Function of endoplasmic reticulum calcium ATPase in innate immunity-mediated programmed cell death

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Programmed cell death (PCD) initiated at the pathogen-infected sites during the plant innate immune response is thought to prevent the development of disease. Here, we describe the identification and characterization of an ER-localized type IIB Ca^{2+} -ATPase (NbCA1) that function as a regulator of PCD. Silencing of NbCA1 accelerates viral immune receptor N- and fungal-immune receptor Cf9-mediated PCD, as well as non-host pathogen *Pseudomonas syringae* pv. tomato DC3000 and the general elicitor cryptogein-induced cell death. The accelerated PCD rescues loss-of-resistance phenotype of Rar1, HSP90-silenced plants, but not SGT1-silenced plants. Using a genetically encoded calcium sensor, we show that down-regulation of NbCA1 results in the modulation of intracellular calcium signalling in response to cryptogein elicitor. We further show that NbCAM1 and NbrbohB function as downstream calcium decoders in N-immune receptor-mediated PCD. Our results indicate that ER- Ca^{2+} -ATPase is a component of the calcium efflux pathway that controls PCD during an innate immune response.

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Introduction

Plant innate immunity can be grouped into two branches of inducible defense systems. The first branch includes an innate immune response, which is triggered on the perception of general pathogen- or microbe-associated molecular patterns by plant pattern recognition immune receptors (PRRs)

(Zipfel, 2008). The second branch of innate immunity is triggered when plant resistance (R) gene-encoded immune receptors recognize pathogen-encoded effector proteins (Caplan *et al*, 2008a). Compared with PRR-initiated defense responses, R immune receptor-mediated defense is often associated with the induction of programmed cell death (PCD) at the site of pathogen infection called the hypersensitive response (HR) (Mur *et al*, 2007). HR-PCD is thought to restrict pathogen proliferation and prevent disease development.

The R immune receptor-mediated HR-PCD is preceded by a rapid burst of secondary messenger molecules such as calcium, reactive oxygen species (ROS) and nitric oxide (Torres *et al*, 2006; Mur *et al*, 2007). As these early cellular responses are initiated in most of the R immune receptor-effector systems, it is believed that these responses are conserved. However, it remains unknown how these signalling molecules control the initiation and progression of HR-PCD.

Calcium has an important function in plant growth, development and responses to biotic and abiotic stresses (Bush, 1995). Calcium homeostasis in plant cells is maintained by the calcium influx and efflux systems (Sanders *et al*, 2002). The influx system controls calcium flux into the cytosol via calcium channels that reside in the plasma membrane and endomembranes. Calcium efflux is controlled by Ca^{2+} -ATPase and calcium transporters that actively transport calcium back to the extracellular space and/or endomembranes to maintain a low calcium level in the cytoplasm. Induction of intracellular calcium levels is well documented as one of the earliest events in PRR and R immune receptor-mediated defense (Lecourieux *et al*, 2006; Ma and Berkowitz, 2007). Calcium channels, such as, the glutamate receptor, two pore channel and cyclic nucleotide-gated channel may facilitate calcium influx during defense responses (Ma and Berkowitz, 2007). It has been hypothesized that an increase in calcium influx rather than a decrease in calcium efflux results in the early calcium rise in the cytoplasm during a defense response (Ma and Berkowitz, 2007). At present, however, there is no direct evidence to link the activity of a specific Ca^{2+} -ATPase or transporter with a specific Ca^{2+} signalling event during defense response. Therefore, it remains unknown whether calcium efflux itself is regulated during plant innate immune response to shape calcium signalling.

Downstream decoders of the initial calcium signal have a function in initiating a complex signalling network that ultimately leads to HR-PCD (Lecourieux *et al*, 2006; Mur *et al*, 2007). However, the precise calcium decoders involved in HR-PCD remain to be identified. A number of proteins involved in calcium regulation such as calmodulin (CaM), plant NADPH oxidases (respiratory burst oxidase homolog, rboh), mitogen-activated protein kinase, and calcium-dependent protein kinase have been implicated to have a function in HR-PCD (Yoshioka *et al*, 2003; Pedley and Martin, 2005; Lecourieux *et al*, 2006; Torres *et al*, 2006). These candidate

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proteins may indeed be calcium decoders; however, it is currently unclear if they function during the decoding of the cytosolic calcium signature.

To study HR-PCD during innate immunity, we use the N-immune receptor that specifically recognizes the p50 effector from the tobacco mosaic virus (TMV) replicases (Burch-Smith *et al*, 2007; Caplan *et al*, 2008b) to initiate HR-PCD and other defense responses. To identify components involved in the induction of HR-PCD, we performed a plant-based RNAi screen using virus-induced gene silencing (VIGS). We found that silencing of NbCA1 that encodes a type BII Ca^{2+} -ATPase accelerated N-mediated HR-PCD. In addition, NbCA1 silencing accelerated cell death induced by the fungal R immune receptor Cf9, the non-host pathogen *Pseudomonas syringae* pv. tomato (Pst) DC3000, and the cryptogin elicitor. Interestingly, silencing of NbCA1 resulted in accelerated HR-PCD even in the absence of Rar1, HSP90 and SGT1 that are required for the function of many R immune receptors (Shirasu, 2009). We show that NbCA1 is an ER-localized type BII Ca^{2+} -ATPase that is rapidly induced during N-immune receptor response to TMV. Using a genetically encoded calcium sensor Case12 (Souslova *et al*, 2007) in intact living tissue, we show that NbCA1 silencing alters the cytosolic and nuclear calcium signature by increasing the amplitude and duration of calcium spikes. Finally, we show that NbCA1 functions upstream of calcium decoders NbCaM1 and NbrbohB. Our findings suggest that the NbCA1 activity is integrated into cellular calcium signalling to modulate cell death during plant innate immunity.

Results

Identification of calcium ATPase (NbCA1) as a modulator of N-immune receptor-mediated HR-PCD

To identify components involved in the R immune receptor-mediated HR-PCD, we performed a high-throughput VIGS-cDNA library screen in N-immune receptor-containing *Nicotiana benthamiana* plants. A search for an enhancement of HR-PCD to TMV infection yielded the library clone, 1D7, which we named NbCA1 due to its high similarity to P-type calcium ATPases (see below for details of the sequence analyses).

TMV-GFP infection resulted in HR that was visible in both NbCA1-silenced N-immune receptor-containing plants and the VIGS-vector control plants at 5 days post-infection (dpi); however, most HR lesion of VIGS-NbCA1 plants showed prominently visible cell death compared with the control plants (Figure 1A, panels 1 and 2). The level of UV-illuminated GFP fluorescence at infection foci was significantly lower in NbCA1-silenced plants compared with the control plants at 5 dpi (Figure 1A, panels 3), implying that the accelerated cell death in NbCA1-silenced plants may lead to a rapid clearing of TMV. To rule out the possibility that the accelerated HR-PCD in the NbCA1-silenced plants is caused by an increase of virus accumulation within the TMV infection sites, we examined the amount of GFP at the site of TMV-GFP infection by western blot analyses. Quantification of GFP levels indicated that in both the control and NbCA1-silenced plants, a comparable amount of GFP protein was detectable at 2 dpi (Figure 1B). The GFP accumulation persisted at 5 dpi in control plants but dropped sharply in NbCA1-silenced plants (Figure 1B).

To further confirm that the accelerated HR-PCD in NbCA1-silenced plants was not due to the enhanced virus replication, we transiently expressed the p50 effector to trigger HR-PCD (Figure 1C). By 3 days post-infiltration, HR-PCD was prominent in NbCA1-silenced plants and barely visible in the control plants (Figure 1C). The observed accelerated HR-PCD in NbCA1-silenced plants is not due to the altered expression level of p50 effector (Supplementary Figure 1). Together, these results indicate that the disruption of NbCA1 function accelerates HR-PCD, which in turn, results in a rapid clearing of TMV from infection sites.

To confirm our VIGS results, we generated stable NbCA1 RNA interference transgenic lines (NbCA1-RNAi). Out of 16 independent lines, we selected one that exhibited the strongest accelerated HR-PCD phenotype for further analysis. In this NbCA1-RNAi line, the level of NbCA1 transcript was significantly reduced compared with the control plants (Figure 1D). Furthermore, the p50-triggered HR-PCD was visible in NbCA1-RNAi plants as early as 40 h post-infiltration compared with the control plants (Figure 1E). These results further indicate that the downregulation of NbCA1 leads to accelerated HR-PCD in response to TMV.

Next, we examined whether the accelerated HR-PCD in NbCA1-RNAi plants was associated with an accelerated induction of defense-related genes. We observed a significantly enhanced accumulation of PR1, slightly increased PR2 and SGT1 transcript levels at 24 h post-infiltration of p50 in NbCA1-RNAi plants compared with the control plants (Figure 1F).

NbCA1 modulates Cf9 immune receptor-, Pst DC3000- and cryptogin elicitor-induced PCD

As NbCA1 modulates N-immune receptor-mediated HR-PCD to TMV, we investigated whether NbCA1 also modulates cell death induced by other elicitors. We co-expressed tomato Cf9 immune receptor with its Avr9 fungal effector and tomato Pto immune receptor with its bacterial-effector AvrPto. In Cf9-Avr9 infiltrated NbCA1-RNAi plants, HR-PCD was accelerated compared with the control plants (Figure 2, panels 1). However, there was no difference in the HR-PCD in plants infiltrated with Pto-AvrPto (Figure 2, panels 2).

Next, we investigated whether the function of NbCA1 was specific to immune receptor-mediated PCD, or whether NbCA1 also modulated PCD induced by non-host resistance or general elicitors. Inoculation of NbCA1-RNAi plants with the non-host pathogen Pst DC3000 and the cryptogin elicitor induced accelerated PCD compared with the control plants (Figure 2, panels 3 and 4). However, general death (non-PCD) induced by methanol treatment was similar in control and NbCA1-RNAi plants (data not shown). Together, these results suggest that in addition to accelerating N-immune receptor-mediated PCD, NbCA1 downregulation also accelerates PCD induced by the Cf9 immune receptor, Pst DC3000 and cryptogin elicitor.

Silencing of NbCA1 rescues loss-of-resistance phenotypes of Rar1 and HSP90 but not of SGT1

Silencing of key immune response modulators Rar1, HSP90 and SGT1 in NN plants leads to loss-of-resistance to TMV (Peart *et al*, 2002; Liu *et al*, 2002a,b, 2004; Lu *et al*, 2003). Interestingly, silencing of NbRar1 and NbHSP90 in NbCA1

Figure 1 Silencing of *NbCA1* accelerates N-immune receptor-mediated HR-PCD. **(A)** TMV-GFP-induced HR-PCD in VIGS-Vector control plants (upper panel) and VIGS-*NbCA1* plants (lower panel) rub inoculated with equal amount of TMV-GFP extract. Images of TMV-GFP-infected leaves (panels 1) and magnified leaf area (panels 2–3) were captured under white light (panels 1 and 2) and UV light (panels 3) at 5 days post-infection (dpi). Experiments were repeated for four times, and four leaves from two individual plants were used in each experiment. **(B)** TMV-GFP accumulation at 0–5 dpi in TMV-GFP-infected leaves of VIGS-Vector plants and VIGS-*NbCA1* plants. Total protein extracted from TMV-GFP-infected leaves was analysed by western blot using GFP antibody. Coomassie staining of Rubisco bands show total protein loading amount. Numbers above the panels indicate days post-infiltration. **(C)** TMV-p50-induced HR-PCD in VIGS-Vector plants (upper panel) and in VIGS-*NbCA1* plants (lower panel) agro-infiltrated with TMV-p50 of $OD_{600}=1.0$. Numbers above the panels indicate days post-infiltration. Experiments were repeated for four times, and four leaves from two individual plants were used in each experiment. **(D)** Expression level of *NbCA1* in *NbCA1*-RNAi plants (1) and control plants (2). Transcription level of *NbCA1* was analysed by Northern blot using *NbCA1* probe. Ethidium bromide (EtBr)-stained ribosomal RNA bands serve as loading control. **(E)** TMV-p50-induced HR-PCD in control plants (top panel) and in *NbCA1*-RNAi plants (bottom panel) agro-infiltrated with TMV-p50 of $OD_{600}=1.0$. hpi, hours post-infiltration. **(F)** Expression of PR1, PR2 and SGT1 in control plants and *NbCA1*-RNAi plants in response to TMV-p50. Transcription levels were analysed by Northern blots using PR1, PR2 and SGT1 probes at 0, 16, 24, 30 and 40 h post-infection (hpi). Ethidium bromide (EtBr)-stained ribosomal RNA bands serve as loading control.

RNAi plants did not abolish the TMV-GFP induced accelerated PCD response (Figure 3A and B). In addition, TMV-GFP was restricted to the infiltrated leaf in *NbCA1*-RNAi plants

(data not shown); whereas, we have reported earlier that TMV-GFP spreads into upper uninfiltrated leaves in control plants (Liu *et al*, 2002a, 2004). These results indicate that

Figure 2 Silencing of NbCA1 accelerates Cf9 immune receptor as well as Pst DC3000- and cryptogeiin elicitor-induced cell death. Control plants (upper panel) and NbCA1-RNAi plants (lower panel) were co-infiltrated with a mixture of Agrobacterium with Cf9 and Avr9 (1), Pto and AvrPto (2) at a 1:1 ratio of OD₆₀₀ = 1.0, 10⁵ cfu of *Pseudomonas syringae* pv. tomato DC3000 (3) and 100 µg/ml of cryptogeiin (4). Photographs of the infiltrated leaves were taken at 3 days post-infiltration. Experiments were repeated four times, and four leaves from two individual plants were used in each experiment.

Rar1- and HSP90-silencing-mediated loss-of-resistance phenotype can be rescued by the downregulation of NbCA1.

Compared with the NbRar1 and NbHSP90, silencing of SGT1 in NbCA1 RNAi plants resulted in a less pronounced accelerated HR-PCD (Figure 3C, columns 3 and 4, upper panels). In addition, the accelerated HR-PCD failed to restrict TMV from spreading into non-infiltrated upper leaves (Figure 3C, columns 3 and 4, middle and bottom panels). In control SGT1-silenced plants, consistent with earlier reports (Peart *et al*, 2002; Liu *et al*, 2002b), we observed greatly reduced HR-PCD (Figure 3C, columns 1 and 2, upper panels); and TMV-GFP is able to spread into the upper non-infiltrated leaves due to loss-of-resistance (Figure 3C, columns 1 and 2, middle and bottom panels). These results suggest that silencing of SGT1 fails to completely abolish PCD response in the absence of NbCA1; however, this accelerated PCD response is insufficient to prevent systemic movement of the virus.

NbCA1 is induced during N-mediated resistance response

To determine whether NbCA1 is regulated during N-immune receptor-mediated HR-PCD, we examined NbCA1 mRNA expression. For this, we used a temperature shift assay (Gianinazzi, 1970) to synchronize HR-PCD in every cell. At temperatures above 28°C, N fails to induce HR-PCD and therefore TMV can replicate and spread throughout the plant. When the plants are shifted to permissive temperature of 22°C, synchronous TMV-triggered HR-PCD is visible within 8 h. RT-PCR analyses using total RNA isolated from the tissue derived from the temperature-shifted NN plants indicate that the NbCA1 message is induced as early as 4 h post-shift (hps) and continues to be induced at the last time point (16 hps) of the experiment (Figure 4). In contrast, we observed no considerable increase of NbCA1 transcript in temperature-shifted plants lacking the N-immune receptor (Figure 4). These results indicate that NbCA1 is upregulated during N-mediated HR-PCD to TMV infection.

NbCA1 is an ER-localized type IIB Ca²⁺-ATPase

As our VIGS-NbCA1 clone contained only the partial sequence, we used rapid amplification of cDNA ends (RACE) to clone the

full-length cDNA of NbCA1. The full-length cDNA of NbCA1 (GU361620 and GU361621) encodes a protein with 1046 amino acid, 10 transmembrane domains and all the functional domains of P-type ATPases (Supplementary Figure 2). In addition, it contains an autoinhibitory CaM-binding domain (CaMBD) at the N-terminus. NbCA1 shares high similarity to the plant type IIB Ca²⁺-ATPase such as Arabidopsis AtACAs, *Brassica oleracea* BoCA1, *Medicago truncatula* MtMCA1, *Oryza sativa* OsMCA4 and *Glycine max* SCA1 (Supplementary Figures 2 and 3). In contrast, NbCA1 has less identity to Arabidopsis type IIA, a typical ER-type Ca²⁺-ATPase family constituted by four ER-localized Ca²⁺-ATPase (AtECA1-4) (Supplementary Figure 3).

To determine whether NbCA1 is a functional Ca²⁺-ATPase, we expressed full-length NbCA1 fused to citrine and a truncated version of NbCA1 (NbCA1ΔN) fused to citrine, in which a 53-amino-acid N-terminal autoinhibitory CaMBD was deleted, in the K616 yeast strain. Both NbCA1 and NbCA1ΔN were driven by a galactose inducible promoter. The K616 yeast strain lacks endogenous Golgi and vacuolar Ca²⁺-ATPases (PMR1 and PMC1), and the calcineurin regulatory subunit B (CNB1). Therefore, the K616 yeast strain fail to grow on calcium-depleted media such as media supplemented with 10 mM EGTA (Cunningham and Fink, 1994; Sze *et al*, 2000). We observed that the K616 cells expressing NbCA1ΔN were able to grow on EGTA-galactose medium, but not full-length NbCA1 or empty vector control (Supplementary Figure 4). We hypothesize that the K616 cells expressing full-length NbCA1 is unable to grow because of the lack of the specific CaM in yeast that can release the autoinhibitory domain. These results suggest that NbCA1ΔN that lacks the autoinhibitory domain is targeted to the proper compartments to pump Ca²⁺ from the cytosol to supply sufficient calcium for their function. The ability of NbCA1ΔN to complement yeast mutant indicates that NbCA1 is an active calcium pump, and that C-terminally fused citrine does not interfere with the activity of NbCA1.

To determine the subcellular localization of NbCA1 in the intact plant tissue, we expressed NbCA1 fused to citrine at the C-terminus (NbCA1-Citrine) or citrine alone under the control of native NbCA1 promoter in *N. benthamiana* leaves via

Figure 3 Silencing of NbCA1 induces accelerated HR-PCD even in the absence of Rar1, HSP90 and SGT1. (A, B) TMV-GFP-induced HR-PCD in RAR1- (A) and HSP90-silenced (B) control plants (1–2) and *NbCA1*-RNAi plants (3–4) agro-infiltrated with TMV-GFP of OD₆₀₀=1.0. All pictures were taken at 6 dpi under white light (1 and 3) and the UV light (2 and 4). (C) TMV-GFP-induced HR-PCD in SGT1-silenced control plants (top row, panels 1 and 2) and *NbCA1*-RNAi plants (top row, panels 3 and 4). TMV-GFP moved to the upper uninoculated leaves of SGT1-silenced control plants (middle and bottom rows, panels 1 and 2) and *NbCA1*-RNAi plants (middle and bottom rows, panels 3 and 4). All pictures were taken at 6 dpi (top and middle row) and 8 dpi (bottom row) under white light (1 and 3) and the UV light (2 and 4).

agro-infiltration method. Citrine alone was observed in cytoplasm and nucleus (Figure 5A, panels 1). In contrast, NbCA1-Citrine was observed as a reticulated network (Figure 5A, panels 2 and 3) that is indicative of ER pattern of localization (Robinson, 2006). To further confirm that NbCA1-Citrine is localized to the ER, we co-expressed NbCA1-Citrine with TagCFP-HDEL that is known to localize to ER. As shown in Figure 5B, NbCA1-Citrine localization pattern overlaps with TagCFP-HDEL expression pattern. Furthermore, the ER localization pattern of NbCA1-Citrine is unaltered in the presence of TMV (Supplementary Figure 5). These results clearly indicate that NbCA1 is an ER-localized Ca²⁺-ATPase.

NbCA1 is a modulator of calcium signatures

We hypothesized that ER-localized NbCA1 might modulate calcium signalling during pathogen-induced HR-PCD. There-

fore, silencing NbCA1 may lead to an alteration in frequency, duration and/or amplitude of calcium signal that contribute eventually to the altered HR-PCD response. To test this, we expressed p50 effector using an inducible promoter to examine TMV-p50-triggered cellular calcium signal in N-immune receptor-containing plants. However, all the inducible systems we tested for controlled expression of p50 resulted in a HR-PCD without the inducer (data not shown). We reasoned that this is due to the leakiness of the available inducible systems. To overcome this problem, we elicited HR-PCD by exogenously supplying cryptogin elicitor because our data indicated that, like TMV-p50, cryptogin induces accelerated HR-PCD in NbCA1-RNAi plants compared with the control plants (see Figure 2). Furthermore, cryptogin elicitor treatment has been shown to induce cytosolic calcium increase in cultured cells within a short timeframe (Lecourieux *et al*, 2002, 2005).

For monitoring cryptogei-induced intracellular calcium, we used a high-dynamic range, genetically encoded fluorescent sensor called Case12, for which changes in the fluor-

Figure 4 Expression of NbCA1 is induced during N-mediated HR-PCD. Total RNA was extracted from temperature-shifted TMV-infected wild-type and N-containing plants at time points indicated (hours post-temperature shift, hps) and analysed by RT-PCR using NbCA1 and EF1 α gene-specific primers. EF1 α was used as an internal control. Pictures are taken from the agarose gels loaded with 25 cycle PCR products. M, DNA marker size ladder.

escent intensity are directly correlated with changes in calcium concentration (Souslova *et al*, 2007). We observed simultaneous cytosolic and nuclear calcium oscillations in individual cells of both the control and NbCA1-RNAi plants expressing Case12 infiltrated with cryptogei but not with water (Supplementary Figure 6). However, depending on the concentration of the cryptogei treatment, the amplitude, duration and frequency of calcium spikes were significantly altered in NbCA1-RNAi plants compared with the control plants (Figure 6A–G; Supplementary Movies 1–4). The amplitude and duration of calcium spikes increased greatly in NbCA1-RNAi plants treated with 10 μ g/ml cryptogei (Figure 6D; Supplementary Movie 3) compared with that of 5 μ g/ml cryptogei (Figure 6C; Supplementary Movie 2). Whereas in 5 μ g/ml cryptogei-treated control plants, we were unable to observe a recordable oscillation (Figure 6A). Furthermore, the amplitude, duration and frequency of oscillation were similar in 10 and 25 μ g/ml cryptogei-treated control plants (Figure 6B and F; Supplementary Movie 1). Interestingly, the treatment of NbCA1 RNAi plants with 25 μ g/ml concentration of cryptogei resulted in single calcium burst or multiple bursts with high frequency (Figure 6E; Supplementary Movie 4). We hypothesized that this high concentration of cryptogei is sufficient to induce rapid calcium burst followed by rapid cell death in NbCA1 RNAi

Figure 5 NbCA1 is localized to ER. (A) Confocal images show that citrine expressed under NbCA1 promoter is localized to the cytoplasm (panel 1) and NbCA1 fused to citrine is expressed in the ER (panel 2) of epidermal (upper panels) and mesophyll (lower panels) cells. Image in the panel 3 is the magnified area of epidermal cell image shown in panel 2. The scale bar represents 20 μ m. (B) NbCA1-citrine co-localizes with ER marker TagCFP-HDEL. Chloroplasts are illuminated by red autofluorescence. Merged image shows the overlay pattern of NbCA1 and TagCFP. The scale bar represents 20 μ m.

Figure 6 Downregulation of NbCA1 alters calcium signature triggered by cryptogein elicitor. (A–E) Calcium oscillation triggered by cryptogein. Nuclear calcium oscillation of 5 $\mu\text{g/ml}$ (A) and 10 $\mu\text{g/ml}$ (B) cryptogein-treated control plant cells. Nuclear calcium oscillation of 5 $\mu\text{g/ml}$ (C), 10 $\mu\text{g/ml}$ (D) and 25 $\mu\text{g/ml}$ (E) cryptogein-treated NbCA1-RNAi plant cells. Red, blue, black, yellow and green colour lines represent different individual cells. (F) Amplitude, duration and frequency of calcium oscillation in control and NbCA1-RNAi plant cells treated with different concentration of cryptogein. (G) Amplitude of nuclear (upper panel) and cytosolic (lower panel) calcium spikes of 10 and 25 $\mu\text{g/ml}$ treated control plant cells and 5 $\mu\text{g/ml}$ cryptogein-treated NbCA1-RNAi plant cells. The individual bar represents amplitude of individual calcium spike from 14 independent control plant cells and 11 independent NbCA1-RNAi plant cells. (H) HR-PCD is induced by 25–500 $\mu\text{g/ml}$ cryptogein in the control plants (upper panel) and NbCA1-RNAi plants (lower panel). Number 1–5 represent 25, 50, 100, 250 and 500 $\mu\text{g/ml}$ cryptogein, respectively.

plants (see below). Although calcium responses varied greatly among individual cells, the average amplitude of cytosolic and nuclear calcium spikes in 5 µg/ml cryptogein-treated NbCA1-RNAi plant cells (Figure 6F and G) are significantly higher ($P < 0.0001$) than the control plant cells treated with 10–25 µg/ml cryptogein (Figure 6F and G). However, the duration and frequency of the calcium amplitude are similar between the control plants treated with 10–25 µg/ml cryptogein and NbCA1-RNAi plants treated with 5 µg/ml cryptogein (Figure 6F). These results suggest that NbCA1 silencing leads to alteration of calcium signature. Furthermore, the outcome of NbCA1 silencing effects depends on the intensity of initial calcium signal triggered by different doses of cryptogein.

To determine whether altered calcium signatures in NbCA1 RNAi plants contribute towards accelerated cell death induction, we treated control and NbCA1 RNAi plants with 25–500 µg/ml cryptogein. In control plants, cell death is visible only at 500 µg/ml of cryptogein treatment; whereas in NbCA1-RNAi plants, cell death is visible in 25 µg/ml of cryptogein treatment. The death becomes more severe in NbCA1-RNAi plants as the concentration of cryptogein is increased. These results support our observation that at high concentrations of cryptogein treatment, single or multiple calcium bursts with high frequency may be sufficient to execute rapid death response in NbCA1 RNAi plants. Taken together, we conclude that NbCA1-encoded ER- Ca^{2+} -ATPase modulates both cytosolic and nuclear calcium signature in response to elicitors.

NbCA1 functions upstream of CaM1 and rbohB calcium decoders and downstream of TPC

The CaM homolog NbCaM1, or the catalytic subunit of gp91phox NbrbohB, or the Ca^{2+} -permeable channel NbTPC were all previously shown to be involved in plant immune response (Yamakawa *et al*, 2001; Yoshioka *et al*, 2003; Kadota *et al*, 2004). To identify calcium decoders and calcium channels involved in NbCA1-modulated calcium signalling, we silenced these genes in control or NbCA1-RNAi plants and examined their function during N-mediated HR-PCD response to TMV. We confirmed efficient silencing of these targeted genes by RT-PCR (Supplementary Figure 7).

Silencing of NbCaM1 or NbrbohB suppressed TMV-p50-triggered HR-PCD in control NN plants (Figure 7A, top panels 1 and 2). Interestingly, silencing of NbCaM1 and NbrbohB in NbCA1-RNAi plants completely suppressed accelerated HR-PCD (Figure 7A, bottom panels 1 and 2). These results indicate that NbCaM1 and NbrbohB are downstream decoders of calcium signalling triggered by N-TMV interaction.

Silencing of the NbTPC in control NN plants did not significantly reduce TMV-p50-induced HR-PCD response (Figure 7A, top panel 3) compared with the VIGS-Vector control (Figure 7A, top panel 4). However, silencing of NbTPC in NbCA1 RNAi plants greatly reduced accelerated HR-PCD (Figure 7A, bottom panel 3) compared with the VIGS-Vector control (Figure 7A, bottom panel 4), but did not completely abolish HR-PCD like NbCaM1 and NbrbohB silencing (Figure 7A, compare bottom panels 1 and 2 versus 3). These results suggest that NbTPC may partially

Figure 7 Effect of silencing NbCAM1, NbrbohB and NbTPC on HR-PCD in NbCA1-RNAi plants. **(A)** TMV-p50-induced HR-PCD in the NbCaM1- (1), NbrbohB (2), and NbTPC- (3) silenced and Vector-alone (4) control plants (upper panel) and NbCA1-RNAi plants (lower panel) agro-infiltrated with TMV-p50 of $\text{OD}_{600} = 1.0$. Pictures were taken at 3 days post-infiltration. **(B)** Avr9, AvrPto and Pst DC3000-induced cell death in NbrbohB-silenced and Vector-alone control plants (upper panels) and NbCA1-RNAi plants (lower panels) infiltrated with Pst DC3000 and agro-infiltrated with Cf9 plus Avr9, and Pto plus AvrPto. Pictures were taken at 3 days post-infiltration.

regulate upstream of NbCA1-mediated calcium signalling triggered during N-immune receptor-TMV interaction.

Future studies should be aimed at resolving the precise localization of TPC in different host plants. Tobacco TPC1 has been described as a plasma membrane calcium channel and suppression of this channel inhibits cryptogei-induced calcium transient and cell death in BY2 suspension cells (Kadota *et al*, 2004). However, the TPC1 homolog in Arabidopsis (AtTPC1) is a vacuolar cation channel that has no major impact on cytosolic calcium homeostasis (Ranf *et al*, 2008). These results suggest that there are different isoforms of TPC1, which may have different mechanisms underlying their contributions to cytosolic calcium signature in different systems. It is most likely that NbTPC1 contributes to initial calcium signal generation, given its probable localization to the plasma membrane. However, our data described here show that silencing NbTPC fails to completely abolish cell death induced in NbCA1 RNAi lines. Therefore, NbTPC may not be the only channel for initial calcium influx.

We also examined whether NbTPC, NbCaM1 and NbrbohB are components involved in modulating Cf9-, Pto- and Pst DC3000-induced cell death. Silencing of NbTPC and NbCaM1 had no effect on Cf9-Avr9, Pto-AvrPto and Pst DC3000-induced cell death response in NbCA1-RNAi and control plants (data not shown). Unexpectedly, silencing of NbrbohB enhanced Cf9-Avr9, Pto-AvrPto and Pst DC3000-induced cell death response in NbCA1-RNAi and control plants (Figure 7B). These results suggest that NbrbohB may have an additional function in mediating cross-talk between ROS and calcium signalling in some host-microbe interactions.

Discussion

Here, we show that NbCA1, an ER- Ca^{2+} -ATPase, has an important function in the control of HR-PCD during a plant innate immune response. Silencing of NbCA1 accelerates N-viral immune receptor- and Cf9 fungal immune receptor-mediated HR-PCD as well as non-host pathogen and general elicitor-induced cell death. Interestingly, downregulation of NbCA1 can rescue loss-of-resistance phenotypes of known modulators of innate immunity Rar1 and HSP90. Our results show that silencing of NbCA1 alters cytosolic and nuclear calcium signature in response to cryptogei elicitor, indicating that NbCA1 functions to modulate cellular calcium signalling during plant immune response. Furthermore, we show that NbCaM1 and NbrbohB are two downstream targets and functions as calcium decoders during HR-PCD. Our findings provide the first example of a loss-of-function phenotype of an ER- Ca^{2+} -ATPase and function of calcium efflux in plant innate immunity.

NbCA1 is ER-type BII Ca^{2+} -ATPase

In animals, type BII Ca^{2+} -ATPase are exclusively localized to the plasma membrane (Nagamune and Sibley, 2006). Three lines of evidence suggest that NbCA1 is an ER-localized type BII Ca^{2+} -ATPase in plants. First, NbCA1 contains a CaMBD at the N-terminus, a characteristic feature of all type BII Ca^{2+} -ATPase. Second, CaMBD-deleted NbCA1 complements the yeast mutant strain K616, indicating that CaMBD functions as an autoinhibitory domain. Third, NbCA1-Citrine fusion under the control of an endogenous promoter localizes to the ER.

Earlier, AtACA2 was the only type BII Ca^{2+} -ATPase shown to localize to the ER in any eukaryote (Geisler *et al*, 2000). It has been inferred that AtACA2 modulates calcium flux in the vicinity of the ER and may serve as a calcium-regulated feedback system to control calcium levels in the cytoplasm (Hwang *et al*, 2000); however, the precise biological function of AtACA2 was not explored. Genevestigator (Zimmermann *et al*, 2004) microarray data indicate that AtACA2 is upregulated in immune receptor Rpm1- and Rps4-mediated defense response in Arabidopsis. Interestingly, our data indicates that NbCA1 is also upregulated during the N-mediated immune response. The transcriptional induction and ER localization of NbCA1 suggests that AtACA2 is the closest Arabidopsis homolog.

NbCA1 negatively regulates plant immune response

A complex signalling network with multiple modes of initiation and progression controls HR-PCD. Hence, we chose to investigate the function of NbCA1 during HR-PCD induced by multiple pathogen elicitors. We show that in addition to N-immune receptor-mediated acceleration of PCD response, suppression of NbCA1 function accelerated fungal immune receptor Cf9-mediated PCD as well as non-host pathogen and general elicitor-induced cell death; however, it had no effect on HR-PCD initiated by the Pto bacterial immune receptor. These results suggest that NbCA1 is a negative regulator of some host-microbe-induced HR-PCD, but there may be parallel pathways that can complement the function of NbCA1 in Pto-mediated HR-PCD.

To further elucidate the pathways required by NbCA1, we assessed the genetic interaction of NbCA1 with Rar1, HSP90 and SGT1—all shown earlier to be required for N function (Peart *et al*, 2002; Liu *et al*, 2002a,b, 2004; Lu *et al*, 2003). Interestingly, accelerated HR-PCD in NbCA1-silenced plants rescued the loss-of-resistance phenotype of Rar1- and HSP90 silencing, and had no effect on the loss-of-resistance phenotype of SGT1 silencing. SGT1-silenced plants have the most robust loss-of-resistance phenotype, and the enhancement of HR-PCD may not be sufficient to rescue the SGT1-silenced phenotype.

The differential requirements of Rar1, HSP90 and SGT1 in NbCA1 RNAi plants for resistance response imply distinct mechanisms by which these proteins function to limit pathogens to infection sites. SGT1 is essential for plant resistance in addition to HR-PCD response. Therefore, our data favour the model that SGT1 may exist in two pools that function upstream and downstream of NbCA1 calcium-initiated signalling. This model would explain the epistatic relationships observed in our co-silencing experiments. There is mounting evidence that Rar1 and HSP90 function either during the accumulation and/or activation of immune receptors (Shirasu, 2009). SGT1 may function upstream in a complex-containing Rar1 and HSP90, which assists in the competent conformation and activation of R protein (Shirasu, 2009). We hypothesize that the initial calcium signal triggered by pathogen perception or recognition is amplified in the absence of NbCA1, which is sufficient to activate downstream components leading to accelerated HR-PCD, and hence, masking the reduction-of-resistance of Rar1 and HSP90 silencing. In addition, SGT1 may have a function downstream of recognition and calcium-initiated signalling by engaging SCF E3 ligase components to execute the degradation of regulators of resistance.

Downregulation of NbCA1 modulates calcium signature in plant immune response

Using a genetically encoded fluorescent calcium sensor, we were able to determine the cytosolic and nuclear calcium signature simultaneously in single cells of living tissue during an immune response. Our data indicate that repetitive calcium oscillations with a similar duration and frequency occur in both cytoplasm and nucleus of cryptogeiin-elicited cells. These observations differ from the earlier studies, which showed that cryptogeiin only triggered a biphasic calcium increase in cytosolic and nuclear compartment within 1 h in suspension cells (Lecourieux *et al*, 2002, 2005), and also different from the studies that showed that elicitors or pathogen effectors triggered a biphasic calcium elevation in intact plants (Grant *et al*, 2000). In both of these studies, the biphasic calcium signature is featured as a transient calcium increase followed by a sustained calcium elevation. Although extracellular calcium concentration and elicitor dose may cause this difference, we believe that the calcium fluctuations derived from the average of stimulated suspension cells or intact tissue does not necessarily reflect calcium signature of individual cells. Furthermore, this difference is compounded by the observed asynchronous calcium oscillation among the cells in elicited tissues (Supplementary Movies 1–4).

So far, there is no evidence supporting a direct function for calcium pump in modulating intracellular calcium signalling events in plants (McAinsh and Pittman, 2009). Given ER is a three-dimensional membrane network in the cell, it is apparent that an ER calcium pump may have a critical housekeeping function in maintaining resting level of cytosolic calcium. However, our results show that downregulation of NbCA1 significantly increases the amplitude of cytosolic and nuclear calcium spikes during cryptogeiin-induced immune response, and it also has effects on duration and frequency of calcium oscillation on application of high elicitor dose. Furthermore, we found that cryptogeiin induces cell death and calcium signature in a dose-dependent manner (Figure 6). These findings clearly indicate that in addition to its housekeeping function, if any, NbCA1 modulates intracellular calcium signature in pathogen-induced calcium-mediated signalling events, and its activity is integrated into intracellular signalling in plant immune response.

Taken together, we propose a model in which recognition of specific effectors or general elicitors by the host immune receptors triggers calcium signalling by activating the calcium influx system in the plasma membrane and/or the endomembrane system to subsequently initiate a calcium release. The initial calcium signal activates ER-localized NbCA1 as well as increases NbCA1 transcription. NbCA1 modulates the magnitude of a calcium signal by controlling the rate of ER calcium efflux. When calcium influx is activated and efflux is reduced in NbCA1-silenced plants, calcium levels remain elevated in the cytoplasm and nucleus. As a consequence, the amplitude of calcium spike is increased, which induces a synergistic effect on the downstream calcium-dependent signalling. When a rapid, large burst of cytosolic calcium is triggered due to high dose of pathogen elicitor or effectors, calcium efflux modulators, like NbCA1, become the determinants of initiation and execution of cell death. This supports the model that HR-PCD can be initiated by early signals released by host–microbe interactions, but the execution of cell death may rely on the amplitude of these signals reaching a threshold required to drive cells to the final stage of death.

Simultaneous cytosolic and nuclear calcium oscillation in single cells revealed in our study suggests that passive calcium diffusions through the nuclear pores, or calcium transport system on nuclear envelopes may contribute to the generation of nuclear calcium oscillation. Given cryptogeiin-triggered calcium transits initially through plasma membrane receptor, the nuclear calcium fluctuation is likely connected to the cytosolic calcium changes via such diffusions and/or calcium-dependent nuclear transport system. However, we do not exclude the possibility that nuclei may perceive directly a stimulus that generates or regulates calcium signalling on its own (Mazars *et al*, 2009).

Molecular targets of calcium signalling

As the disruption of NbCA1 function alters calcium signalling, this provided an excellent system to dissect calcium-signalling components involved in immune response. Earlier studies showed that manipulation of CaM expression altered HR-PCD and basal resistance responses (Heo *et al*, 1999; Chiasson *et al*, 2005; Takabatake *et al*, 2007). These findings lead to the assumption that CaMs are involved in plant defense by sensing the initial signal of cytosolic calcium elevation. Here, we provide *in vivo* evidence that NbCaM1 participates in HR-PCD by directly transmitting the cytosolic calcium signal. In addition, our results imply NbrbohB is another downstream target of calcium signalling in N-immune receptor-mediated HR-PCD. ROS produced by NbrbohB functions as a positive regulator of calcium-dependent HR-PCD in N-mediated immune response. We conclude that NbrbohB and NbCaM1 are two downstream decoders specifically involved in N-TMV-triggered calcium signalling.

Interestingly, we observed the opposite effect of NbrbohB silencing on immune receptor Cf9-, Pto- and non-host pathogen-induced cell death, compared with N-mediated PCD. These findings support the notion that ROS generated by NbrbohB can have opposite functions during HR-PCD, depending on host–microbe systems as indicated in earlier studies (Torres *et al*, 2006). It appears that ROS produced by NbrbohB negatively regulate calcium-dependent cell death in Cf9, Pto and non-host pathogen-induced immune responses. In these systems, ROS appears to be calcium antagonist. These findings imply that relationships between calcium and ROS signalling pathways are more complex than anticipated earlier. The interaction between these two signalling pathways can be both stimulatory and inhibitory, depending on the type of host–microbe interaction system.

In conclusion, here we show that calcium-dependent cell death is involved in plant innate immunity, and the interruption of ER calcium efflux results in accelerated HR-PCD. Furthermore, we show that the ER-Ca²⁺-ATPase actively controls intracellular calcium signalling in addition to contributing to ER calcium storage. As HR-PCD is correlated with resistance during plant immune response in most host–microbe system, understanding how HR-PCD is related to resistance should not only provide insights into the mechanisms enabling resistance but also offer a new avenue for engineering this resistance.

Materials and methods

Plant materials, bacteria and virus strains

NbCA1-RNAi plants were generated by introducing the dsNbCA1 construct into NN plants using Agrobacterium-mediated leaf disc

transformation (Horsch *et al*, 1985). TMV and TMV-GFP were described earlier (Liu *et al*, 2002a). 35S:Cf9 and 35S:Avr9, 35S:Pto and 35S:AvrPto, *P. syringae* pv. *tomato* DC3000 were kindly provided by P De Wit, G Martin and B Staskawicz, respectively.

Plasmid constructs

Full-length NbCA1 cDNA sequence from *N. benthamiana* was amplified by RT-PCR using gene-specific primers. The NbCA1 promoter was amplified using GenomeWalker Universal Kit (Clontech, Palo Alto, CA). NbCA1 cDNA/genomic DNA hybrids (NbCA1cg) was generated by cloning a genomic fragment containing three introns into NbCA1 cDNA clone. The dsNbCA1 construct was created by inserting 1 kb of sense and antisense NbCA1 cDNA flanking a 1.3-kb spacer fragment derived from the first intron of the Arabidopsis potassium transporter into pYL400. The p50-TAP construct was generated by cloning into pZXH103 using GATEWAY cloning system (Invitrogen, CA).

VIGS-cDNA library screening and VIGS of candidate genes

A normalized cDNA library construction in the pTRV2-GATEWAY vector and screening has been described earlier (Liu *et al*, 2005). Silenced plants were infected with TMV-GFP and monitored for HR-PCD or altered resistance by tracking the movement of GFP from the infection sites. GFP imaging was captured under UV illumination using an Olympus Camedia E10 digital camera. We screened about 3000 individual clones, and focused on the clone associated with the accelerated HR-PCD. NbCAM1, NbTPC and NbrbohB cDNA fragments were amplified from the pTRV2-cDNA library using the gene-specific primers and cloned into the pTRV2-GATEWAY vector. pTRV2-SGT1, -Rar1 and -HSP90 constructs were described earlier (Liu *et al*, 2002a, b, 2004). VIGS of NbCAM1, NbTPC, NbrbohB, SGT1, Rar1 and HSP90 were conducted as described (Liu *et al*, 2002b). TMV-p50 or TMV-GFP was transiently expressed using agro-infiltration method in the silenced and vector-infected plants to trigger HR-PCD.

Temperature shift assay

Fully expanded leaves of six-leaf-stage wild-type and *NN* plants were rub infected with TMV-GFP inoculum and were kept at 30°C for 40 h in a growth chamber. Then the infected plants were transferred to 20°C. Samples were collected at different time points.

Quantification of mRNA levels and protein levels

Total RNA prepared from temperature-shifted tissue were isolated using RNeasy Mini Kit (Qiagen, Valencia, CA). First-strand cDNA was synthesized using 2 µg of total RNA, SuperScript reverse transcriptase and Oligo d(T) (Invitrogen, Carlsbad, CA). The transcripts of NbCA1 and EF1α were amplified using primers of NbCA1 and NbEF1α (Supplementary Table 1). PCR products of 25 cycles were separated on agarose gel and documented using FOTO/Analyst Express system.

To quantify GFP and p50 levels in the TMV-GFP infected or p50-expressed plants, leaf discs were sampled from the infiltrated area of each leaf at 0–5 dpi (for TMV-GFP-infected leaves) or at 0–40 h post-infiltration (for p50-expressed leaves). Total protein was extracted from the leaf discs pooled from four leaves at each time point using buffer containing 150 mM NaCl, 20 mM Tris/HCl, 1 mM EDTA, 1% Triton X-100, 0.1% mercaptoethanol, 1 mM PMSF, and complete protease inhibitors (Roche, Indianapolis, IN). The loading of each sample was adjusted as the same leaf disc area per microliter extraction buffer (cm²/ml). Proteins were transferred to PVDF membranes (Millipore, Billerica, MA) and probed using mouse anti-GFP or anti-Myc (Covance) as primary antibody and anti-mouse horseradish peroxidase conjugate (Sigma, St Louis, MO) as secondary antibody.

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Fluorescence microscopy

Confocal imaging analysis was conducted on a Zeiss Axiovert 200 M light microscope equipped with a LSM 510 NLO confocal microscope (Carl Zeiss, Inc., Thornwood, NY). Tissue samples were prepared from *N. benthamiana* leaves transiently expressing given constructs at ~48 h post-infiltration. The 458 and 514 nm laser lines of a 25-mW argon laser (Coherent, Inc., Santa Clara, CA) and 543 nm laser line of a 1-mW helium neon laser (LASOS Lasertechnik GmbH, Germany) with appropriate emission filters were used to image citrine and chloroplast autofluorescence, respectively.

Complementation of yeast mutants

NbCA1 and NbCA1ΔN were cloned into pYES2. Yeast strain K616 (MATa, pmr1::HIS3, pmc1::TRP1, cnb1::LEU2, ura3) and strain K601 (MATa, leu2, his3, ade2, trp1, ura3) was transformed with pYES2-NbCA1-citrine, pYES2-NbCA1ΔN-citrine and the pYES2 vector. The transformants were selected for uracil prototrophy by plating on synthetic media lacking uracil (SD-Ura). For complementation experiments, Ura⁺ colonies were streaked on SD-Ura/Gal and SD-Ura/Glu plates containing either 10 mM CaCl₂ or 10 mM EGTA (pH 5.5).

Expression and purification of cryptogein

Expression and purification of cryptogein was performed according to O'Donohue *et al* (1996) by using the *Pichia pastoris* bearing the plasmid pLEP3 (kindly provided by Professor Jean-Claude Pernollet).

Measurement of calcium in single cells of cryptogein-inoculated leaves

Case12 sensor (Axxora, LLC, CA) was cloned into expression vector pYL400 and transiently expressed in *NN* and NbCA1-RNAi plants using agro-infiltration. Case12-expressed leaves were inoculated with 5–25 µg/ml of cryptogein just before confocal imaging. Green fluorescent emission of Case12 was collected at 500–540 nm in the same excitation and time series setting for all samples. The amplitude of calcium spike was calculated as the value of the peak intensity minus resting intensity from 11 to 14 representative cells in three independent experiments. For each cell, the amplitude of nuclear calcium spike represents an average of multiple spikes, and the amplitude of cytosolic calcium spike represents an average of multiple spikes from 3 to 4 regions of interest in the cytoplasm. Duration was calculated as an interval of time between the initiation and the completion of spikes. Frequency was expressed as an interval of time between the peaks of two spikes. *t*-test was used to compare the means.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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