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The chimeric cyclic nucleotide-gated ion channel ATCNGC11/12 constitutively induces programmed cell death in a Ca^{2+} dependent manner

William Urquhart · Arunika H. L. A. N. Gunawardena · Wolfgang Moeder · Rashid Ali · Gerald A. Berkowitz · Keiko Yoshioka

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Abstract The hypersensitive response (HR) involves programmed cell death (PCD) in response to pathogen infection. To investigate the pathogen resistance signaling pathway, we previously identified the *Arabidopsis* mutant *cpr22*, which displays constitutive activation of multiple defense responses including HR like cell death. The *cpr22* mutation has been identified as a 3 kb deletion that fuses two cyclic nucleotide-gated ion channel (CNGC)-encoding genes, *ATCNGC11* and *ATCNGC12*, to generate a novel chimeric gene, *ATCNGC11/12*. In this study, we conducted a characterization of cell death induced by transient expression of *ATCNGC11/12* in *Nicotiana benthamiana.* Electron microscopic analysis of this cell death showed similar characteristics to PCD, such as plasma membrane shrinkage and vesicle formation. The hallmark of animal PCD, fragmentation of nuclear DNA, was also observed in *ATCNGC11/12*-induced cell death. The development of cell death was significantly suppressed by caspase-1 inhibitors, suggesting the involvement of caspases in this process. Recently, vacuolar processing enzyme (VPE) was isolated as the first plant caspase-like protein, which is involved in HR development. In *VPE*-silenced plants development of cell death induced by *ATCNGC11/12* was much slower and weaker compared to control plants, suggesting the involvement of VPE as a caspase in *ATCNGC11/12*-induced cell death. Complementation analysis using a Ca^{2+} uptake deficient yeast mutant demonstrated that the *ATCNGC11/12* channel is permeable to Ca^{2+}. Additionally, calcium channel blockers such as $GdCl_3$ inhibited *ATCNGC11/12*-induced HR formation, whereas potassium channel blockers did not. Taken together, these results indicate that the cell death that develops in the *cpr22* mutant is indeed PCD and that the chimeric channel, ATCNGC11/12, is at the point of, or up-stream of the calcium signal necessary for the development of HR.

Keywords *Arabidopsis* · Cyclic nucleotide-gated ion channel (CNGC) · Programmed cell death · Hypersensitive response · HR · Caspase · Calcium · VPE

W. Urquhart · A. H. L. A. N. Gunawardena · W. Moeder · K. Yoshioka (✉)
Department of Cell and Systems Biology, University of Toronto, 25 Willcocks Street, Toronto, ON, Canada M5S 3B2
e-mail: yoshioka@csb.utoronto.ca

W. Urquhart · W. Moeder · K. Yoshioka
Center for the Analysis of Genome Evolution and Function (CAGEF), University of Toronto, 25 Willcocks Street, Toronto, ON, Canada M5S 3B2

Present Address:
A. H. L. A. N. Gunawardena
Biology Department, Life Science Center, Dalhousie University, 1355 Oxford Street, Halifax, NS, Canada B3H 4J1

R. Ali · G. A. Berkowitz
Department of Plant Science, University of Connecticut, Storrs, CT 06269, USA

Introduction

To fight against numerous pathogenic microbes, plants have evolved a large number of defense systems. Generally, a strong and rapid defense response is triggered by the direct or indirect interaction between the products of a plant resistance (*R*) gene and a cognate pathogen avirulence (*avr*) gene (Flor 1971; Jones and Dangl 1996; Hammond-Kosack and Jones 1996).

One of the defense mechanisms frequently activated following this *R* gene-mediated pathogen recognition is the

hypersensitive response (HR), which is manifested as rapid cell death at the site of, or surrounding the point of, pathogen entry (Heath 2000; Hammond-Kosack and Jones 1996). The HR cell death can be associated with resistance and in some cases it can prevent the growth and spread of pathogens into healthy tissue (Dangl et al. 1996). Extensive studies have revealed that in general this HR-cell death is a type of programmed cell death (PCD), which is an organized and active destruction of cells (Heath 2000). PCD is involved in basic physiological processes that control development and various stress responses in plants and animals. Several studies have shown that PCD in plants, yeast, and animals share similar characteristics, such as chromatin condensation and DNA fragmentation (Lam 2004; van Doorn and Woltering 2005). Furthermore, vacuolar processing enzyme (VPE) was recently identified as the first plant enzyme which possesses caspase like activity and is involved in the induction of HR in tobacco after tobacco mosaic virus infection (Hatsugai et al. 2004). Since it has been well known that caspases, which are specialized cysteine proteases, are a prominent control point of apoptosis in animals (Baehrecke 2002), the finding of VPE further demonstrated the conservation of cell death signaling components among various organisms.

The early events after pathogen recognition, before the HR development, have been studied intensely. Two of these events, a rapid bi-phasic increase in reactive oxygen species, known as the oxidative burst, and ion flux changes, are frequently observed after pathogen infection (Grant et al. 2000). An influx of H^+ and Ca^{2+} and an efflux of K^+ and Cl^- were reported by Atkinson et al. (1996). In particular Ca^{2+} influx was shown to play a role in activating the oxidative burst following elicitor treatment (Chandra et al. 1997; Jabs et al. 1997; Sasabe et al. 2000; Kadota et al. 2004), and in activating pathogen or elicitor-induced cell death in tobacco, cowpea, and soybean (Atkinson et al. 1990; Levine et al. 1996; Xu and Heath 1998; Sasabe et al. 2000). Moreover, Ca^{2+} has been implicated in signaling SA-induced *PR* gene expression and other resistance responses (Raz and Fluhr 1992; Doke et al. 1996).

Over the last decade, considerable efforts have been made to understand the signal transduction pathway that activates the aforementioned hallmark events. These efforts have revealed many important factors in the plant defense signaling pathway. For example, screening of enhanced susceptibility mutants led to the discovery of *PAD4*, *EDS1* and *NDR1* as key components of *R* gene-mediated resistance to distinct groups of pathogens (Century et al. 1995; Glazebrook et al. 1996; Parker et al. 1996). Conversely, enhanced resistance mutants were identified by their constitutive expression of *PR-1* gene, elevated SA levels and heightened resistance to virulent and avirulent pathogens (Yu et al. 1998; Glazebrook 1999, 2001; Jambunathan et al. 2001; Yoshioka et al. 2001; Balague et al. 2003). However, despite this remarkable progress, a complete signaling pathway that activates resistance responses has yet to be outlined.

To identify novel signaling components involved in activating defense responses after pathogen attack, we previously screened T-DNA tagged lines from the *Arabidopsis* Wassilewskija ecotype for individuals that constitutively expressed the marker gene *PR-1*. *cpr22* was identified through this screening and it falls into the category of lesion mimic mutants (Yoshioka et al. 2001). Lesion mimic mutants are a broad class of morphological mutants that exhibit spontaneous lesion formation in the absence of a pathogen (Lorrain et al. 2003). A variety of lesion mimic mutants have been isolated in maize, rice, barley and *Arabidopsis*. These mutants exhibit not only spontaneous lesion formation resembling that of pathogen-induced HR cell death, but also exhibit alterations in pathogen resistance and expression of some defense related genes in the absence of pathogens. Therefore, it has been hypothesized that some of these mutants have alterations in genes that play important roles in the pathogen resistance signaling pathway (Lorrain et al. 2003). Thus, lesion mimic mutants could be useful tools to identify the key components in pathogen resistance signaling (Lorrain et al. 2003).

Recently we have identified the *cpr22* mutation as a 3 kb deletion that fuses two cyclic nucleotide-gated ion channel (CNGC)-encoding genes, *ATCNGC11* and *ATCNGC12*, generating a novel chimeric gene, *ATCNGC11/12* (Yoshioka et al. 2006). Genetic, molecular and complementation analyses suggest that ATCNGC11/12, as well as ATCNGC11 and ATCNGC12 form functional CNGCs, and that the *cpr22* phenotype is due to expression of *ATCNGC11/12*. Furthermore, we have demonstrated that transient expression of this chimera gene, but not *ATCNGC11* or *ATCNGC12* genes, induces rapid cell death in *Nicotiana benthamiana* leaves (Yoshioka et al. 2006).

The objective of this work is to further characterize the cell death induced by ATCNGC11/12 in order to better understand the molecular mechanisms underlying this induced cell death and explore the possible usage of *ATCNGC11/12* for research on PCD as well as on pathogen resistance signaling. For this purpose we have used *Agrobacterium*-mediated transient expression of *ATCNGC11/12*, since this system provides us with a more synchronized development of cell death in the infiltrated area. Here, we demonstrate that the cell death induced by ATCNGC11/12 exhibits clear characteristics of PCD and a caspase-like enzyme is involved in cell death development. Furthermore, we show that ATCNGC11/12 can function as a Ca^{2+}-conducting channel and calcium ions appear to play a crucial role in the cell death induced by ATCNGC11/12.

Materials and methods

Plant material

Nicotiana benthamiana plants were grown on Pro-Mix soil (Premier Horticulture) in a greenhouse under a 14:10 h light:dark regimen at 25°C (day) 20°C (night) with ~65% humidity.

Construction of plasmids

The cDNAs of *ATCNGC11*, *ATCNGC12* and *ATCNGC11/12* were cloned from *Arabidopsis* ecotype Wassilewskija wild type and *cpr22* mutant plants. These cDNAs were subsequently sub-cloned into the plant expression vector pMBP3 as described previously (Yoshioka et al. 2006).

Agrobacterium-mediated transient expression

Agrobacterium tumefaciens-mediated transient expression in *Nicotiana benthamiana* was performed as described by Sessa et al. (2000). The *A. tumefaciens* strain GV2260 (final density of 0.5 OD_{600}) was used to syringe-infiltrate *N. benthamiana* leaves.

Transmission electron microscopy

Tissue samples from leaves were harvested 48 h after *Agrobacterium* infiltration and fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 6.9, overnight under vacuum (20 p.s.i.). The rest of the procedure was performed as described previously (Gunawardena et al. 2004).

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay

Leaf samples were harvested from the *Agrobacterium* infiltrated area 36 h after infiltration and immediately fixed in formol acetic alcohol (FAA) solution for 12 h with vacuum infiltration being used for the first 3 h. Subsequently, samples were washed by 70% ethanol and sliced by a microslicer. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was performed according to the manufacture's instructions (Roche Diagnostics, Mannheim, Germany) and nuclei were stained by incubating in 2 μg/ml 4′-6-Diamidino-2-phenylindole (DAPI) solution for 10 min. A negative control was performed without terminal deoxynucleotidyl transferase enzyme, and a positive control was performed using DNase1. Samples were observed under a Zeiss Imager Z1 AXIO inverted compound microscope (Carl Zeiss Canada, Toronto, Ontario) fitted with the following configuration: excitation at 365 nm and emission at 445/59 nm (bandpass) for DAPI, and excitation 470/40 nm (bandpass) and emission 525/50 nm (bandpass) for TUNEL. Images were captured using a CCD camera (Hamamatsu ORCA.ER C4742-80). *ATCNGGC11/12* without GFP tag was used to prevent interference by GFP fluorescence.

RNA extraction and RT-PCR

Small-scale RNA extraction was performed using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

RT-PCR analyses for expression of three genes, *ATCNGC11/12*, *VPE* and *EF-1* were performed with primers *ATCNGC11/12*, cDNA-13-Fwd17 (5′-AGAAGG TTTACTGGAGG-3′) and F11C10-14-R3 (5′-GGAAGG CGAGAACCATT-3′); *VPE*, VPE-f (5′-CAACTGTTGAA GAAGGGTGGTCTC-3′) and VPE-r (5′-GATACTCGCA AGGAATTGTCATCC-3′); *EF-1*, Nb-EF-1-f (CTTCCT ACCTCAAGAAGGTAGGATACAAC) and Nb-EF-1-r (TGCCTCCTGAAGAGCTTCGTGGTGCAT), respectively using cDNA generated by Super-Script II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Pharmacological analyses

One millimolar caspase-I inhibitor, Ac-YVAD-CMK and Ac-YVAD-CHO (CALBIOCHEM) were made in a 0.5% DMSO solution and co-infiltrated with *Agrobacterium* carrying the *ATCNGC11/12* gene. The same DMSO solution was used as the control solution. One millimolar protease inhibitor, PMSF (Sigma) was used as a non specific protease inhibitor.

To study the effect of the ion channel inhibitors, we used Tetraethylammonium chloride (TEA) as a K^+ channel blocker and $LaCl_3$ and $GdCl_3$ (all from Sigma) as Ca^{2+} channel blockers. Concentrations are specified in the Figure legends.

Development of VPE silenced *Nicotiana benthamiana* using virus-induced gene silencing (VIGS)

The VIGS assays were performed using the tobacco rattle virus (TRV) system (Liu et al. 2002). A 408 bp DNA fragment of the *VPE* gene was amplified using the primers

VIGS-VPE-f (5′-CGGGATCCGGAGTTATCATTAATAG CCC-3′) and VIGS-VPE-r (5′-ATCTCGAGCCCAGCTAC TCTCTTCAGC-3′) and cloned into the TRV2 vector (pYL156). The vector was transformed into the *Agrobacterium tumefaciens* strain GV2260. Young *N. benthamiana* plants were inoculated by pricking the cotyledons and the lower leaves (2–3 times each) with *Agrobacterium* strains carrying TRV1 and either the empty TRV-vector or the TRV::VPE construct. The plants were used for transient expression analysis about 3 weeks after the VIGS treatment.

Complementation of *cch1*, *mid1* response to mating pheromone

The cDNAs encoding plant ATCNGC11, ATCNGC12, and ATCNGC11/12 were subcloned into the pSM1052 vector with a GFP tag for expression and functional analysis in yeast as described previously (Ali et al. 2006). The subcloning was done using PCR and sequence-specific primers with Not1 and Kpn1 restriction sites at the 5′ and 3′ ends, respectively, Vent DNA polymerase (Stratagene; La Jolla, CA), and AtCNGC cDNAs (described above) as template. The Ca^{2+} uptake deficient yeast (*Saccharomyces cerevisiae*) strain ELY151 (*mid1, cch1*) was transformed with the empty vector or the pSM1052 vector containing the GFP-tagged channel coding sequences. This assay was performed as previously described (Ali et al. 2006). Yeast cells were grown in selective SC medium (SC-trp, -leu, -ura) overnight, washed three times with sterilized Milli Q water, and then resuspended in liquid YPD medium to an O.D. 600_{nm} of 4. An aliquot (100 µl) of this resuspension was added to 4 ml of top agar (YPD with 0.7% agar), mixed, and overlaid on YPD agar plates. Immediately after solidification of the top agar-containing cells, sterile cellulose discs (0.6 cm; Schleicher & Schuell, Keene, NH, USA) with 50 µg of synthetic α-factor were placed on the nascent lawn. The plates were incubated at 30°C and photographed after 72 h. Fluorescence and bright field images of *mid1, cch1* yeast were recorded using an Olympus IX70 (Olympus, Melville, NY) inverted microscope and CCD camera as described previously (Ali et al. 2006).

Results

Timing of cell death development by ATCNGC11/12 in *N. benthamiana*

In the homozygous state the *cpr22* mutation leads to a lethal phenotype unless the plants are grown under high relative humidity (RH 95%, Yoshioka et al. 2001). Therefore, the homozygous mutant is not convenient to conduct experiments to observe cell death development. On the other hand, *cpr22* heterozygous plants develop many clusters of spontaneous cell death constitutively under normal conditions (Fig. 1A) and complete their normal life cycles. However cell death development is not well synchronized. Thus, the investigation of the cell death process in lesion mimic mutants generally has been hindered by this lack of synchronicity. To assess the development of *cpr22*-mediated cell death, a system that can induce cell death synchronously is required. *Agrobacterium*-mediated transient expression in *N. benthamiana* fulfills these requirements (Fig. 1B and Yoshioka et al. 2006). Previously we already showed that expression of *ATNGC11/12* induces cell death in *N. benthamiana* (Yoshioka et al. 2006). However, here we utilized the synchronicity of cell death induction to investigate the development of ATCNGC11/12-induced cell death in a time course experiment. First, to visualize the correlation between cell death development and the expression of *ATCNGC11/12*, an *ATCNGC11/12*-green fluorescent protein (GFP) fusion gene under the control of the constitutive CaMV35S promoter was generated. To assess whether the addition of GFP protein to the carboxy terminus of ATCNGC11/12 causes alterations in its ability to induce cell death, both *ATCNGC11/12* and *ATCNGC11/12-GFP* were expressed via *Agrobacterium* infiltration in *N. benthamiana.* As expected, there was no difference in cell death development in terms of timing and intensity (data not shown). Therefore, the *ATCNGC11/12-GFP* fusion gene was used in subsequent experiments except the TUNEL assay. Visual observation indicated that approximately 12 h after *Agrobacterium* infiltration, infiltrated areas of the leaf began to display discernable weak green fluorescence, indicating expression of *ATCNGC11/12*. However, there was no visible change to the infiltrated area (Figs. 1C-1 and 2, 12 hpi). The intensity of this green fluorescence gradually increased and at 36 h after infiltration, almost all the infiltrated area displayed observable green fluorescence. Around this time, the infiltrated area started to turn brown in color (Fig. 1C-1, 36 hpi). This brownish area gradually expanded until around 48 h after infiltration when the entire infiltrated area had become brown and started to desiccate. The expansion of the desiccated brownish area was restricted to the infiltrated region and was well synchronized (Fig. 1C-1, 48 hpi). The texture of the desiccated leaf section resembled that of HR induced by *R*-gene mediated resistance responses, as opposed to necrotic cell death, during which a water-soaked texture is often observed. Trypan Blue staining was performed to assess whether this browning of tissue was actually cell death. As we expected, the browned regions were extensive areas of dead cells which appeared as dark stained dots under the microscope and the development of this cell death was well synchronized (Fig. 1C-3). As the

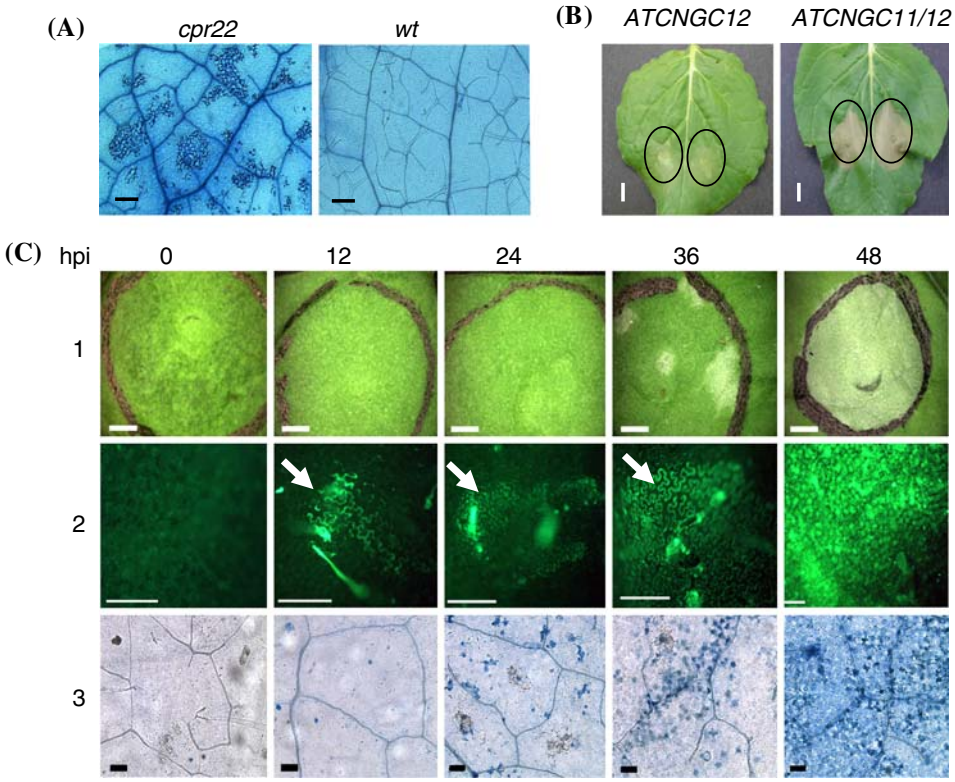

Fig. 1 Transient expression *of ATCNGC11/12* induces cell death in *N. benthamiana*. (**A**) Microscopic analysis of Trypan Blue-stained leaves of 3-week old *Arabidopsis cpr22* mutant plants revealed patches of intensely stained dead cells, which were not visible in wt plants. Bars = 50 µm. (**B**) Development of cell death in *N. benthamiana* plants after transient expression of *ATCNGC11/12* but not *ATCNGC12*. Bars = 1 cm. (**C**) Development of cell death in *N. benthamiana* plants after transient expression of *ATCNGC11/12*. (1) Visual lesion formation in *N. benthamiana* leaves at 0, 12, 24, 36 and 48 h post inoculation (hpi) of *Agrobacterium* carrying *ATCNGC11/12*. Bars = 0.25 cm. (2) Expression of *ATCNGC11/12::GFP* in *N. benthamiana* leaves was monitored by fluorescence microscopy at 0, 12, 24, 36 and 48 hpi. Arrows indicate GFP fluorescence. Bars = 2.5 mm. (3) Microscopic analysis of Trypan Blue stained in *ATCNGC11/12*-infiltrated leaves at 0, 12, 24, 36 and 48 hpi indicated progressing cell death in the infiltrated area. Bars = 10 µm

infiltrated area became progressively browner, a significant increase in the extent and intensity of green fluorescence was observed (Fig. 1C-2, 48 hpi). This increased fluorescence in the late stage was tightly correlated with the browning area and was later determined to be auto-fluorescence of dying cells. In contrast, infiltration of *Agrobacterium* carrying an empty vector (only GFP) or wild type genes (*ATCNGC11 or ATCNGC12*) did not induce cell death (data not shown; Yoshioka et al. 2006). Taken together, we concluded that the expression of *ATCNGC11/12* induces cell death 36–48 h after *Agrobacterium* infiltration in a synchronized manner and this system is useful to study the process of the cell death development.

Morphology of ATCNGC11/12-induced cell death

As already mentioned above, the *Arabidopsis cpr22* mutant which carries the novel chimeric gene *ATCNGC11/12* develops spontaneous lesions and falls into the category of "lesion mimic mutants" (Yoshioka et al. 2001; Lorrain et al. 2003). Several lines of evidence indicated that the expression of *ATCNGC11/12* induces HR-cell death (PCD) similar to the process seen in an incompatible plant pathogen interaction (Yoshioka et al. 2006). Additionally, as we mentioned above, the texture of the dead tissue induced by *ATCNGC11/12* expression resembled that of the HR induced by *R*-gene mediated resistance responses, further supporting this hypothesis. However, the possibility that the expression of *ATCNGC11/12* perturbs cellular physiology and causes necrotic cell death could not be ruled out. To explore this possibility, the morphological characteristics of the cell death caused by *ATCNGC11/12* in *N. benthamiana* leaves were investigated by transmission electron microscopy. The characteristics of PCD during development and defense have been studied well and hallmarks of PCD were well documented (Levine et al. 1996; Groover et al. 1997; Fukuda 2000; Mittler et al. 1997). A number of these hallmarks of PCD were observed in *ATCNGC11/12* expressing cells 48 h after

Agrobacterium infiltration. Control cells showed normal ultrastructure with intact plasma membrane, vacuole, nuclei and other organelles (Fig. 2A). In dying cells expressing *ATCNGC11/12*, retraction of the plasma membrane from the cell wall, vacuoles filled with granular material and degradation of the tonoplast (Fig. 2D), invagination of plasma membrane with abundant vesicles present near the plasma membrane (Fig. 2G) and condensed cytoplasm were observed (Fig. 2H). All these characteristics were similar to PCD observed in tobacco cells after tobacco mosaic virus infection (Mittler et al. 1997). At higher magnification, degrading organelles were observed in the dying cells (Fig. 2E), but not in control cells (Fig. 2B). Nuclei were intact, with abundant condensed chromatin in dying cells (Fig. 2F) but not in the control cells (Fig. 2C). This type of condensation of chromatin was also observed previously in soybean cell suspension cells treated with an avirulent strain of *Pseudomonas syringae* pv. *glycinea* (Levine et al. 1996). In dying cells degrading organelles such as chloroplasts were observed; however they remained mostly intact (Fig. 2H, E). In some cells that displayed a more progressed stage of cell death, collapsed organelles and other remnants of cytoplasmic components were present (Fig. 2I). Plasmodesmata were visible within the cell wall but were disrupted by cytoplasm retraction. The cell walls were distorted, but appeared to remain intact (Fig. 2I).

Although we have seen these characteristics of different stages of cell death, the majority of dying cells was at a similar stage of PCD, indicating that cell death was induced synchronously as we expected. Taken together, the cell death induced by the expression of *ATCNGC11/12* in *N. benthamiana* leaves showed morphological characteristics of PCD.

ATCNGC11/12 induces DNA fragmentation

Genomic DNA fragmentation is a common phenomenon occurring during plant and animal PCD. There is a well characterized 180-mer laddering pattern that occurs in animal cells during PCD. This laddering of genomic DNA is also present in several types of plant PCD including cell death caused by viral and fungal infection (Ryerson and Heath 1996; Mittler et al. 1997). To better characterize the cell death induced by the expression of *ATCNGC11/12*, the possible presence of genomic DNA fragmentation and laddering was first investigated over 48 h after *Agrobacterium* infiltration by agarose gel electrophoresis and a subsequent Southern blot analysis. An increase in DNA

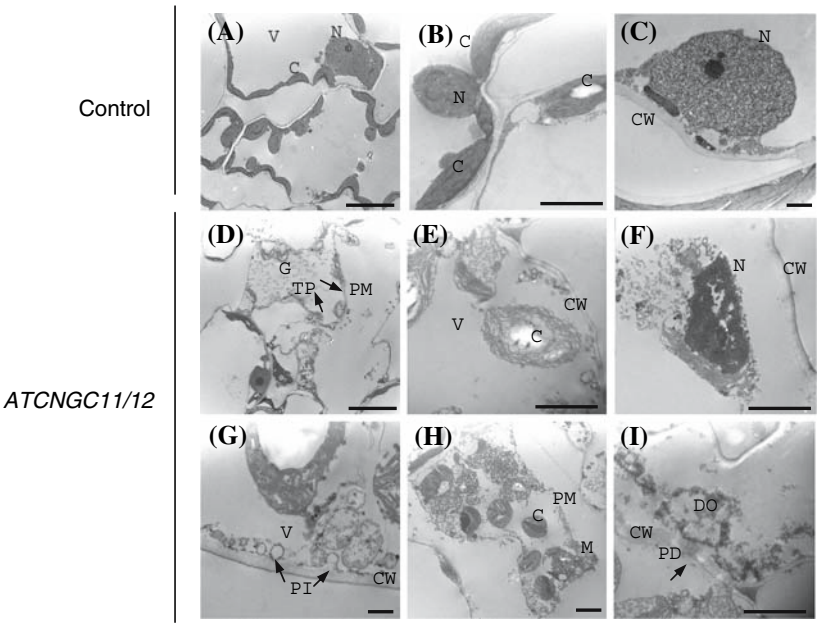

Fig. 2 *ATCNGC11/12* expressing *N. benthamiana* leaves exhibit microscopic characteristics of programmed cell death. Transmission electron micrographs of *N. benthamiana* tissue 48 hpi with *Agrobacterium* carrying *ATCNGC11/12* (**D**–**I**) or uninfiltrated control tissue (**A**–**C**). In *ATCNGC11/12*-expressing tissue the plasma membrane retracted from the cell wall and the tonoplast started to degrade (**D**), whereas control cells exhibited normal ultrastructure (**A**). Chloroplasts started to degrade in *ATCNGC11/12*-expressing (**E**) but not control tissue (**B**). Nuclei of *ATCNGC11/12*-expressing but not control tissue contained large amounts of condensed chromatin (**C** and **F**). Invagination of the plasma membrane, vesicle formation (**G**) and condensed cytoplasm (**H**) were observed in *ATCNGC11/12*-expressing tissue. Plasmodesmata were visible within the cell wall but were disrupted by cytoplasm retraction (**I**). C, Chloroplast; CW, cell wall; DO, Degraded organelles; G, Granular material; M, Mitochondria; N, Nucleus; PD, Plasmodesmata; PI, Plasma membrane Invagination; PM, Plasma membrane; TP, Tonoplast; V, vacuole. Bars = 10 μm (**A**, **B**), 500 nm (**G**), 2 μm (**C**, **D**, **E**, **F**, **H** and **I**)

smearing was observed at 36 and 48 h in *ATCNGC11/12*-expressing tissues; however we were unable to identify a clear laddering pattern at the time points taken (data not shown).

The degradation of genomic DNA in the late stages of PCD generates DNA strands with exposed 3′-hydroxyl ends. The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay is commonly used to detect these 3′ hydroxyl ends in such plant processes as development, senescence, and response to abiotic and biotic stresses (Gunawardena et al. 2001, 2004; Tada et al. 2001; Pasqualini et al. 2003; Coupe et al. 2004). In order to further asses the presence of DNA fragmentation, a TUNEL assay was conducted 36 h after *Agrobacterium* infiltration. As shown in Fig. 3, TUNEL-positive nuclei were only observed in samples infiltrated with *Agrobacterium* carrying *ATCNGC11/12*, but not in control samples that were infiltrated with empty *Agrobacterium*, indicating that *ATCNGC11/12* expression induced the observed DNA fragmentation (Fig. 3A, B). Furthermore, to assess if this fluorescence was due to the TUNEL staining, not auto-fluorescence, we also examined a control sample that was infiltrated by *Agrobacterium* carrying *ATCNGC11/12* but was treated without terminal deoxynucleotidyl transferase enzyme. TUNEL-positive nuclei were not observed in these samples confirming that the positive result by *ATCNGC11/12* was authentic (Fig. 3C). 4′-6-Diamidino-2-phenylindole (DAPI) staining was used to identify all nuclei and overlay images showed co-localization of TUNEL- and DAPI-positive spots. A non-infiltrated leaf sample that had been treated with DNase served as a positive control for the procedure (Fig. 3D).

Involvement of caspases and vacuolar processing enzyme (VPE)

Caspases are conserved cysteine proteases that regulate animal PCD (Whyte 1996). A growing body of evidence suggests that caspase-like proteases are an important signaling step in the development of PCD in plants (Lam 2004; Hara-Nishimura et al. 2005). To further characterize the cell death induced by *ATCNGC11/12*, the requirement of cysteine proteases for the development of cell death was assessed. For this assay the caspase1-specific synthetic inhibitors Ac-YVAD-CMK and Ac-YVAD-CHO were used. Ac-YVAD-CMK and Ac-YVAD-CHO have been effectively used to abolish HR-cell death development through inhibiting caspase like protease activity in tobacco inoculated with pathogens (del Pozo and Lam 1998; Hatsugai et al. 2004). When Ac-YVAD-CMK or Ac-YVAD-CHO was infiltrated together with *ATCNGC11/12*-carrying *Agrobacterium*, development of cell death was clearly suppressed compared to controls (Fig. 4A). This suppression was not observed in the samples in which only 0.5% DMSO or the non-specific protease inhibitor, PMSF were co-infiltrated with *Agrobacterium*. Trypan Blue staining also revealed similar amounts of dead cells in DMSO control and PMSF-treated samples, whereas a significantly lower number of dead cells were detected in the caspase inhibitor-treated samples (Fig. 4A). This result was also confirmed by ion-leakage measurements where a significant reduction was observed in the caspase-inhibitor-treated samples but not in the PMSF or DMSO-treated samples (Fig. 4C). To verify whether this suppression was

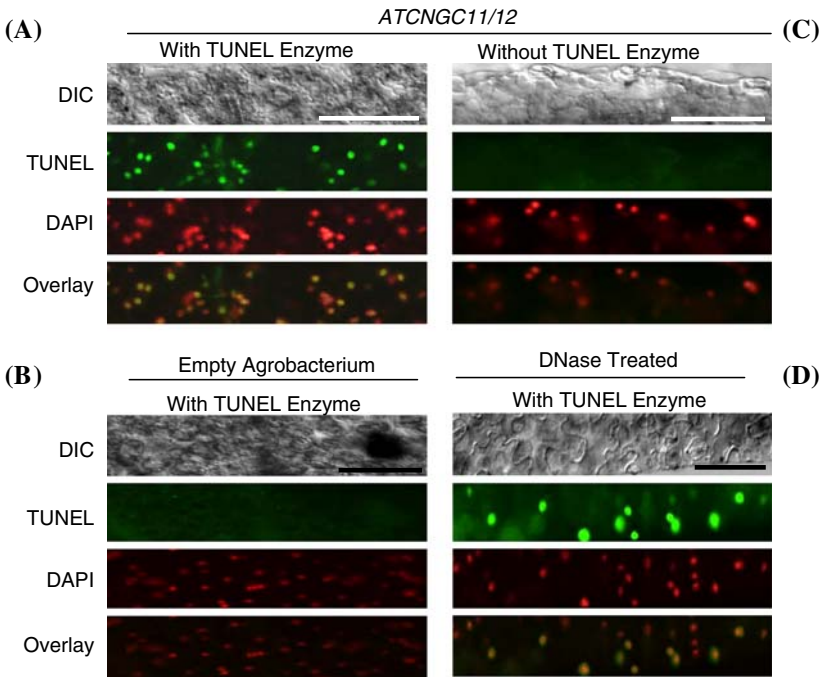

Fig. 3 Confocal microscopy detected DNA fragmentation by TUNEL assay. Leaf tissue was harvested at 36 hpi with *Agrobacterium* carrying *ATCNGC11/12* and nuclei with fragmented DNA were visualized by TUNEL stain (green). DAPI stain depicts all nuclei for comparison (red) which over layed with the TUNEL stained nuclei (yellow, **A**). Leaf tissue infiltrated with empty *Agrobacterium* (**B**) or a control without the TUNEL enzyme (**C**) did not show green fluorescence. Tissue that had been treated with DNase served as a positive control for the procedure (**D**). For better visualization of overlay pictures, the color of DAPI staining is artificially modified from blue to red. Bars = 0.1 mm

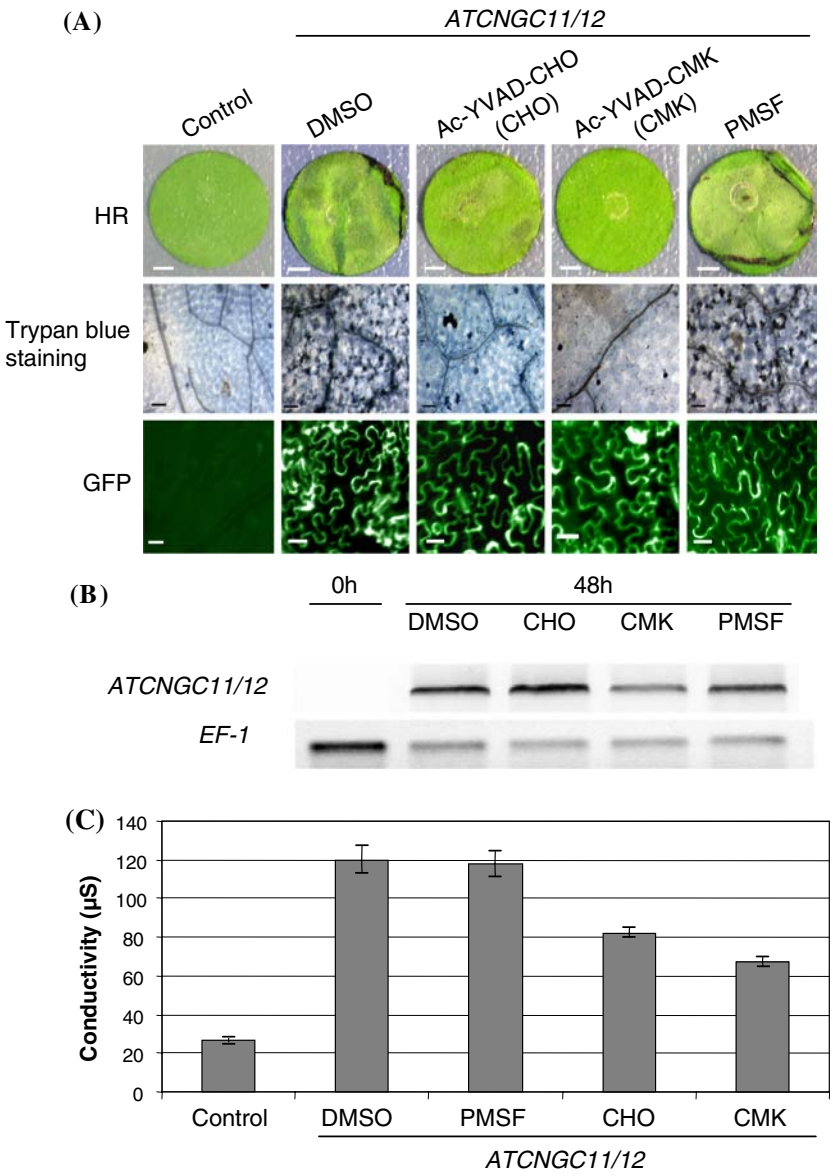

Fig. 4 Caspase 1 inhibitors suppress cell death development induced by *ATCNGC11/12* expression in *N. benthamiana* leaves. (**A**) Photographs of *N. benthamiana* leaves 48 hpi with *Agrobacterium* carrying *ATCNGC11/12* with caspase 1 inhibitors, Ac-YVAD-CHO (CHO) and Ac-YVAD-CMK (CMK), and a non-specific protease inhibitor, PMSF (all 1 mM). 0.5% DMSO served as a buffer control. Tissue without *ATCNGC11/12* expression was used as a control sample. Trypan Blue staining indicates cell death development in samples with DMSO and PMSF, but not in samples with caspase 1 inhibitors. GFP analysis of epidermal peels showed no effect on translation of *ATCNGC11/12* with any treatments. Scale bars for leaf discs = 0.5 cm. Scale bars for Trypan Blue = 12.5 µm. Scale bars for GFP = 5.5 µm. (**B**) RT-PCR of leaf discs from *ATCNGC11/12*-expressing *N. benthamiana* leaves 0 hpi and samples with various treatments at 48 hpi. EF-1, elongation factor 1, served as a loading control. (**C**) Electrolyte leakage of leaf discs from *ATCNGC11/12*-expressing *N. benthamiana* leaves at 48 hpi

not due to the inhibition of *Agrobacterium*-mediated transient expression, transcription and translation of *ATCNGC11/12* was confirmed by RT-PCR and GFP fluorescence. As shown in Fig. 4A and B, respectively, no difference in GFP fluorescence or *ATCNGC11/12* transcript levels were observed in Ac-YVAD-CMK, Ac-YVAD-CHO or PMSF treated compared to control samples. Thus, this data suggests the involvement of caspase-like activity in *ATCNGC11/12*-induced cell death.

Vacuolar processing enzyme (VPE) was recently identified as the first plant caspase-like protein, which is essential for virus-induced HR-cell death development (Hatsugai et al. 2004). To investigate whether VPE is involved in ATCNGC11/12-induced cell death, we silenced *VPE* using virus-induced gene silencing (VIGS) in *N. benthamiana* plants. A tobacco rattle virus-based vector (TRV, Liu et al. 2002) was used. RT-PCR analysis showed that the expression of the *VPE* gene was significantly lower or completely silenced in these plants (Fig. 5A). The primers used for this RT-PCR analysis can detect two VPE genes of *N. benthamiana*, *NbVPE-1a* and *NbVPE-1b* that correspond to the most abundant *VPEs* found in *N. tabacum* in tobacco mosaic virus-infected leaves (Hatsugai et al. 2004). *NbVPE1a/b* also show the highest homology to *VPEγ*, which is the most strongly induced isoform in *Arabidopsis* after pathogen infection (Rojo et al. 2004). In *VPE*-silenced plants the development of *ATCNGC11/12*-induced cell death was much slower and weaker compared to control plants (Fig. 5B). This visual suppression of HR was also supported by reduced Trypan Blue stained cells and reduced electrolyte leakage in leaves silenced for *VPE* (Fig. 5B, C).

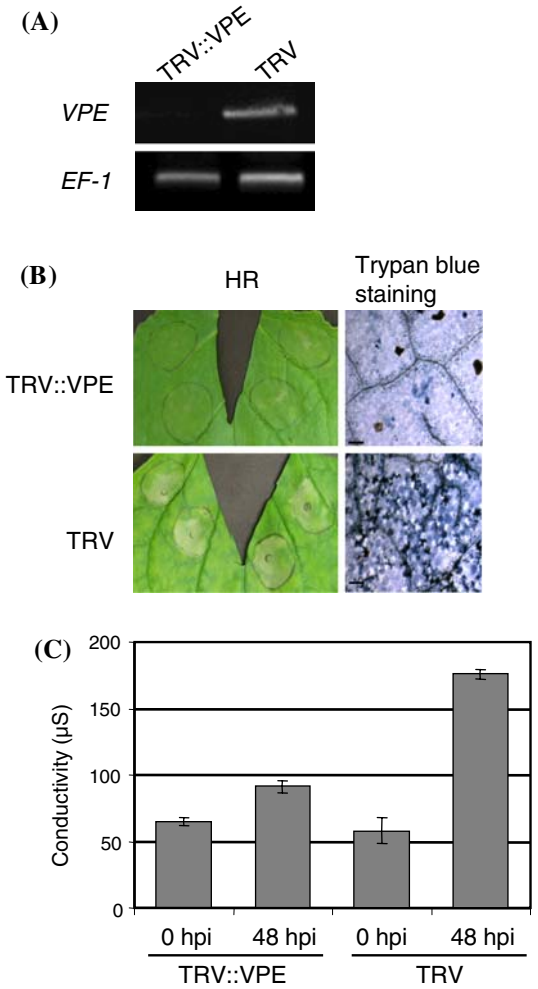

Fig. 5 Virus-induced gene silencing (VIGS) of vacuolar processing enzyme (VPE) attenuates *ATCNGC11/12* induced cell death in *N. benthamiana*. (**A**) RT-PCR analysis of *VPE* gene expression in 5-week old control (TRV) and *VPE*-silenced (TRV::VPE) plants. (**B**) Photograph of lesion formation and Trypan blue staining in control (TRV) and *VPE*-silenced (TRV::VPE) plants 48 hpi of *Agrobacterium* carrying *ATCNGC11/12*. Bar = 15.5 µm. (**C**) Electrolyte leakage of control and *VPE*-silenced plants at 0 and 48 hpi

Thus, together with the result from the caspase1 inhibitor assay, we concluded that caspase-like activity is required for the development of *ATCNGC11/12*-induced cell death.

ATCNGC11, 12 and ATCNGC11/12 can function as Ca^{2+} channels in yeast

Several *Arabidopsis* CNGCs have been shown to conduct monovalent cations (K^+, and/or Na^+) upon expression in heterologous systems, including ATCNGC1, 2, 3, 4, 10, 11, and 12 (Köhler et al. 1999; Leng et al. 1999, 2002; Li et al. 2005; Yoshioka et al. 2006; Gobert et al. 2006). We have previously demonstrated that the chimera ATCNGC11/12 can also function as an inwardly-conducting channel permeable to K^+ (Yoshioka et al. 2006). Ca^{2+}, which has been implicated in cell death signaling (Levine et al. 1996; Groover and Jones 1999), permeates ATCNGC1 and 2 (Leng et al. 2002; Ali et al. 2006, 2007). It is not currently known if other ATCNGCs conduct Ca^{2+}. We used the yeast double mutant, *mid1, cch1* to evaluate Ca^{2+} conduction by ATCNGC11, 12, and 11/12. Haploid yeast cells generate cytosolic Ca^{2+} signals in response to mating pheromone 'α' factor (Muller et al. 2001). Yeast mutants with translational arrest of the endogenous Ca^{2+} transporters CCH1 and MID1 fail to generate the Ca^{2+} signals in response to this factor and can not grow well on solid medium plates in the 'diffusion halo' surrounding a filter disc containing α factor (Ferrando et al. 1995). We transformed *ATCNGC11*, *12*, *11/12* and empty vector into this yeast mutant. Growth of the *mid1, cch1* yeast mutant in the halo surrounding a filter disc containing α factor was used as an assay to assess whether these CNGCs can function as inwardly Ca^{2+}-conducting channels. As shown in Fig. 6A, expression of *ATCNGC11*, *12* or *11/12* significantly increased the number of yeast colonies in this halo compared to the empty vector. These results suggest that Ca^{2+} can permeate through ATCNGC11, 12 and 11/12 pores.

Subcellular localization of ATCNGC11, 12 and 11/12

Through the use of immuno-detection and GFP fusion gene expression *in planta* or in yeast, NtCBP4 (a CNGC isolated from *Nicotiana tabacum)*, HvCBT1 (a CNGC isolated from *Hordeum vulgare* as a CaM-binding transporter), ATCNGC1, ATCNGC2, ATCNGC3 and ATCNGC10 have been demonstrated to be localized to the plasma membrane (Arazi et al. 2000; Schuurink et al. 1998; Mercier et al. 2004; Gobert et al. 2006; Ali et al. 2006; Borsics et al. 2007). However, the subcellular localization of ATCNGC11, 12 and 11/12 has not been determined. Moreover, any difference in the localization of ATCNGC11/12 from that of the wild type proteins, ATCNGC11 or 12, may cause the induction of the observed cell death associated with *ATCNGC11/12* expression (Fig. 1). In order to determine the subcellular localization of these proteins, GFP fusion constructs of *ATCNGC11* and *12* were created in addition to the earlier mentioned *ATCNGC11/12-GFP* construct. All three constructs were subsequently transiently expressed in *N. benthamiana* leaves and localization of the fusion proteins was investigated using confocal imagery. All three ATCNGC-GFPs were clearly observed at the periphery of the cell, suggesting plasma membrane localization (data not shown). There is, however, very little cytosolic space

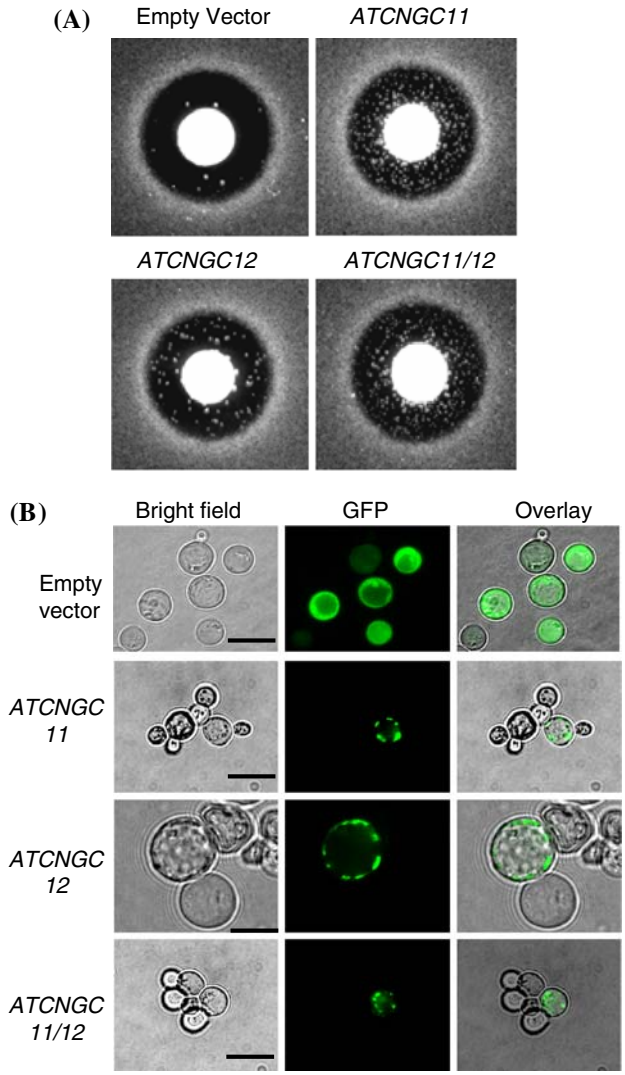

Fig. 6 ATCNGC11/12, 11, and 12 are able to channel Ca^{2+} and are localized to the plasma membrane. (**A**) Expression of *ATCNGC11, 12*, or *11/12* complemented growth sensitivity of a Ca^{2+} uptake-deficient yeast mutant to mating pheromone. Filter discs containing 50 µg of the mating pheromone α factor were placed on nascent lawns of *cch1, mid1* yeast mutant. Haploid yeast, which lacks endogenous inwardly conducting Ca^{2+} transporters CCH1 and MID1, fail to grow in a halo around the disc. Growth of the yeast transformed with either the empty vector, *ATCNGC11*, *ATCNGC12* or *ATCNGC11/12* were recorded after 48 h. (**B**) Photomicrographs of *trk1,2* yeast transformed with GFP fusion protein constructs. Bright field, fluorescence (GFP), and overlays are shown for transformed yeast. GFP fusion proteins of ATCNGC11/12, 11, and 12 localized to the plasma membrane whereas GFP alone (empty vector) localized in the cytosol. Bar = 10 µm

separating the plasma membrane and the tonoplast in mature cells, making it impossible to definitively conclude which membrane the channels are localizing to. Additionally, the expression of GFP fluorescence of these fusion proteins *in planta* was significantly lower than the vector control, which expresses only GFP. This made it more difficult to analyze the localization of these proteins using this approach.

For better visualization of the subcellular localization of the three ion channel proteins, all three fusion genes were expressed in yeast and their GFP expression were monitored by fluorescence microscopy. Although the expression was again weak, all three fusion proteins clearly localized to the plasma membrane in yeast and their fluorescence was observed as a cluster (Fig. 6B). By contrast, GFP expressed alone resulted in accumulation in the yeast cell cytosol. Thus, we concluded that ATCNGC11 and 12 are plasma membrane-localized proteins, as is ATCNGC11/12. Therefore, the cell death induced by expression of *ATCNGC11/12* is not due to a change in subcellular localization.

A calcium channel blocker suppresses cell death development by ATCNGC11/12

Calcium ions (Ca^{2+}) have been demonstrated to play an active role in the transduction of pathogen invasion signals (Grant et al. 2000) and also developmental cell death signaling (Groover and Jones 1999). A blockage of this Ca^{2+} signal causes a disruption in the ability of the plant to perceive the pathogen invasion resulting in a failure of the plant to develop HR (Xu and Heath 1998).

Since ATCNGC11, 12 and 11/12 function as Ca^{2+} channels as shown in Fig. 6, it was of prime interest to determine if Ca^{2+} plays a role in *ATCNGC11/12*-induced cell death. To study the possible involvement of Ca^{2+}, we took a pharmacological approach. First, the effect of Gd^{3+} and La^{3+}, which block plant Ca^{2+}-conducting channels (Grant et al. 2000; Lemtiri-Chlieh and Berkowitz 2004), and tetraethylammonium (TEA), a K^+ channel blocker (Mercier et al. 2004) (all supplied as chloride salts), were studied. The chemicals were applied by foliage spraying 12 h after *Agrobacterium* infiltration. Strikingly, $GdCl_3$ treatment suppressed cell death development completely in all samples (Fig. 7A). At 36–48 h after *Agrobacterium* inoculation, the degree of cell death was analyzed by electrolyte leakage assay confirming the suppression of cell death by $GdCl_3$ (Fig. 7B). The same reduction of electrolyte leakage was also observed in the samples treated with La^{3+} (Fig. 7B). In contrast, the K^+ channel blocker TEA had no effect on the development of cell death, indicating less or no involvement of K^+ in the actual induction of cell death, even though ATCNGC11/12 can function as a K^+-conducting channel (Fig. 7B, Yoshioka et al. 2006). As shown in Fig. 7C, Gd^{3+} suppressed cell death in a dose-dependent manner. These data indicate the involvement of Ca^{2+} in cell death development facilitated by ATCNGC11/12.

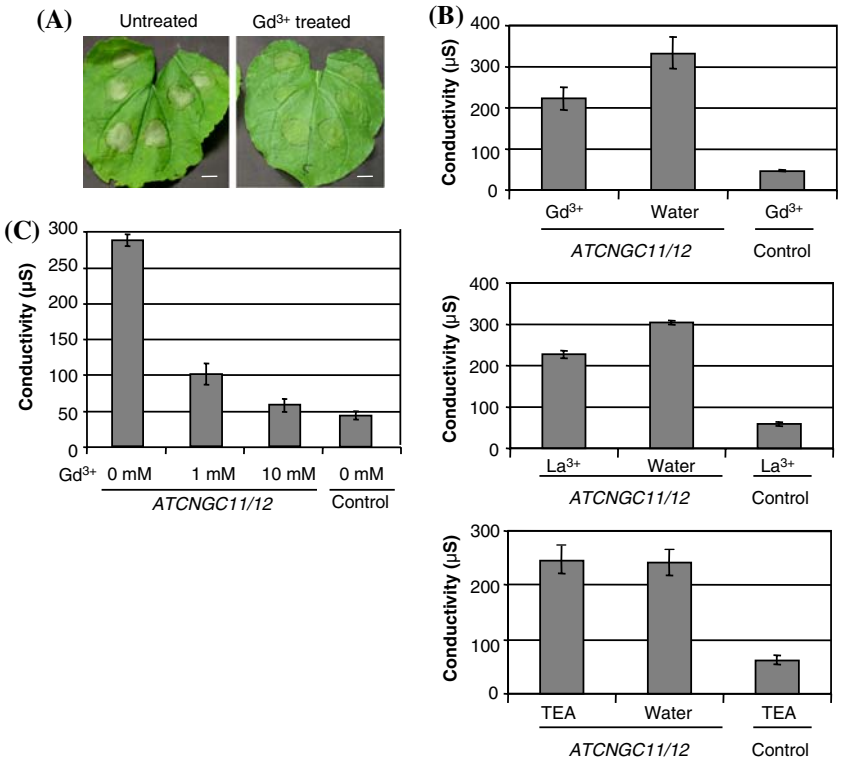

Fig. 7 Calcium channel blockers inhibit cell death development in *ATCNGC11/12* expressing *N. benthamiana* leaves. (**A**) Photographs of *ATCNGC11/12* expressing *N. benthamiana* leave 48 hpi that were sprayed with either water or $GdCl_3$ 12 hpi. Bar = 1 cm. (**B**) Electrolyte leakage of leaf discs from *ATCNGC11/12* expressing *N. benthamiana* leaves 48 hpi. The leaves were sprayed with either water, the Ca^{2+} channel blockers $GdCl_3$ (1 mM) and $LaCl_3$ (0.1 mM) or the K^+ channel blocker tetraethylammonium chloride (TEA, 10 mM). Control leaves were just sprayed with the above chemicals without *Agrobacterium* infiltration. (**C**) Dose dependency of $GdCl_3$ to inhibit *ATCNGC11/12*-induced cell death, shown as electrolyte leakage

Discussion

Plant cyclic nucleotide ion channels (CNGCs) are a recently discovered family of ion channels. The first plant CNGC, HvCBT1 (*Hordeum vulgare* CaM-binding transporter), was identified as a calmodulin (CaM)-binding protein in barley (Schuurink et al. 1998). Subsequently, several CNGCs were identified from *Arabidopsis* and *Nicotiana tabacum* (Köhler and Neuhaus 1998; Köhler et al. 1999; Arazi et al. 1999). The completion of the *Arabidopsis* genome sequencing project revealed a large family of CNGC genes, consisting of 20 members (Mäser et al. 2001). Considering that only six CNGC genes were found in mammalian genomes (Kaupp and Seifert 2002), the size of this gene family in plants suggests that plant CNGCs have a large diversity in physiological functions (Talke et al. 2003; Kaplan et al. 2007). Bioinformatics studies revealed that plant CNGCs can be categorized into five different subgroups (1–4B); however, the knowledge of the relationship among and within these groups, in terms of function and expression patterns, is fragmented and as of yet unclear (Mäser et al. 2001; Kaplan et al. 2007). So far, possible physiological functions of group I and group 4B members have been reported (Köhler et al. 2001; Sunkar et al. 2000; Balague et al. 2003; Chan et al. 2003; Li et al. 2005; Ma et al. 2006; Borsics et al. 2007; Gobert et al. 2006; Yoshioka et al. 2006; Ali et al. 2007). *ATCNGC1*, a member of group I, has been reported to contribute to Ca^{2+} uptake into plants, and is involved in root growth (Ma et al. 2006). On the contrary, another group I member, *ATCNGC3*, did not complement a Ca^{2+} uptake yeast mutant, and *Arabidopsis* knockout mutants did not show any specific sensitivity to Ca^{2+} (Gobert et al. 2006). *ATCNGC2, 4, 11* and*12* have defined physiological roles in *Arabidopsis* where they function as components in plant pathogen resistance responses including HR cell death (Clough et al. 2000; Balague et al. 2003; Yoshioka et al. 2006; Ali et al. 2007). Of these ATCNGCs, *ATCNGC2* was further indicated to be involved in development and senescence (Köhler et al. 2001). In prior work, we have shown that the novel chimeric gene *ATCNGC11/12* induces multiple pathogen resistance responses, including HR-like cell death; the wild type genes *ATCNGC11* and *ATCNGC12* are also involved in pathogen resistance (Yoshioka et al. 2006). The aim of the study we report here was to characterize the cell death induced by *ATCNGC11/12* in detail to understand its mechanism.

Transmission electron microscopic and TUNEL analyses as well as electrophoresis of genomic DNA revealed similar characteristics in the cell death induced by *ATCNGC11/12* to typical PCD. PCD is a genetically encoded, active process that leads to rapid cell death. For multicellular organisms like animals and plants, PCD is essential for proper development and defense (Lam 2004). One well-studied example of plant PCD is its involvement in the differentiation of specialized cells, such as tracheary

elements in the xylem, during development (Mittler and Lam 1995; Groover et al. 1997; Fukuda 2000; Obara et al. 2001). Another well known plant PCD occurs during defense where an avirulent pathogens leads to hypersensitive cell death (HR) (Mittler et al. 1997; Heath 2000; Lam 2004; Hofius et al. 2007). The cytological events during cell-death activation have been studied comprehensively in both model systems and it has been revealed that they share a high degree of similarity. For example, morphological changes in mitochondria and chloroplasts, formation of vesicles from the plasma membrane and chromatin condensation were observed in both cases (Levine et al. 1996; Mittler et al. 1997; Gunawardena et al. 2001, 2004; Greenberg and Yao 2004). Furthermore, Levine and colleagues reported that these characteristics were observed when cultured soybean cells were infected with the avirulent pathogen, *Pseudomonas syringae* pv. *glycinea* (*Psg*), but not after treatment with syringomycin, a phytotoxin from *Pseudomonas syringae* pv. *syringae* that acts on plasma membrane ATPases by a detergent like mechanism, suggesting that avirulent pathogen-induced HR is PCD and differs from disease-related cell death (Levine et al. 1996). These cytological characters were clearly observed in cell death induced by *ATCNGC11/12* transient expression. Additionally we have investigated if DNA cleavage occurred during development of cell death. DNA cleavage resulting in nucleosomal fragments, which are multiples of 180-bp in size, is a hallmark of PCD in animal apoptosis (Wyllie et al. 1980; Oberhammer et al. 1993). Although this DNA cleavage was observed during HR development in various plants, it is not common (Mittler et al. 1997; Navarre and Wolpert 1999; Ryerson and Heath 1996; Kiba et al. 2006). In our analysis, although we have not seen clear DNA laddering, we have demonstrated evidence of DNA fragmentation by electrophoresis and TUNEL assay. At this moment, it is not clear whether 180-mer DNA laddering is absent in this case or if the lack of DNA laddering is due to improper timing or detection sensitivity. However, considering the fact that *ATCNGC11/12* was expressed under the powerful constitutive promoter CaMV 35S synchronously and that Southern hybridization also did not reveal a clear laddering pattern sensitivity may not be the issue. Therefore, it is highly likely that the DNA laddering does not occur in this case.

Caspases are aspartate-specific cysteine proteases that play an essential role in executing PCD in metazoans (Ho and Hawkins 2005). It has been shown that caspase-like activity is required for the development of PCD in plants (del Pozo and Lam 1998; Lam and del Pozo 2000; Lam 2004; van Doorn and Woltering 2005). Recently, vacuolar processing enzyme (VPE), a plant cysteine protease, was identified as a potential plant counterpart to animal caspases that is essential for pathogen-induced HR (Hatsugai et al. 2004; Rojo et al. 2004). Although VPE does not share significant sequence similarity to animal caspases, it exhibits caspase-1 like activity and disrupts the vacuole during pathogenesis and development, consequently contributing to PCD (Hatsugai et al. 2004; Kuroyanagi et al. 2005; Hatsugai et al. 2006). Groover and Jones (1999) have suggested in a developmental cell death model that influx of Ca^{2+} initiates cell death and leads to vacuole collapse and cessation of cytoplasmic streaming. Based on TEM analysis, tonoplast rupture and vacuole collapse was also observed in the cell death induced by *ATCNGC11/12*. Considering the importance of caspases and vacuole collapse for plant cell death, it is of interest to evaluate the involvement of VPE in *ATCNGC11/12* induced cell death. Interestingly, our data using caspase-1 inhibitors and *VPE*-silenced plants suggests that caspase activity, especially VPE, may play an important role in cell death induced by *ATCNGC11/12*. Further investigations on the effect of VPE using genetic tools, such as the analysis of double mutants of *Arabidopsis* VPE knockout lines and *cpr22*, could provide us with more insight.

Finally, to study the possible involvement of Ca^{2+} in cell death induction, we have conducted pharmacological experiments using a variety of channel blockers. Strikingly, Ca^{2+} channel blockers, but not a K^+ channel blocker, significantly suppressed the induction of cell death in *ATCNGC11/12*-expressing leaves. The same effects were reported by Ali et al. (2007) for *ATCNGC2*. These data indicate that Ca^{2+} plays a crucial role to facilitate *ATCNGC11*/12-induced cell death. It has been reported that ATCNGC1 and ATCNGC2 can function as Ca^{2+} channels (Leng et al. 1999; Ma et al. 2006; Ali et al. 2007) and Ca^{2+} flux is known as an early event after pathogen infection. Recently Ali et al. (2007) showed that *ATCNGC2* is an inward rectified Ca^{2+} conducting channel that is involved in innate immunity. Interestingly however, in the *dnd1* mutant the disruption of *ATCNGC2* (as well as for *dnd2/hlm1* for *ATCNGC4*) leads to enhanced pathogen resistance and reduced HR formation after infection with avirulent pathogens (Yu et al. 1998; Balague et al. 2003; Jurkowski et al. 2004). On the other hand *ATCNGC11/12* is a functional channel and presumably its altered function leads to the observed constitutive cell death and pathogen resistance (Yoshioka et al. 2006).

As mentioned above, it has been suggested that an influx of Ca^{2+} initiates cell death and leads to vacuole collapse (Groover and Jones 1999). Thus, in the work reported here, we evaluated if ATCNGC11/12 is permeable to Ca^{2+}. Expression of *ATCNGC11/12* complemented the Ca^{2+}-uptake deficient phenotype of the *mid1, cch1* yeast mutant, a result consistent with ATCNGC11/12 functioning as a Ca^{2+} channel *in planta*. Taken together, these results

suggest that *ATCNGC11/12* is a constitutively active Ca^{2+} channel *in planta*, causing uncontrolled Ca^{2+} ion influx that induces spontaneous cell death formation. Given this possibility and the fact that ATCNGC11 and 12 also can function as Ca^{2+} channels, it is tempting to speculate that the authentic role of ATCNGC11 and 12 might be as Ca^{2+} channels that induce pathogen resistance responses. In fact, null mutations in *ATCNGC11* or *12* partially affect *R*-gene mediated resistance to *Hyaloperonospora parasitica* (Yoshioka et al. 2006). It is of interest to explore the authentic function of these genes and investigate the role of Ca^{2+} for the induction of pathogen resistance. For further understanding, visualization of Ca^{2+} currents in the *cpr22* mutant and relevant knockout lines are under investigation.

Mittler et al. (1995) showed that ectopic expression of a bacterium proton channel, bacterioopsin (bO) in tobacco resulted in a HR-like lesion-mimic phenotype. bO transgenic plants showed enhanced resistance to different types of pathogens and exhibited an activation of resistance responses. A recent study by Wendehenne et al. (2002) also reported that an anion transporter plays an essential role for elicitor induced HR cell death in tobacco. Strikingly, resistance responses induced by bO including HR-like cell death, were suppressed by high temperature. A similar suppression of resistance responses has been observed in *R*-gene related mutants and transgenic plants (Xiao et al. 2003; Zhou et al. 2004; Yoshioka et al. unpublished data). Furthermore, *cpr22* mutant phenotypes attributed to *ATCNGC11/12* including cell death were also abolished by high temperature (Yoshioka et al. 2001, unpublished data) similar to the TMV-induced HR in tobacco plants carrying the *N*-gene (Kassanis 1952). These observations indicate the importance of ion flux regulation in the execution of HR cell death after pathogen infection and the signal may include an environmentally sensitive factor. Further investigation on the signal transduction in abiotic and biotic stress responses will give us more insight in the robustness of an intricate signal-transduction network.

Over the last decade, a significant amount of research has been conducted in order to understand the mechanism of plant PCD. Particular focus has been placed on developmental PCD, which has been studied extensively. It has been suggested that at least three major cytological variants of the PCD process exists in plants (Fukuda 2000; van Doorn and Woltering 2005). However, the signaling pathway that leads to PCD is not yet known at the molecular level. Here we have shown that transient expression of *ATCNGC11/12* induces PCD in a Ca^{2+} dependent manner in *N. benthamiana* and it has been reported that the expression of this gene induces multiple pathogen resistance responses in *Arabidopsis*. Thus, ATCNGC11/12 induced cell death can be a useful model system to investigate and identify the signal transduction pathway of cell death development at the molecular level. Furthermore, the chimeric ATCNGC11/12 is a unique tool, since it may be a constitutively active channel. It will be useful to analyze the structurally and/or functionally important parts of this channel protein, including animal CNGCs.

Further physiological characterization, identification of down-stream components in the signal transduction, and CNGC structural-functional relationships using ATCNGC11/12 are in progress.

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