 2010). Extrusion of Ca\(^{2+}\) from cytosol to external cell and intercellular store compartment is believed to be achieved by Ca\(^{2+}\)-ATPases (Ca\(^{2+}\) pumps) and Ca\(^{2+}\)/H\(^{+}\) antiporters (CAXs) driven by ATP and proton motive force, respectively (Geisler et al., 2000; Sze et al., 2000; Shigaki and Hirschi, 2006). Ca\(^{2+}\) pumps fall into two groups: type IIA or ECA for ER type Ca\(^{2+}\)-ATPases and type IIB or ACA for autoinhibited Ca\(^{2+}\)-ATPase (Geisler et al, 2000). ACAs
have an N-terminal calmodulin-binding autoinhibitory domain that inhibits the ATPase activity in the C-terminus and they are localized to diverse membrane compartments (Sze et al., 2000; Boursiac and Harper, 2007). Calcium pumps in vacuole, ER, and nucleus were found to be important for calcium signal generation in response to environmental stimuli. The loss of function (LOF) of PCA1, a vacuole localized ACA, resulted in sustained rather than transient elevated Ca$^{2+}$ in cytosol under salt treatment in the moss *Physcomitrella patens* (Qudeimat et al., 2008). In tobacco, knock-down of NbCA1, an ER localized ACA, greatly increased the amplitude and duration of calcium spikes induced by cryptogein (Zhu et al., 2010). The LOF of MCA, a nucleus localized EAC, resulted in greatly reduced nuclear calcium spiking in response to Nod factor in *Medicago truncatula* (Capoen et al., 2011). The involvement of PM localized calcium pumps in calcium signal is not known yet in plants.

Calcium signaling has recently been genetically connected with plant immunity. Distinct calcium signatures are rapidly induced upon pathogen invasion (Lecourieux et al., 2006; Ma and Berkowitz, 2007). Disruption of putative calcium channels such as PM-localized CNGCs and GLR either enhance or compromise plant immune responses (Clough et al., 2000; Ford and Roberts 2014). The PM-localized calcium pumps ACA8 and ACA10 are found to be associated with immune receptor FLS2 and their LOF mutants were susceptible to bacterial pathogens (Freid Frey et al., 2012). Multiple calcium binding proteins are also involved in plant immunity regulation. For instance, a calmodulin (CaM)-binding protein MLO negatively regulates resistance to powdery mildew in barley (Kim et al., 2002), and a Ca$^{2+}$- and CaM-binding transcription factor AtSR1/CAMTA3 is a negative regulator of salicylic acid (SA)-dependent defense responses in Arabidopsis (Du et al., 2009). A CaM gene in *Nicotiana benthamiana* (*N. benthamiana*) is required for immune responses triggered by silencing of the ER calcium pump.
CA1 (Zhu et al., 2010), and overexpression of a pepper CaM gene induces programmed cell death (PCD) and enhances disease resistance (Choi et al., 2009). A chloroplast calcium regulated protein CAS also plays an important role in plant immunity (Nomura et al., 2012). In addition, calcium-dependent protein kinases (CDPK or CPK) are critical regulators of plant immune responses both to pathogen-associated molecular patterns (PAMP) and effectors (Boudsocq and Sheen, 2013). Four CDPKs (CPK4/5/6/11) are found to be critical for transcriptional reprogramming and ROS production in responses to PAMPs (Boudsocq et al., 2010). CPK1/2/4/5/6/11 are shown to be involved in downstream events including transcriptional reprogramming and reactive oxygen species (ROS) production after activation of plant immune receptor NLR (NOD1 like Receptors) genes in response to pathogen effectors (Gao et al., 2013). Recently, CPK28 is shown to phosphorylate BIK1, a substrate of multiple PAMP receptors, and therefore attenuating PAMP signaling (Monaghan et al., 2014).

One intriguing component involved in calcium signaling and plant immunity is the Arabidopsis \textit{BON1} gene. \textit{BON1} is a member of an evolutionarily conserved copine family found in protozoa, plants, nematodes, and mammals (Creutz et al., 1998). The copine proteins have two calcium-dependent phospholipid-binding C2 domains at their amino (N)-terminus and a putative protein-protein interaction VWA (von Willebrand A) or A domain at their carboxyl (C)-terminus (Rizo and Sudhof, 1998; Whittaker and Hynes, 2002). The \textit{BON1} protein resides on the PM mainly through myristoylation of its second residue glycine (Hua et al., 2001; Li et al., 2010). Mutating residue glycine (G) 2 to alanine (A) abolishes PM localization of \textit{BON1} and results in the loss of \textit{BON1} activity (Li et al., 2010). \textit{BON1} has conserved aspartate residues important for calcium binding in the two C2 domains. Mutating all these Asp residues in either C2A or C2B domains abolishes \textit{BON1} activity in rescuing the \textit{bon1-1} defects indicating that calcium binding
is required for BON1 function (Li et al., 2010).

BON1 is a negative regulator of plant immune responses and a positive regulator of stomatal closure response. In the LOF mutant bon1-1 in Col-0 background, a plant immune receptor NLR gene \textit{SNC1} (\emph{suppressor of NPR1, constitutive 1}) is increased at transcript level through initial upregulation followed by SA-mediated amplification (Yang and Hua, 2004; Li et al., 2007). The \textit{bon1-1} mutant thus has constitutive defense (autoimmune) responses and is consequently dwarf. However, these defects observed is dependent on a Col-0 specific NB-LRR gene \textit{SNC1}, and the LOF \textit{bon1-2} allele in the Wassilewakija (Ws) accession does not exhibit constitutive defense activation because there is no functional \textit{SNC1} in Ws (Yang and Hua, 2004). In addition to the function in negatively regulating \textit{SNC1}, \textit{BON1} is recently shown to promote stomatal closure in response to stimuli including abscisic acid (ABA) and bacterial pathogen (Gou et al., 2015). This function is independent of \textit{SNC1} or NB-LRR signaling, and the \textit{bon1} mutant is defective in stomatal closure when autoimmunity is suppressed by the \textit{SNC1} LOF mutation \textit{snc1-11} (Gou et al., 2015). Therefore, \textit{BON1} has a more general role in regulating signaling events in addition to NLR gene expression.

It is intriguing that an evolutionarily conserved calcium binding copine protein can regulate stomatal closure and impact NLR gene expression. Here we identified calcium pumps ACA10 and ACA8 as interacting proteins of BON1 both physically and genetically. The LOF mutants of these genes have altered calcium signature and calcium homeostasis and are defective in stomatal closure and plant immunity. Thus we have uncovered a critical role for PM-localized calcium pumps ACA10/8 in calcium signature generation as well as their regulation by an evolutionarily conserved BON1 protein in Arabidopsis.
Results

BON1 and the calcium ATPase ACA10 interact physically

To further understand how BON1 functions in immunity, we searched for BON1 co-expressed genes as they might function together with BON1. Among the top 300 genes with high co-expression values in the ATTED-II database (Obayashi et al., 2009), 33 genes were annotated as calcium binding proteins including calcium channels, pumps, calmodulin and calcium dependent kinases (Supplementary Table 1). Five of them encode calcium efflux pumps including ACA1, ACA2, ACA10, ACA11 and ACA1. Previously, ACA1 was identified as a putative BON1 interacting protein by BON1 immunoprecipitation followed by Mass Spectrometry (Gou et al., 2015), suggesting that BON1 could physically interact with ACA proteins. Because ACA1, 2 and 11 are localized to plastid envelope, ER, and vacuole respectively (Huang et al., 1993; Harper et al., 1998; Lee et al., 2007), we chose the PM-localized ACA10 for further analysis because BON1 is PM localized. This is also due to the fact that the loss of function (LOF) mutant of ACA10 in the Nossen-0 (No-0) accession had a phenotype reminiscent to that of bon1 (see below section).

We first analyzed potential physical interaction between BON1 and ACA10. We were not able to clone the full length of ACA10 cDNA without mutations in E. coli, which was also reported for the full-length cDNAs of ACA9, the close homologue of ACA10 (Schiott et al., 2004). We therefore used a cDNA/genomic DNA chimera (where the genomic fragment is used after the 22nd exon) for ACA10 for expression analysis. When expressed in N. benthamiana, the ACA10-GFP fusion protein showed signals only on the PM (Supplementary Fig. 1), confirming that ACA10 is indeed a PM-localized protein (Bonza et al., 2000; Schiott et al., 2004). Bimolecular Fluorescence Complementation (BiFC) (Bracha-Drori et al., 2004) assays were used.
to determine the interaction of BON1 and ACA10 in *N. benthamiana*. Strong fluorescence signals were observed when both the ACA10 fusion with N-terminal of YFP and the BON1 fusion with C-terminal of YFP were expressed (Fig. 1A; supplemental Fig. 2); and the signals
were on the PM where both BON1 and ACA10 are localized (Supplemental Fig. 1A). The positive BiFC signal of ACA10 with BON1 is not a non-specific interaction of BON1 with any PM protein, as another PM protein OPT3 (oligopeptide transporter 3) did not give a positive signal when co-expressed with BON1 (Fig. 1A and supplemental Fig. 2).

We subsequently used BiFC assay to test which segment of ACA10 (Supplemental Fig. 1B) interacts with BON1, and found that BON1 interacts with the N-terminal segment I that includes the autoinhibited domain of the ACA10 (Fig. 1B). Four other segments (II to IV) were also analyzed, but no positive signals were observed (Fig. 1B). Further test will determine their expression levels in order to determine whether or not these segments interact with BON1 in addition to segment I. The interaction between BON1 and the segment I of ACA10 was analyzed further by split–LUC (luciferase) assay (Fig. 1C). Co-expression of the fusion of BON1 and the N-terminal LUC (BON1-NLUC) with the fusion of C-terminal LUC and segment I of ACA10 (CLUC-ACA10I) in N. benthamiana gave a strong luminescence signal from the LUC activity, while the controls without co-expressing the two proteins showed no luminescence (Fig. 1C). This interaction was further verified by the yeast two hybrid (Y2H) assay where the C-terminal A domain of BON1 (BON1-A) was found to interact with the segment I of ACA10 (Fig. 1D). The full-length BON1 did not exhibit interaction with segment I (Fig. 1D), likely because the N-terminal part of BON1 targets to the PM and inhibits the fusion protein to go to the nucleus to activate gene expression. Because the segment I of ACA10 contains the putative autoinhibitory motif that confers auto-inhibition of many ACA proteins (Bonza et al, 2000; Curran et al., 2000; Geisler et al., 2000), BON1 may regulate ACA10 activity by binding to this motif or its nearby sequences.
ACA10 mutant in the Nossen-0 (No-0) background has an autoimmune phenotype

We found that ACA10, like BON1, is a negative regulator of plant immunity. An aca10 mutant in No-0 accession, named cif1-1, was reported to have a compact inflorescence (George et al., 2008). This aca10 mutant allele will be referred as aca10-cif1 to be consistent with other mutant names. We suspected that the growth phenotype of aca10-cif1 is at least partially due to constitutive activation of immune responses induced by plant immune receptor NB-LRR genes based on the following observations. Under long day growth condition, the young leaves of aca10-cif1 display water soaked phenotype that is similar to the autoimmune mutant bon1-1 (Fig. 2A; Yang and Hua 2014). In addition, the compact inflorescence phenotype of aca10-cif1 is reminiscent of that of bon1 bon2 bon3 pad4 mutant (Yang et al., 2006). Phenotype of water-soaked leaf was suppressed by a higher growth temperature of 28°C (Fig. 2A), reminiscent of the suppression of NB-LRR mediated plant defense responses by an elevated temperature (Wang and Hua, 2009). Furthermore, the aca10-cif1 phenotype is only present in No-0 but not in Col-0 or Ws, which was due to an accession difference at the CIF2 locus (George et al., 2008). The CIF2 gene is not yet cloned, and it is likely to be a NB-LRR gene as the accession specificity is often the property of such genes (Noel et al., 1999; Clark et al., 2007).

We therefore assayed the growth of virulent pathogen Pseudomonas syringe pv tomato (Pst) DC3000 in aca10-cif1. The bacterial growth was reduced by 10 times in the aca10-cif1 mutant compared to that in the wild-type No-0 plants at 22°C but not 28°C (Fig. 2B). This enhanced resistance likely results from a constitutive upregulation of defense responses at 22°C. The defense marker gene PRI, which is induced by pathogen in the wild type, is upregulated in aca10-cif1 in the absence of pathogen inoculation and this upregulation of PRI expression was observed at 22°C but not at 28°C (Fig. 2C). To further test the hypothesis that growth defect is
due to activation of NB-LRR mediated defense, we introduced in aca10-cif1 mutant the LOF mutation in the PAD4 gene that plays an important role in such a defense (Wiermer et al., 2005).

The pad4-1 mutant in Col-0 background was crossed to aca10-cif1 in No-0 and F2 plants were
genotyped at the \textit{ACA10}, \textit{CIF2}, and \textit{PAD4} loci. All plants with genotypes of \textit{aca10-cif1 cif2-n} (No-0 allele of \textit{CIF2}) exhibited water-soaked leaf phenotype while all \textit{aca10-cif1 cif2-n pad4} plants exhibited a wild-type phenotype (Fig. 2D). The suppression of \textit{cif1} mutant phenotype by \textit{pad4} is further confirmed by analyzing progenies from F2 plants with \textit{aca10-cif1 cif2-n pad4/+} genotype. In addition to the growth phenotype, \textit{pad4} mutation also reduced \textit{PR1} expression in \textit{aca10-cif1} (Fig. 2E). Taken together, ACA10 is a negative regulator of disease resistance in a \textit{PAD4}-dependent manner in the No-0 accession and constitutive immune responses result in growth retardation in \textit{aca10-cif1} mutant.

\textbf{ACA10 and ACA8 are negative regulators of plant immune responses}

Earlier study reported that ACA8, the closest homolog of ACA10, interacts with a PAMP receptor FLS2 and is a positive regulator of plant immune response (Freidt Frey et al., 2012). It was also reported that, using surface inoculation method in which bacteria enter the plants through stomata, the \textit{aca8}, \textit{aca10}, and \textit{aca10aca8} mutants in the Col-0 background were more susceptible to virulent pathogen \textit{Pst} DC3000 compared to the wild type Col-0 (Freidt Frey et al., 2012). However, our characterization of the \textit{aca10-cif1} mutant as well as the genetic and physical interaction of ACA10 and BON1 indicate that ACA10 is a negative regulator of immune responses. To investigate this discrepancy, we analyzed the disease phenotype of \textit{aca10} and \textit{aca8} mutants in the Col-0 background. The single LOF mutants of \textit{aca10-2} or \textit{aca8-2} in Col-0 did not exhibit obvious growth defects in early developmental stage, but the double mutant had water-soaked leaves and exhibited dwarfism (Fig. 3A, B). When analyzed for resistance to the virulent pathogen \textit{Pst} DC3000, the \textit{aca10-2} and the \textit{aca10-2aca8-2} mutants consistently exhibited enhanced resistance compared to the wild-type Col-0 (Fig. 3C, D, E). Using syringe infiltration
and dipping methods, aca10-2 but not aca8-2 had reduced pathogen growth while aca10 aca8 mutant had a further reduction of pathogen growth compared to the wild-type Col-0 (Fig. 3C, D).

We subsequently used the same spray method as described in the prior study (Frei dit Frey et al.,...
2012) for inoculation, and found a smaller but significant reduction of pathogen growth in the aca10-2 and aca10-2aca8-2 mutants (Fig. 3E). We could not explain the discrepancy between our result and the prior result by different inoculation methods or plant growth conditions although we cannot exclude the possibility that a difference in an undefined plant growth condition or pathogen preparation caused a difference in disease resistance. We conclude that ACA10 and ACA8 are negative regulators of defense responses to bacterial pathogen Pst DC3000 from our analysis of their mutants. This is supported by the upregulation of a defense response marker gene PRI in the aca10 and aca10aca8 mutants compared to the wild-type Col-0 (Fig. 3F).

Mutants of ACA10 and BON1 have synergistic interactions

The physical interaction of BON1 and ACA10 proteins as well as a similar phenotype in immunity indicates that they might function together. To further test this hypothesis, we constructed double mutant of aca10 and bon1 in the Ws background as neither of the LOF mutants aca10-1 nor bon1-2 in the Ws background exhibited visible developmental defects (Fig. 4A and Supplemental Fig. 3A). The double mutant had smaller, curled and water soaked leaves and produced a more compact inflorescence compared to the wild type or the single mutants at 22 but not 28°C (Fig. 4A and Supplemental Fig. 3A-D). Pathogen growth assay demonstrated that the disease resistance was increased in the aca10-1bon1-2 double mutant compared to wild-type and single mutant under 22 but not 28°C (Fig. 4b). The aca10-1bon1-2 double mutant had a significant increase of PRI expression compared to wild-type and the single mutants at 22 but not 28°C (Fig. 4C). All the defects in the double mutant were suppressed by either pad4 or eds1. The two triple mutants aca10-1 bon1-2 eds1-1 and aca10-1 bon1-2 pad4-5, in contrast to aca10-
A bon1-2, had a wild-type leaf and inflorescence morphology (Fig. 4D and Supplemental Fig. 3E). These triple mutants also had the wild-type level of disease resistance and PR1 upregulation (Fig. 4E, F). Thus, the aca10-1 and bon1-2 has synergistic effect on immune responses in the Ws background.

**Figure 4**: Genetic interaction between BON1 and ACA10 in Wassilewskija (Ws) accession background.

A. Growth phenotypes of wild-type Ws, aca10, aca8, and aca10 aca8 in Ws background at 22°C and 28°C.

B. Pst DC3000 bacterial growth in above genotypes assayed by the syringe infiltration method.

C. PR1 expression level in above genotypes grown at 22°C and 28°C by RNA blotting. Same amount of total RNAs were loaded on gels and the blots were hybridized at the same time.

D. Growth phenotypes of aca10 aca8 mutants and their eds1 and pad4 combination mutants grown at 22°C.

E. Growth of Pst DC3000 in genotypes in D assayed by the syringe infiltration method.

F. PR1 expression level in genotypes in D assayed by real-time RT-PCR. Different letters indicate statistical difference (P-value <0.001 by Bonferroni test) among genotypes.

Genotypes: a/b=aca10-1 bon1-2, a/b/eds1-1=aca10-1 bon1-2 eds1-1, a/b/pad4-5=aca10-1 bon1-2 pad4-5.

Letters in (B) and (D) indicate statistical difference (P-value <0.001 by Bonferroni test) among genotypes.
background. We hypothesize that NLR genes other than SNC1 are activated in the bon1-2 aca10-
I mutant in the Ws background similar to SNC1 activation in the bon1-1 mutant in the Col-0
background.

Overexpression of BON1 suppressed the aca10-cif1 but not the aca10aca8 defects

The synergistic genetic interaction between BON1 and ACA10 single mutants is not
apparently consistent with the hypothesis that BON1 and ACA10 function in the same protein
complex and/or regulate each other in a signaling pathway. However, this can be explained if
members of the BON1 or the ACA10 family each have similar functions. In this case, over-
expression of one family member of the upstream component might be able to compensate for
the loss of one but not all family members of the downstream component. To test this hypothesis,
we overexpressed BON1 in aca10-cif1 mutant. More than 20 transgenic lines were generated,
and majority of the lines showed a rescued phenotype with wild-type leaf and inflorescent
morphology (Fig. 5A and Supplemental Fig. 4A). In addition, bacterial growth was increased and
the PRI expression was reduced in BON1-OE/aca10-cif1 compared to aca10-cif1 (Fig. 5B, C).

In contrast, when we overexpressed BON1 in the double mutant aca10aca8, none of the over
10 transgenic lines of BON1-OE/aca10aca8 exhibited any difference from the aca10aca8 mutant
(Fig. 5D). To exclude the possibility that the non-rescue was due to lower expression of BON1 in
aca10aca8 than in aca10-cif, we analyzed the BON1 protein level by protein blot. BON1 protein
level was in general lower in BON1-OE/aca10aca8 than in BON1-OE/aca10-cif1, however, one
BON1-OE/aca10-cif1 line exhibiting a rescued phenotype had a lower expression than several
BON1-OE/aca10aca8 lines with a non-rescued phenotype (Supplemental Fig. 4B). To confirm
the non-rescue phenotype in aca10aca8, we crossed a high BON1-expression line in aca10-cif1

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(in No-0) with aca10aca8 (in Col-0) and analyzed the F2 progenies. The aca10aca8 plants (now in mixed No-0 and Col-0 background) exhibited a similar growth defect irrespective of the presence of the BONI-OE transgene. The non-rescuing of aca10aca8 defects by BONI-OE was
further confirmed in the F3 progenies of several lines of BON1-OE/aca10aca8 in mixed background (Fig. 5E). Therefore, BON1-OE rescues the defects of aca10 single mutant but not the aca10 aca8 double mutant, which suggests that BON1 functions upstream of both ACA10 and ACA8.

**ACA8 and BON1 have physical interaction**

We subsequently tested the hypothesis that BON1 physically interacts with ACA8 as well as ACA10. In the BiFC assay in N. benthamiana, co-expression of the full length ACA8 and BON1 exhibited a strong fluorescence signal while co-expression of BON1 and a control PM protein did not (Fig. 6A, supplemental Fig. 2). The interaction between ACA8 and BON1 was verified by a positive signal when the N-terminal segment I of ACA8 and BON1 were co-expressed in the split-LUC assay (Fig. 6B). Therefore, both ACA8 and ACA10 interact with BON1 and the BON1-interacting domains in ACA8 and ACA10 are localized in the same region of the protein.

**ACA10, ACA8 and BON1 affect calcium homeostasis and calcium signals**

Because ACA10 and ACA8 are calcium pumps, we hypothesize that the loss of the pump function or its regulation will alter calcium homeostasis. To test this hypothesis, we monitored calcium homeostasis and signature in plant cells using a FRET reporter Yellow Cameleon (YC). A plant version of this calcium sensor YC3.6 under the control of the constitutive 35S promoter (Yang et al., 2008) was introduced into aca10-2, aca8-2, and bon1-1 mutants. Steady levels of calcium were measured in guard cells where YC3.6 has a strong expression. Calcium concentration was measured higher in the aca10 mutants compared to the wild type in both the
Ws and No-0 backgrounds (Fig. 7A), which is consistent with earlier report based on another calcium reporter (Frei dit Frey et al., 2012). Interestingly, the bon1 mutants also exhibited an increase of calcium at steady status in both Ws and Col-0 backgrounds (Fig. 7A).
Furthermore, cytosolic calcium signals generated in response to imposed calcium were altered in the aca10 and bon1 mutants. In wild-type Col-0, an external application of 10 mM calcium rapidly induced cytosolic calcium oscillation in guard cells (Fig. 7B) as reported earlier.
(Allen et al., 2000; Hubbard et al., 2012). In aca10-2, aca8-2, and bon1-1 LOF mutants, only one calcium spike was observed, and no more calcium peaks followed (Fig. 7B). Moreover, the decreasing of calcium level from the first spike in the three mutants was drastically delayed compared to the wild-type Col-0 (Fig. 7B). Therefore, the initial influx of calcium ions happened normally but the calcium oscillation pattern was lost. This observation indicates an essential role of BON1, ACA10 and ACA8 in cytosolic calcium oscillation and supports the hypothesis that BON1 functions closely with ACA10 and ACA8 in regulating calcium signature.

ACA10, ACA8 and BON1 regulate stomatal movement

Calcium signaling is critical in controlling stomatal movement (Kim et al., 2010). The altered calcium signature in bon1, aca10 and aca8 guard cells suggests that these mutants might have defects in stomata closure in response to environmental stimuli. Indeed, the bon1 mutant does not close stomata in response to calcium, ABA, or pathogen, and this defect is not a consequence of autoimmunity as it is independent of SNC1 or PAD4 (Gou et al., 2015) (Fig. 8A). None of the aca10, aca8 and aca10aca8 mutants in No-0, Ws, or Col-0 closed their stomata in response to 10 mM calcium either (Fig. 8A). Furthermore, ACA10 and ACA8, similar to BON1, functions in pathogen induced stomata closure control as well. When applied with a coronatine-deficient (COR–) Pst DC3000 strain which does not induce stomata reopening (Melotto et al., 2006), none of the aca10 and aca8 single or double mutants closed their stomata, in contrast to the wild type (Fig. 8B). We further examined the stomatal movement over a time course in response to Pst DC3000 COR– as well as Pst DC3000 which causes reclose of stomata in the wild type Col-0. As expected, the wild type Col-0 opened stomata at 1.5 hours and 3 hours in response to both Pst DC3000 COR– and Pst DC3000 and closed its stomata at 4.5 hours when applied with Pst
DC3000 but not *Pst* DC3000 COR\(^{-}\) (Fig. 8C). In contrast, the *aca8 aca10* mutant kept its stomata closed at 1.5, 3, and 4.5 hours when applied with either of the pathogen strains except for a slight opening at 3 hours when applied with *Pst* DC3000 (Fig. 8C). This further supports that the...
"aca10 aca8" double mutant is insensitive or less sensitive to pathogens in stomatal closure compared to the wild type. Therefore, the BON1 and ACA10/8 regulate stomata closure and plant immune responses in a similar fashion, and they might do so through modulating calcium signatures.
Calcium signaling is universal and complex. Despite rapid progress in deciphering calcium signaling and network in various developmental and environmental responses, it remains largely unknown in many processes what calcium transporters are used to generate calcium signals and how these transporters are regulated. Here we identified Ca\(^{2+}\)-ATPases ACA10 and ACA8 and calcium-binding protein BON1 as important regulators of calcium signature (Fig. 9). They are essential to generate calcium oscillation in response to externally applied calcium; and without their functions, only one single calcium spike is produced instead of multiple repeated spikes. This could be due to a failure to produce additional spikes or, more likely, to terminate the first spike due to the lack of ACA10/8 function. Prior studies also found an effect of ACAs on calcium signatures. For instances, mutations of tonoplast-localized PCA in moss and ER-localized NbCA1 in tobacco caused elevated calcium concentration and increased the amplitude and duration of calcium spikes, respectively (Qudeimat et al., 2008; Zhu et al., 2010). Ca\(^{2+}\)-ATPases are high affinity but low-capacity transporters whereas Ca\(^{2+}\)-exchangers are low affinity high-capacity transporters. Based on their biochemistry characteristics, it is hypothesized that Ca\(^{2+}\)-ATPase is the primarily component that terminates Ca\(^{2+}\) signaling, whereas Ca\(^{2+}\)-exchanger is the primarily factor that removes Ca\(^{2+}\) following cytosolic Ca\(^{2+}\) elevation (Sze et al., 2000). The finding here that the loss of ACA10/8 function compromises cytosolic calcium oscillation not only establishes a critical role of PM-localized ACAs in calcium signals but also indicates a broader role of ACAs in calcium signature generation than previously thought.

Environmental signals such as PAMPs trigger opening of calcium permeable channels for calcium influx leading to the cytosolic calcium spike and sometimes multiple spikes forming an oscillation (Thor and Peiter, 2014). Our study indicates that calcium pumps ACA10 and ACA8
are needed for the oscillation but not the first spike. It is likely that calcium level in cytosol are reset after the first induction before subsequent spikes can be generated, and calcium pumps are needed for this resetting. BON1, whose activity is promoted by calcium binding (Li et al., 2010),
potentially modulates calcium oscillation through its binding to and activating ACA10 and ACA8. ACA10 and ACA8 both have an auto-inhibitory domain that overlaps with the calmodulin binding motif, and calmodulin binding is thought to activate ACAs (Tidow et al., 2012). The binding of BON1 to this auto-inhibitory domain of ACA10/8 may alter the protein conformation of ACA10/8 or facilitate calmodulin binding of ACA10/8, leading to the release of auto-inhibition of ACA10/8 (Fig. 9). Because BON1 and calmodulin are both calcium binding proteins, the ACA10/8 pump activity could be controlled by calcium itself, thus forming a feedback regulation. Future analysis of the interaction among ACA10/8, BON1 and calmodulin will reveal further the regulatory mechanisms of calcium efflux conferred by ACA10/8.

Calcium oscillation is generated in response to a number of stimuli in plants including NOD factor in a variety of legume species (Granqvist et al., 2015). In legume, calcium oscillation occurs in nucleus in response to NOD factor, and an ER- or nuclear envelope- localized calcium ATPase was found to be essential to generate nuclear calcium oscillation (Capoen et al., 2011). The finding of a role of PM-localized calcium ATPases in calcium oscillation in cytosol indicate that calcium ATPases have general roles in calcium signature generation in various cellular compartments. Further study should determine whether or not ACA10/8 and BON1 affect calcium signatures in response to other stimuli as well. The role of BON1 in calcium signature generation also has implications for the copine protein family where BON1 is a member. Some copines in animals were identified as genetic modifiers of channel or receptor mutants (Church and Lambie, 2003; Gottschalk et al., 2005), and they have also been implicated in receptor mediated signaling from cell culture analysis (Tomsig et al., 2004; Heinrich et al., 2010). It would be interesting to test if copines have a regulatory role of calcium signals involved in those processes.
This study also finds opposing functions of ACA10 and ACA8 in two layers of plant immunity similarly to BON1. On one hand, the aca10 and aca8 mutants are insensitive to calcium and pathogen in stomatal closure response similarly to the bon1 mutants. This feature presumably makes these mutant plants more susceptible to pathogen at the invasion stage. An earlier report did find a more susceptible phenotype for the aca8 and aca10 mutants (Frey et al., 2012). However, we found in this study that the aca10 and aca10 aca8 mutants have enhanced resistance when bacterial propagation in plants is assayed by either infiltration or surface inoculation methods. This discrepancy might have resulted from differences in unidentified plant or pathogen growth conditions because resistance phenotype was observed even when we replicated the experiment reported in Frey et al., 2012. Nevertheless, the enhanced resistance we observed is temperature- and PAP4-dependent, indicating of activation of plant immune receptor NLR genes reminiscent of the activation of NLR genes in the bon1 mutant. Apparently, this layer of regulation over-rides the stomatal layer of immunity regulation as both bon1 and aca10 mutants have enhanced resistance measured by pathogen growth.

How do BON1 and ACA10/8 regulate two layers of plant immunity? Conceivably, their positive role in stomatal closure control is directly tied with their roles in calcium signature generating and this might represent a more direct role of these proteins. Their roles in controlling pathogen growth likely results from their negative regulation of immune receptor gene expression which could be associated with calcium homeostasis. Altered steady state of calcium might mimic signals from sustained pathogen invasion and therefore upregulate or activate NLR genes. Indeed, disruption of calcium channels and pumps have been shown to affect plant immunity (Clough et al., 2000; Zhu et al., 2010). For instances, two vacuole-localized ACA members, ACA4 and ACA11 repress cell death and innate immunity in Arabidopsis (Boursiac et
Although these molecules are located in different parts of the cell including plasma membrane, ER, or vacuole, they could all regulate calcium levels in cytosol and perhaps nucleus as well.

Although both bon1 and aca10aca8 have autoimmunity, their growth phenotypes are not identical. This might be due to specific functions these proteins have in addition to their overlapping roles. It could also be due to overlapping functions among the BON1 family members or the ACA10 family members. BON1 has two other homologs BON2 and BON3 in Arabidopsis, while ACA10 has ACA8 and ACA9 as close homologs as well as ACA12 and ACA13 as homologs. It is likely that BON1 family members interact with ACA10 family members, each pair with a different regulatory strength. Therefore a partial loss of the family activity could result in different statuses of calcium homeostasis. This variation in calcium steady level might be associated with activation of different NLR genes. Identifying such NLR genes activated in various mutants of calcium channels and pumps will lead to a further understanding of the mode of action of calcium signatures as well as their roles in plant immunity.

Materials and Methods

Arabidopsis Mutants and Plant Growth

The seeds of aca10-cif1 and aca10-1 were kindly provided by R. Sharrock (George et al., 2008). The aca10-2 (GK-044H01) and aca8-2 (GK-688H09) lines were obtained from the Arabidopsis Stock Centre (http://arabidopsis.info/). For growth phenotyping, the Arabidopsis plants were grown under constant light condition with 100 mmol m^-2 s^-1 and relative humidity at 50% to 70%. For pathogen growth assays, plants were grown under 12 or 16 hour light condition. N. benthamiana plants were grown in the greenhouse at 24°C for 4 to 6 weeks before
use for transient expression studies.

Protein Subcellular Localization Assay

An ACA10 genomic/cDNA hybrid was constructed and cloned into the Gateway entry vector pCR8 TOPO TA vector (Invitrogen). For localization of ACA10 protein, the ACA10 gene was transferred from the entry vector to the Hpt-psat1-ACA10 (GW) vector, and transformed into the Agrobacterium tumefaciens (A. tumefaciens) strain GV3101 for infiltration into the abaxial surface side of 4- to 6-week-old N. benthamiana plant leaves as previously described (Gou et al., 2015). Fluorescence of the epidermal cell layer of the lower leaf surface was examined at 2 to 4 days post inoculation (DPI). Images were captured by a Leica TCS SP2 Confocal Microscope with excitation wavelength at 488 and 496 nm and emission wavelength between 520 and 535 nm for GFP signals.

BiFC Assay

The full-length complementary DNA (cDNA) fragments (without stop codon) of BON1, ACA8, OPT3 (Zhai et al., 2014) and ACA10 (genomic/cDNA hybrid) were amplified using primers in Supplemental Table S2 and cloned into the Gateway entry vectors pCR8 TOPO TA or pENTR/D TOPO (Invitrogen). For BiFC experiments, BON1 was cloned into pSPYCE-35S GW (Schutze et al., 2009) using LR clonase (Invitrogen) to generate BON1:cYFP constructs, while ACA8, ACA10 and OPT3 were cloned similarly into pSPYNE-35S GW to generate corresponding nYFP constructs, respectively. A previously described protocol (Schutze et al., 2009) was followed to observe BiFC signals with minor modification. The constructs were transformed into the A. tumefaciens strain GV3101. Overnight cell cultures were collected and
resuspended in 1 mL of AS medium (10 mM MES-KOH pH5.6, 10 mM MgCl₂ and 150 μM acetosyringone) to optical density at 600 nm (OD₆₀₀) to 0.8. The working suspensions were prepared by mixing appropriate clones containing the BiFC constructs and the gene-silencing inhibitor pBA-HcPro plasmid (Menke et al., 2005) at 1:1:1 ratio, and let them stand for 2 to 4 hours. The *A. tumefaciens* suspensions were then co-infiltrated into the abaxial surface of 4-week-old *N. benthamiana* plant leaves. Fluorescence of the epidermal cell layer of the lower leaf surface was examined at 3 to 4 DPI. Images were captured by a Leica SP5 confocal microscope with excitation wavelength at 514 nm and emission wavelength between 500 and 550 nm for YFP signals.

**Split-Luc Assay**

The full length cDNA of *BON1* was amplified using the oligos listed in Supplemental Table 2. The PCR fragment of BON1 was ligated into the pCAMBIA-NLuc vector (Chen et al., 2008) digested by BamH1 and SalI using the ClonExpress™ MultiS One Step Cloning Kit (Vazyme) to generate BON1-NLuc. The N-terminal parts of ACA8 and ACA10 were each amplified using the oligos listed in Supplemental Table 2. The PCR fragments of ACA8 and ACA10 were ligated into the pCAMBIA-CLuc vector (Chen et al., 2008) digested by KpnI and SalI using a similar strategy as above to generate ACA8I-CLuc and ACA10I-CLuc. These constructs were transformed into *A. tumefaciens* strain GV3101.

*A. tumefaciens* GV3101 strains containing recombinant constructs were grown in liquid Luria-Bertani medium with rifampicin and kanamycin for 2 days, pelleted, and resuspended in Murashige and Skoog with 10 mM MES medium containing 200 μM acetosyringone to a final concentration of OD₆₀₀=0.6. After 2 hours’ induction, the bacterial suspensions were infiltrated
into young expanded leaves of *N. benthamiana* plants with a needleless syringe. After infiltration, the plants were covered with dark bag at 23°C for 48 hours. The plants were then kept under light for 16 hours, sprayed with 1 mM luciferin in 0.01% Triton X100 solution, and kept in dark for 5 min to quench the fluorescence. A deep cooling CCD imaging apparatus (DU934P-BV, Andor) was used to capture the fluorescence image. The camera was cooled to -80°C and all images were taken after 3 min exposure.

**Pathogen Growth Assay**

*Pst* DC3000 grown on plates with King’s B medium were washed, collected, and diluted with 10 mM MgCl₂ and 0.02% Silwet L-77. Syringe infiltration was used for inoculation unless stated otherwise where dipping or spray methods were used. For syringe infiltration, bacteria were diluted to OD₆₀₀ of 0.002 and syringe-infiltrated on leaves of 3 to 4 week old plants. Six inoculated leaf were collected as one sample, weighed, ground in 1 ml of 10 mM MgCl₂, and shaken at room temperature for 1 hour. Dipping inoculation was performed as previously described (Gou et al., 2015), and bacteria were diluted to OD₆₀₀=0.05 for dipping seedlings of 2 weeks old. Three whole seedlings were collected as one sample. Spray inoculation was performed as described previously (Frei dit Frey et al., 2012), and the bacteria were diluted to OD₆₀₀=0.2 for spray.

To determine bacterial growth (for all inoculation methods), serial dilutions of the ground tissue solution were spotted on KB medium, and the number of cfu (colony forming unit) per fresh weight was calculated. Three to four samples were analyzed for each genotype and time point.
Yeast Two Hybrid Assay

The yeast (Saccharomyces cerevisiae) two-hybrid constructs were made in the pDEST-GBKT7 and pDEST-GADT7 Gateway vectors (Rossignol et al., 2007). The DNA fragment of the N-terminal segment I of ACA10 was cloned into the pCR8 TOPO TA vector and transferred to the pDEST-GBKT7 vector to obtain the BD:ACA10-I construct. The AD:BON1, AD:BON1-A, and BD-BAP1 constructs were previously described (Hua et al., 2001). The yeast two-hybrid assay was performed as previously described (Hua et al., 2001; Li et al., 2010).

Northern Blotting and Real-time RT-PCR

Total RNA was extracted from 3-week old seedling using Trizol reagent (Invitrogen) as instructed. RNA of 20 μg was resolved in a 1.2% gel containing formaldehyde. RNA blots were hybridized with gene specific and 32P labeled single strand DNA probe.

For Real-time PCR, SuperScript II Reverse Transcriptase (Invitrogen) was used to synthesize cDNA from isolated RNA. Real time RT-PCR was performed using PRI primers listed in Supplemental Table S2. The ACTIN2 gene was used as internal controls. Advanced Universal SYBR Green Supermix (Bio-Rad) was used for Real time RT-PCR.

Western Blotting

The vector of 35S::BON1-HA used to overexpression BON1 was reported previously (Gou et al., 2015). Arabidopsis leaves grown in soil under constant light for 3 weeks were used for protein extraction and western blotting with anti-HA antibody (Sigma Aldrich, Catalog: H3663) following a previously described method (Gou et al., 2015).
Stomatal Closure Assay

Stomatal aperture assay was performed as described (Nomura et al., 2012). Young rosette leaves from 17- to 25-day-old Arabidopsis plants were detached, floated on the stomatal opening buffer containing 10 mM Tris-Mes (pH 6.15), 5 mM KCl and 50 μM CaCl$_2$, and incubated for 2.5 hours under white light (200-250 μmol m$^{-2}$ s$^{-1}$) at 22°C. Each leaf was then clung onto a cover slip with a medical adhesive (Hollister, Libertyville, USA) and their mesophyll cells were removed by a razor blade. The epidermal strips were then transferred into the stomatal closing buffer containing 10 mM Tris-Mes (pH 6.15), 5 mM KCl and 10 mM CaCl$_2$, and incubated for another 2 hours under white light. Then the epidermal strips were observed under an inverted microscopy (Model D1, Carl Zeiss) before and after the closing buffer treatment. The stomatal aperture was calculated as the ratio of the inner width/outer length of each pair of stomata. For each sample more than 50 guard cells were calculated, and the experiments were repeated for 4 times.

Calcium Level Measurement

For calcium oscillation experiment, the epidermal strips after the light treatment was acquired as described above. Cover slips were then placed in a perfusion chamber which was fit to an inverted microscopy (Model D1, Carl Zeiss, Germany) equipped with an emission filter wheel (Lambda XL, Novato, USA) and a CCD camera (Andor™ Technology, Belfast, Northern Ireland). Imaging calcium in the guard cells was conducted by monitoring the ratio (535nm/442nm) of YC3.6 using the MetaFluor fluorescence ratio imaging software. The epidermal strips were firstly measured for approximately 100 seconds, and then the opening
buffer was changed into the closing buffer by an injector when the same epidermal strips were measured for 700 seconds. The interval of image acquisition was 3 seconds. For each genotype more than 30 guard cells were measured, and the experiments were repeated for 3 times.

Measurement of steady level of Ca$^{2+}$ concentration using the YC3.6 system was performed with ZESS LSM710 confocal laser scanning microscope following the protocol previously described (Krebs et al., 2012).

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Figure legends

Figure 1: Protein-protein interaction between ACA10 and BON1.

A. BiFC assay of ACA10 and BON1 interaction. ACA10 fused with N-terminal part of YFP
(ACA10-NE) and BON1 fused with C-terminal part of YFP (BON1-CE) were transiently expressed in *Nicotiana benthamiana* (*N. benthamiana*) by Agrobacterium mediated transformation. A control plasma membrane protein OPT3 co-expressed with BON1 is shown at the bottom panel. Bar=50µm.

B. BiFC assay of interaction between BON1 and each of the five segments of ACA10: I, II, III, IV, and V (supplemental Fig. 1B). Bar=50µm.

C. Split-LUC assay of interaction of BON1 and the first segment of ACA10. Fusion of segment I of ACA10 with C-terminus LUC (ACA10I-Cluc) was co-expressed with fusion of BON1 with N-terminus LUC (BON1-Nluc) in *N. benthamiana* (upper left panel). Co-expressions of ACA10I-Cluc with Nluc (upper right), Cluc with BON1-Nluc (lower left), and Cluc with Nluc (lower right) were used as controls.

D. Yeast-two-hybrid assay between the first segment of ACA10 and the A domain of BON1. BAP1 was used as a positive control. Shown is growth of serial dilutions of yeasts on synthetic medium without leucine or tryptophan (SC-LT) or synthetic medium without leucine, tryptophan, adenine, or histidine (SC-LTAH). AD: Activation domain of GAL4; BD: DNA-binding domain of GAL4.

Figure 2: The loss of *ACA10* in Nossen-0 (No-0) background leads to autoimmune responses.

A. Growth phenotype of *aca10-cif1* and wild-type No-0 plants at 22°C and 28°C.

B. *Pst* DC3000 bacterial growth in *aca10-cif1* and No-0 plants under 22°C and 28°C. "***" indicates significant difference by student’s t-test (*P*-value<0.001).

C. *PR1* expression level in *aca10-cif1* and No-0 plants at 22°C and 28°C analyzed by Northern blotting.
D. Growth phenotype of No-0, *aca10-cif1* and *aca10-cif1 pad4* plants grown at 22°C.

E. *PR1* expression level in No-0, *aca10-cif1* and *aca10-cif1pad4* plants analyzed by real-time RT-PCR assay. Actin2 is used as a control gene. “***” indicates significant difference by student’s t-test (P-value<0.001).

Figure 3: The growth and defense phenotypes of *aca10* and *aca8* mutant in Col-0 accession background.

A. The growth phenotypes of Col-0, *aca10-2, aca8-2*, and *aca10-2 aca8-2* at seedling stage.

B. The growth phenotypes of Col-0, *aca8-2, aca10-2*, and *aca10-2 aca8-2* at seed setting stage.

C-D-E. Growth of virulent pathogen *Pst* DC3000 in the above genotypes. Different inoculation methods were used: syringe infiltration (C), dipping (D) and spray (E). Different letters indicate statistical difference (P-value <0.001 by Bonferonni test) among genotypes.

F. *PR1* expression level in Col-0, *aca10, aca8* and *aca10aca8* plants analyzed by real-time RT-PCR assay. Actin2 is used as a control gene.

Figure 4: Genetic interaction between *BON1* and *ACA10* in Wassilewakija (Ws) background.

A. Growth phenotypes of wild type, *aca10-1, bon1-2*, and *aca10-1 bon1-2* in Ws background at 22°C and 28°C.

B. *Pst* DC3000 bacterial growth in above genotypes assayed by the syringae infiltration method.

C. *PR1* expression level in above genotypes grown at 22°C and 28°C by RNA blotting. Same amount of total RNAs were loaded on gels and the blots were hybridized at the same time.

D. Growth phenotypes of *aca10-1 bon1-2* mutants and their *eds1-1* and *pad4-5* combination mutants grown at 22°C.
E. Growth of *Pst* DC3000 in genotypes as in D assayed by the syringae infiltration method.

F. *PR1* expression level in genotypes as in D assayed by real-time RT-PCR.

Genotypes: \(a/b=aca10-1bon1-2\), \(a/b/eds1-1=aca10-1bon1-2eds1-1\), \(a/b/pad4-5=aca10-1bon1-2pad4-5\).

Different letters in (B) and (D) indicate statistical difference (\(P\)-value < 0.001 by Bonferonni test) of various genotypes.

Figure 5: Overexpression of *BON1* rescued defects of the *aca10-cif1* mutant but not the *aca10aca8* mutants.

A. Growth phenotype of wild-type No-0, *aca10-cif1* and *BON1* overexpression in *aca10-cif1* at 22°C.

B. Growth of *Pst* DC3000 in genotypes above assayed by the dipping inoculation method.

C. *PR1* expression level in above genotypes by real-time RT-PCR assay. *Actin2* is used as a control gene.

D. Growth phenotype of *aca10-2 aca8-2* (in Col-0) and *BON1* overexpression in *aca10 aca8* at 22°C.

E. Growth phenotype of *BON1* overexpression in *aca10 aca8* double mutant in a mixed Col-0 and No-0 background. Shown as the second and the last plant respectively are the two parental lines used for the cross: the *BON1-OE* line in *aca10-cif1* and the *aca10-2 aca8-2* in Col-0. The two plants in the middle are F3s from the same F2 parent from the cross, with one carrying the *BON1-OE* transgene.

Different letters in (B) and (C) indicate statistical difference (\(P\)-value < 0.001 by Bonferonni test) of various genotypes.
Figure 6. Physical interaction of ACA8 and BON1.

A. BiFC assay of ACA8 and BON1. ACA8 fused with N-terminal part of YFP (ACA8-NE) and BON1 fused with C-terminal part of YFP (BON1-CE) were transiently expressed in N. benthamiana by Agrobacterium-mediated transformation. The plasma membrane protein OPT3 was used as a negative control. Graphs show YFP-mediated fluorescence derived from the protein-protein interaction, chlorophyll autofluorescence (chlorophyll), and superimposed images of chlorophyll auto-fluorescence and YFP (Merge). Bar=100 µm.

B. Split-LUC assay of BON1 with N-terminal segment I of ACA8. Fusion of segment I of ACA8 with C-terminus LUC (ACA8I-Cluc) was co-expressed with fusion of BON1 with N-terminus LUC (BON1-Nluc) in N. benthamiana (upper left panel). Co-expressions of ACA8I-Cluc with Nluc (upper right), Cluc with BON1-Nluc (lower left), and Cluc with Nluc (lower right) were used as controls.

Figure 7: Calcium homeostasis and calcium oscillation are altered in the bon1 and aca10 mutants.

A. Steady state calcium levels in guard cells of No-0, aca10-cif1, Ws, bon1-2, aca10-1, bon1-2 aca10-1, Col-0, and bon1-1 assayed by the YC3.6 reporter. Shown are the average and standard deviation of ratios of FRET/CFP from at least 30 guard cells (from 5 plants, 2 leaves/plant, and 3 guard cells/leaf) of each genotype. “**” and “*” indicate significant difference between the wild type and the mutant at P-value<0.001 and P-value<0.05, respectively, by Bonferroni test.

B. Calcium signals induced by exogenous application of calcium in Col-0, bon1-1, aca10-2, and aca8-2 plants. Shown is the representative image of calcium signals in guard cells of each
The genotype measured as FRET/CFP ratio using the YC3.6 reporter. The two numbers underneath each genotype indicate the number of cells with the representative calcium pattern versus the number of total cells analyzed.

Figure 8: Stomatal movement is compromised in the bon1, aca10, and aca8 mutants.

A. Stomatal closure induced by exogenous application of CaCl₂ in wild type, aca10, aca8, and bon1 in No-0, Ws and/or Col-0 backgrounds.

B. Stomatal closure induced by exogenous application of Pst DC3000 COR⁻ in wild type, aca10, and bon1 in the Col-0 background.

C. Time course of stomatal movement by application of Pst DC3000 COR⁻ and Pst DC3000 in the wild type and the aca8aca10 plants.

“**”and “*” indicate significant difference before and after treatment by student’s t-test at P-value<0.001 and P-value<0.05 respectively.

Figure 9: Working model for the role of ACA10/8 and BON1 in calcium signature and immunity responses.

Extra-cellular calcium was released into cytosol by plasma membrane localized calcium channels when the host cells use receptors to detect pathogen features. The transient increase of Ca²⁺ concentration activates BON1 and ACA10/8 complex which pumps calcium from cytosol to extracellular space. This Ca²⁺ exclusion is necessary to activate the following Ca²⁺ increase in cytosol. Ca²⁺ oscillation in cytosol is generated coordinately by Ca²⁺ channels that released calcium from extracellular medium and subcellular compartments into cytosol and by Ca²⁺
pumps that export Ca\(^{2+}\) from the cytosol. The information within Ca\(^{2+}\) spiking was decoded by Ca\(^{2+}\) binding proteins and transmitted to control stomatal movement and the expression of defense response genes.

Supplementary tables

Supplementary Table 1. List of BON1 co-expressed genes that code for calcium signaling molecules.

Supplementary Table 2. Primers used in this study.

Supplementary Figures

Supplemental Figure 1: Subcellular localization and structure of the ACA10 protein.

Supplemental Figure 2: Physical interaction of ACA8/10 with BON1 assayed by BiFC.

Supplemental Figure 3: The growth defect of aca10-1 bon1-2 is dependent on temperature, EDS1 and PAD4.

Supplemental Figure 4: Overexpression of BON1 in the aca10 mutants.


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