Journal of Experimental Botany, Vol. 67, No. 3 pp. 809–819, 2016
doi:10.1093/jxb/erv500 Advance Access publication 25 November 2015

RESEARCH PAPER

AtCNGC2 is involved in jasmonic acid-induced calcium mobilization

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Received 17 August 2015; Revised 16 October 2015; Accepted 26 October 2015

Editor: Angus Murphy, University of Maryland

Abstract

Calcium (Ca^{2+}) mobilization is a central theme in various plant signal transduction pathways. We demonstrate that *Arabidopsis thaliana* cyclic nucleotide-gated channel 2 (AtCNGC2) is involved in jasmonic acid (JA)-induced apoplastic Ca^{2+} influx in *Arabidopsis* epidermal cells. Ca^{2+} imaging results showed that JA can induce an elevation in the cytosolic cAMP concentration ($[cAMP]_{cyt}$), reaching a maximum within 3 min. Dibutyryl cAMP (db-cAMP), a cell membrane-permeable analogue of cAMP, induced an increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$), with a peak at 4 min. This $[Ca^{2+}]_{cyt}$ increase was triggered by the JA-induced increase in $[cAMP]_{cyt}$. W-7[*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], an antagonist of calmodulin, positively modulated the JA-induced increase in $[Ca^{2+}]_{cyt}$, while W-5[*N*-(6-aminohexyl)-1-naphthalenesulfonamide], an inactive antagonist of calmodulin, had no apparent effect. db-cAMP and JA positively induced the expression of primary (i.e. *JAZ1* and *MYC2*) and secondary (i.e. *VSP1*) response genes in the JA signalling pathway in wild-type *Arabidopsis thaliana*, whereas they had no significant effect in the AtCNGC2 mutant 'defense, no death (*dnd1*) plants. These data provide evidence that JA first induces the elevation of cAMP, and cAMP, as an activating ligand, activates the AtCNGC2 channel, resulting in apoplastic Ca^{2+} influx through AtCNGC2.

Key words: *Arabidopsis thaliana*, Calcium influx, cAMP, CNGC, Cytosolic Ca^{2+}, Jasmonic acid.

Introduction

Calcium (Ca^{2+}) is involved in nearly all aspects of plant development and in many regulatory processes (Tegg *et al.*, 2005), including plant responses to biotic and abiotic stress. Nearly all signals (developmental, hormonal, and stress) cause changes in cellular Ca^{2+} (Ali *et al.*, 2006), although each response appears to have a specific signature determined by the spatial location (address), amplitude, and duration of the Ca^{2+} signal.

Plant cyclic nucleotide-gated channels (CNGCs) are ligand- and voltage-gated channels proposed to conduct extracellular Ca^{2+} into the cytosol (Britten *et al.*, 1992; Pouliquin *et al.*, 1999; Lemtiri-Chlieh and Berkowitz, 2004; Tegg *et al.*, 2005; Qi *et al.*, 2010). They play an important role in modifying plant development and in integrating plant responses to pathogens and abiotic stress (Lemtiri-Chlieh and Berkowitz, 2004; Ali *et al.*, 2007).

In *Arabidopsis*, there are 20 annotated CNGCs (AtCNGCs), with at least one isoform expressed in each of the major tissues (Frietsch *et al.*, 2007; Konrad and Hedrich, 2008). Plant CNGCs are involved in the control of growth processes and responses to abiotic and biotic stresses (Kugler *et al.*, 2009). AtCNGC2 localizes to the plasma membrane and has been shown to translocate Ca^{2+}, suggesting that it transports Ca^{2+} from the apoplast into the cell (Urquhart *et al.*, 2007). AtCNGC2 is constitutively expressed in leaves (Talke *et al.*, 2003) and is a key determinant for growth under physiologically relevant cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_{cyt}$) (Dutta and Robinson, 2004). It has also been suggested to participate in pathogen-mediated Ca^{2+}signalling and leaf development/senescence processes (Britten *et al.*, 1992; Kudla *et al.*, 2010). The *Arabidopsis* 'defense, no death' (*dnd1*) mutant lacks a plasma membrane-localized cation channel (CNGC2), and *dnd1* mutants show increased sensitivity to Ca^{2+} (White and Broadley, 2003; Ali *et al.*, 2007; Nakagawa *et al.*, 2007). Mutation of *Arabidopsis* CNGC2 (in the *dnd1* mutant) results in impaired hypersensitive response to avirulent pathogens and impaired cAMP/cGMP-dependent cytosolic Ca^{2+} elevation (Ma and Berkowitz, 2011). Generation of the signalling molecule nitric oxide in response to the pathogen-associated molecular pattern lipopolysaccharide is also impaired in *dnd1* cells (Ma and Berkowitz, 2011). AtCNGC1, 2, 3 and 4 have an apparent role in ion homeostasis, uptake, and transport, including of calcium and sodium, as well as in stress-related signal transduction (Kaplan *et al.*, 2007; Kugler *et al.*, 2009); AtCNGC2 and AtCNGC4 are involved in programmed cell death (Kaplan *et al.*, 2007), and they have a unique role in controlling the timing of flowering (Chin *et al.*, 2013); AtCNGC6 mediates heat-induced Ca^{2+} influx and the acquisition of thermotolerance (Saand *et al.*, 2015); AtCNGC7, 8, 16, and 18 are involved in different physiological roles throughout plant development (Kaplan *et al.*, 2007); AtCNGC10 is part of a light signal transduction pathway (Kaplan *et al.*, 2007); AtCNGC11 and12 are involved in plant defence against pathogens (Kaplan *et al.*, 2007); and AtCNGC19 and 20 may help the plant to cope with toxic effects caused by salt stress through re-allocation of sodium within the plant (Kugler *et al.*, 2009). However, the molecular mechanisms and signal components associated with CNGC-mediated biological processes are still to be elucidated.

Cyclic nucleotides, such as cAMP and cGMP, are potential activating ligands of plant CNGCs, including CNGC2 (Qi *et al.*, 2010). cAMP activates an inward Ca^{2+} current through the plasma membrane in wild-type (WT) leaf epidermal cells; however, this current is absent in leaves of the CNGC2 loss-of-function mutant *dnd1* (Britten *et al.*, 1992; Talke *et al.*, 2003; Ma *et al.*, 2006). Mutation of AtCNGC2 (in the *dnd1* mutant) results in impaired cAMP-dependent and cGMP-dependent cytosolic Ca^{2+} elevation (Ali *et al.*, 2007; Ma *et al.*, 2009a; Qi *et al.*, 2010).

Jasmonic acid (JA) is a member of the jasmonates, which act as plant growth and stress regulators. They play important roles in plant development and stress responses, including wounding and pathogen attacks (León *et al.*, 2001; Boter *et al.*, 2004), and in defence against pathogens (Farmer *et al.*, 2003; Sun *et al.*, 2006). Jasmonates act both locally and systemically to orchestrate the plant defence signalling network (Adie *et al.*, 2007; Truman *et al.*, 2007; Fernández-Calvo *et al.*, 2011; Wasternack and Hause, 2013) through the activation of transcription factors (Ma *et al.*, 2010) such as AtMYC2 (Chini *et al.*, 2007; Thines *et al.*, 2007). Jasmonoyl-l-isoleucine has been found necessary for binding of the JA ZIM-domain (JAZ) repressors to the F-box COI1 jasmonate receptor and subsequent ubiquitination, leading to 26S proteasome-mediated degradation of the JAZ repressor and activation of jasmonate signalling (Sheard *et al.*, 2010).

Ca^{2+} is involved in the regulation of JA biosynthesis and signalling (Wasternack and Hause, 2013), and our previous work also demonstrates that JA can induce an influx of extracellular Ca^{2+} (Sun *et al.*, 2006).

Methyl-jasmonate (MeJA)-induced stomatal closure is inhibited by Ca^{2+} channel blockers and calmodulin (CaM) inhibitors (Suhita *et al.*, 2003; Suhita *et al.*, 2004). Furthermore, *Pseudomonas syringae* virulence factor coronatine, a jasmonate isoleucine mimic (Fonseca *et al.*, 2009), is necessary to reopen stomata following virulent *Pseudomonas* infection (Melotto *et al.*, 2006). Additionally, MeJA activates guard cell plasma membrane Ca^{2+}-permeable cation channels, and this activation is abolished in the MeJA-insensitive mutant *coi1* (Munemasa *et al.*, 2007; Munemasa *et al.*, 2011). Activation of Ca^{2+}-permeable channels was proposed to contribute to $[Ca^{2+}]_{cyt}$ elevation via guard cell abscisic acid (ABA) signalling (Hamilton *et al.*, 2000; Pei *et al.*, 2000; Munemasa *et al.*, 2011). This suggests that cytosolic Ca^{2+} serves as an important second messenger in MeJA signalling in *Arabidopsis* guard cells (Munemasa *et al.*, 2011). Cross-talk between cytosolic ABA receptors and JA repressors was recently demonstrated (Lackman *et al.*, 2011). To explore the mechanism of how JA induces extracellular Ca^{2+} influx, WT *A. thaliana* (ecotype 'Columbia') and the *dnd1* mutant were used to evaluate the relationship between JA and JA-induced extracellular calcium mobilization. Our results showed that AtCNGC2 is involved in JA-induced extracellular mobilization.

Jasmonates regulate a wide range of plant processes, including defence against biotic and abiotic stress (Dar *et al.*, 2015). Ca^{2+} is thought to be an early key player of JA signalling (Matschi *et al.*, 2015). The pathway of JA-induced calcium mobilization could help elucidate the role of JA in plant. To our knowledge, this is the first report to describe such a model.

Materials and methods

Plant growth

Seeds of WT *A. thaliana* (ecotype 'Columbia') and the *dnd1* mutant were surface-sterilized and plated on Murashige and Skoog (MS) medium (pH 5.8). The seedlings were grown in ~150 μmol m^{-2} s^{-1} white light under a photoperiod cycle of 16 h light (26°C) and 8 h dark (22°C) with 60% relative humidity.

Measurement of $[Ca^{2+}]_{cyt}$

The leaf epidermal cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) was measured according to Sun *et al.* (2006). Ten-day-old seedlings were sliced into strips (approximately 2 mm), rinsed with MS

medium three times, and then used for dye loading. Fluo-3 acetoxymethyl (AM) ester, a Ca^{2+}-sensitive fluorescent dye, was purchased from Molecular Probes (Eugene, OR, USA). The leaf strips were first incubated in MS medium containing 20 µM Fluo-3/AM. After 2 h incubation at 4°C, the leaf strips were washed twice with fresh Fluo-3/AM-free MS medium, followed by incubation in fresh MS medium for another 2 h at room temperature in the dark. Fluorescence from the leaf strips loaded with Fluo-3/AM was detected by laser scanning confocal microscopy (LSCM; MRC-1024 with a four-line argon laser box, Bio-Rad Laboratories, Hercules, CA, USA). Imaging of leaf cells was obtained at an excitation of 488 ± 10 nm and emission of 530 ± 40 nm. The XY-T three-dimensional scan mode was used, and the change in fluorescence intensity was recorded using the Lasersharp 2000 time-lapse program (Bio-Rad Laboratories). Laserpix 4.0 software was used to measure the fluorescence intensity kinetics. Data were analysed using Graphpad Prism 5.

Detection of membrane potential

Membrane potential was detected using DiBAC4(3), a lipophilic anionic membrane potential probe, according to Konrad and Hendrich (2008) with minor alterations. *Arabidopsis* stem epidermis was sliced into strips, and the strips were constantly superfused at a flow rate of 120–150 µl min^{-1} to prevent photobleaching of the dye. This is necessary to calculate membrane potential values accurately from DiBAC4(3) fluorescence intensities.

Fluorescence from the stem epidermal strips loaded with DiBAC4(3) was detected using LSCM (Leica SP5). Imaging of leaf cells was obtained at an excitation of 480 nm and emission of 520 nm. The XY-T three-dimensional scan mode was used, and the change in fluorescence intensity was recorded and measured using the LASAF program. Images were saved every 15 s. All measurements were corrected for background fluorescence recorded from reference regions close to the analysed cells.

Real-time PCR analysis

Ten-day-old seedlings grown on MS medium were treated with 100 µM JA or different concentrations of the lipophilic cAMP analogue db-cAMP (Sigma). The seedlings were collected for total RNA isolation using the UNIQ-10 Column Trizol Total RNA Isolation Kit (Sangon Biotech, Shanghai, China).

Quantitative PCR experiments were performed according to the methods described by Yan *et al.* (2003), using an Applied Biosystems ABI 7300 with SYBR Green I as the fluorescent dye to quantify DNA, using actin as the endogenous control. The $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate the C_T values of *JAZ1*, *MYC2*, and *VSP1* relative to the endogenous controls. First-strand cDNA was prepared using the PrimeScript™ RT Reagent Kit (Takara, Japan). To amplify gene-specific products, *JAZ1* (AGI No. At1g19180), *MYC2* (AGI No. At1g32640), *VSP1* (AGI No. AT5G24780), and actin (AGI No. AT3G46520), specific primers were used as follows: *JAZ1* forward primer: GCCAATCCAATCCTCCCCAA, *JAZ1* reverse primer: TGCCTGTGGTTTGAGGGTTT; *MYC2* forward primer: AGGAGGTGACGGATACGGAA, *MYC2* reverse primer: TAGACGGGTCGTTCTCACCT; *VSP1* forward primer: CGCCAAAGGACTTGCCCTAA, *VSP1* reverse primer: ATCCGTTTGGCCTGCGTTTC; actin forward primer: ACCCAATTTCAGGGCACGAT, actin reverse primer: CTGCAAAACCAGCCTTGACC. The significance of the differences among the different treatments was evaluated using a one-way ANOVA ($p < 0.05$).

Leaf cAMP determination

We used the method of Ma *et al.* (2009a). Detached leaves (from WT and *dnd1* plants) were treated with 100 µM JA. Leaves were ground in liquid N_2, and frozen leaf powder was extracted using $HClO_4$ and neutralized using KOH. The extract was clarified by centrifugation. The supernatant was lyophilized, and the cAMP content was quantified using an enzyme-linked immunoassay kit (cAMP-Glo™ Assay, Promega). Data were analysed using Graphpad Prism 5.

Measurement of stomatal aperture

The stomatal aperture was measured according to Liu *et al.* (2005) with minor revision. The fully expanded leaves of 3–4 week old *A. thaliana* were kept under light to induce stomatal opening, then epidermal strips were peeled from the abaxial side and mesophyll cells that had adhered to the peels were removed by careful brushing. Peels were incubated in 2-(N-morpholino)ethanesulfonic acid–KOH buffer with the addition of JA and kept under light continuously (light flux intensity 200 µmol m^{-2} s^{-1}) for 3 h. Stomatal aperture was measured by cellSens software (Olympus) using an inverted microscope (Nikon TE2000-U) at room temperature. Data were analysed using Graphpad Prism 5. Six fields of vision were randomly picked to determine stomatal apertures in each treatment, and each experiment was repeated at least three times. Each data point is expressed as mean ± standard error of the mean ($n = 60$).

Results

Apoplastic Ca^{2+} influx through AtCNGC2

The $[Ca^{2+}]_{cyt}$ rises either as a result of uptake from the extracellular space through plasma membrane channels or from the release of internal stores, such as the endoplasmic reticulum or vacuoles (Gilroy *et al.*, 1990; Cessna *et al.*, 1998; Sun *et al.*, 2009). Extracellular Ca^{2+} is involved in the JA-induced elevation of $[Ca^{2+}]_{cyt}$, and JA was most effective at a concentration of 100 µM (Sun *et al.*, 2006; Sun *et al.*, 2009). Two methods are usually employed to detect $[Ca^{2+}]_{cyt}$ changes in plants directly. The first is to measure $[Ca^{2+}]_{cyt}$ using the bioluminescent Ca^{2+}-reporter aequorin, which can be transformed or microinjected into plant cells (Knight *et al.*, 1991; Fisahn *et al.*, 2004). The second is to examine fluorescence images or intensity using LSCM after loading Ca^{2+}-sensitive dyes, such as Fluo-3/AM (Zhang *et al.*, 1998; Sun *et al.*, 2006) or calcium orange-AM (Lam *et al.*, 2005), into the cell. In a previous experiment, Fluo-3 localized in the cytoplasm and did not move into the vacuole or apoplast (Liu *et al.*, 2003). In our study, we loaded Fluo-3/AM at 4°C into *A. thaliana* leaf epidermal cells according to a previous method (Sun *et al.*, 2006). When treated with 100 µM JA, the fluorescence intensity in the *A. thaliana* leaf cells increased gradually (Fig. 1A, C), whereas there was no significant change seen in the *dnd1* mutant (Fig. 1B, C).

Effects of the CaM antagonist W-7 on JA-induced $[Ca^{2+}]_{cyt}$

Plant CNGCs have a C-terminal CaM-binding domain (Talke *et al.*, 2003). W-7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide], an antagonist of CaM, significantly increased JA-induced $[Ca^{2+}]_{cyt}$ ($p < 0.05$) in WT *Arabidopsis* (Fig. 2A, 100 µM JA + W-7), whereas W-5[*N*-(6-aminohexyl)-1-naphthalenesulfonamide], an inactive structural analogue of W-7, had no significant effect on $[Ca^{2+}]_{cyt}$ with JA treatment (Fig. 2A, 100 µM JA + W-5). The results showed that W-7 activates

JA-induced Ca^{2+} mobilization in WT *A. thaliana*. In contrast, in the *dnd1* mutant, W-7 had no significant effect on $[Ca^{2+}]_{cyt}$ (Fig. 2B).

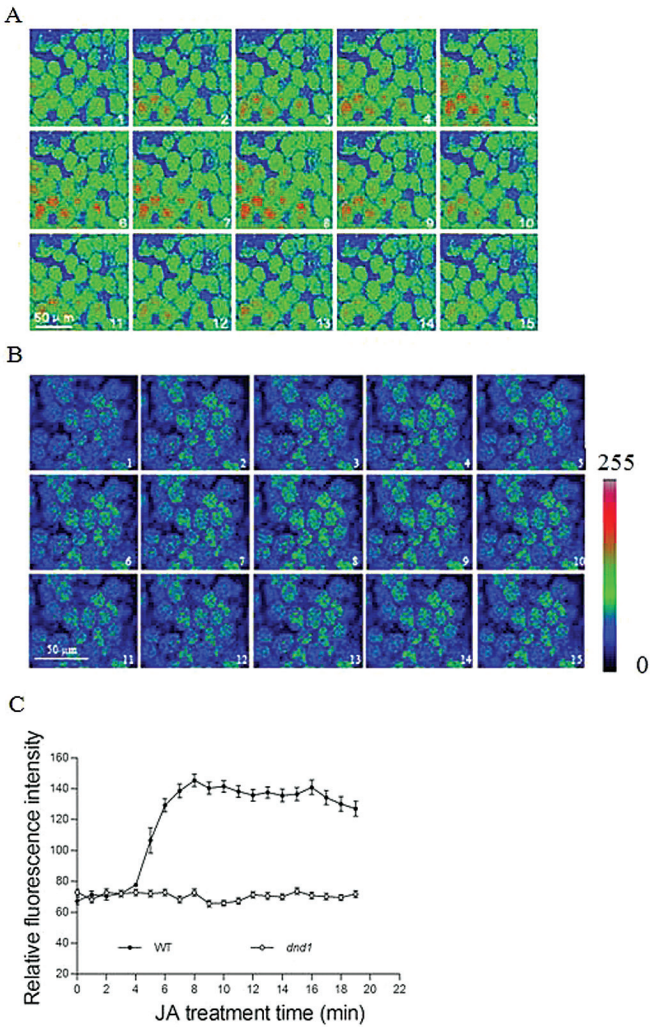

Fig. 1. Effect of JA on $[Ca^{2+}]_{cyt}$ in (**A**) WT *A. thaliana* and (**B**) *dnd1* mutant leaf cells. Ten-day-old leaves were sliced into strips. The strips were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM, then treated with the addition of JA to the medium. Excitation (488 ± 10 nm) and emission (530 ± 40 nm) filters were used. The scan mode was three-dimensional (XY-T). The fluorescence intensity was recorded every minute. The colour bar on the right side shows the minimum (0) and maximum intensities (255). (**C**) The characteristic curve of the cytosolic calcium dynamic after JA treatment. Data were collected from individual cells from leaf strips. Data are means ± SE (n = 6).

Effect of db-cAMP on Ca^{2+} mobilization

Plant CNGCs have C-terminal cyclic nucleotide-binding and CaM-binding domains (Talke *et al.*, 2003). cAMP is a potential activating ligand of plant CNGCs, including CNGC2 (Qi *et al.*, 2010). The *dnd1* mutant lacks a functional cyclic nucleotide gated (non-selective) cation channel (CNGC2).

db-cAMP is a cell-permeable cAMP analogue (Yamauchi *et al.*, 2011) that stimulates cAMP-dependent protein kinases, such as PKA. db-cAMP has no effect on cAMP content (Bagg *et al.*, 2009). When treated with db-cAMP, $[Ca^{2+}]_{cyt}$ increased in a concentration-dependent manner (Fig. 3A). $[Ca^{2+}]_{cyt}$ increased within 1 min after db-cAMP treatment, reached a maximum at about 5 min, and maintained high levels for the next 15 min (Fig. 3A). $[Ca^{2+}]_{cyt}$ elevation did not occur in *dnd1* (CNGC2 mutant) plants when treated with different concentrations of db-cAMP (Fig. 3B). These data show that the cAMP-induced $[Ca^{2+}]_{cyt}$ elevation is dependent on AtCNGC2.

Impact of JA and db-cAMP on A. thaliana *plasma membrane potential*

The membrane potential is widely used to report early events associated with changes in light regime, hormone action, or pathogen attack (Konrad and Hedrich, 2008). DiBAC4(3), a lipophilic anionic membrane potential probe that carries a delocalized negative charge, is suitable for non-invasive monitoring of the PM potential (Sivaguru *et al.*, 2005; Konrad and Hedrich, 2008). DiBAC4(3), which has been successfully used in plant cell experiments (Britten *et al.*, 1992; Johannes *et al.*, 1992; Pouliquin *et al.*, 1999; Sivaguru *et al.*, 2005; Konrad and Hedrich, 2008), permeates the plasma membrane and accumulates in the cytosol upon membrane depolarization. Redistribution of the dye as a result of depolarizing or hyperpolarizing stimuli in cells causes changes in fluorescence (Baxter *et al.*, 2002). An upward deflection of the DiBAC4(3) fluorescence intensity represents a depolarization event (Konrad and Hedrich, 2008). To our knowledge, the effect of JA on the plasma membrane potential has not been reported in intact *Arabidopsis* stem epidermal cells. Changes in membrane potential in response to JA were measured in *A. thaliana* and *dnd1* stem epidermal cells using DiBAC4(3). In the absence of JA treatment, the relative fluorescence intensity in both WT *Arabidopsis* and *dnd1* mutant plants showed no significant change (Fig. 4A, ckwt, ck dnd1). Following treatment with 100 μM JA, the relative fluorescence

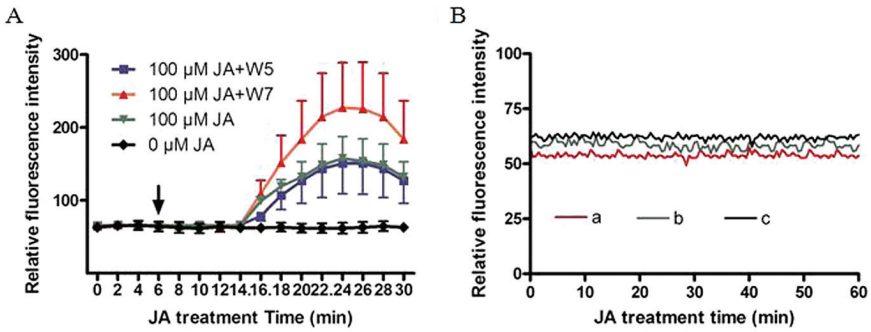

Fig. 2. Effect of W7 and W5 on JA-induced $[Ca^{2+}]_{cyt}$ increase in WT and *dnd1* leaves. The data were collected as in Fig.1. Leaf strips were pre-treated for 1 h with 200 μM W-7 or W-5. (**A**) Wild-type; 100 μM JA were added at the time indicated by the arrow. Data are means ± SE (*n* = 6). (**B**) *dnd1*; a, b, and c represent single cells. (This figure is available in colour at *JXB* online.)

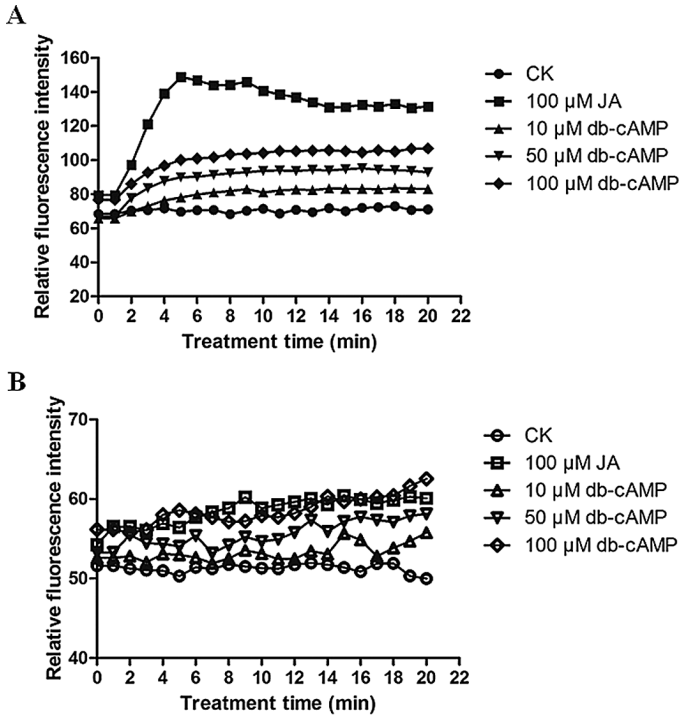

Fig. 3. Effect of db-cAMP on $[Ca^{2+}]_{cyt}$ in WT and *dnd1* leaves. The data were collected as in Fig.1. The seedlings of (**A**) WT and (**B**) *dnd1* mutant plants were treated with 0, 10, 50, or 100 μM db-cAMP. The control (CK) was not treated with db-cAMP or JA.

intensity increased significantly in WT *Arabidopsis* (Fig. 4A, 100 μM JA wt) and *dnd1* cells (Fig. 4A, 100 μM JA dnd1). Thus, JA induces depolarization of the plasma membrane in WT and *dnd1* cells; however, the fluorescence intensity was more than 1.7-fold greater in WT than *dnd1* cells (Fig. 4A).

Application of db-cAMP, a cell-permeable cAMP analogue, which selectively activates cell membrane-localized non-selective cation channels (Clough *et al.*, 2000; Ma *et al.*, 2009a), also induced an increase in DiBAC4(3) fluorescence intensity. As indicated in Fig. 4B, db-cAMP induced depolarization of *Arabidopsis* plasma membrane (Fig. 4B), whereas it had no significant effect on *dnd1* plasma membrane potential (Fig. 4C).

Effect of JA on $[cAMP]_{cyt}$ in A. thaliana *leaf epidermal cells*

JA elevated the cytosolic cAMP concentration ($[cAMP]_{cyt}$) in *A. thaliana* but had no effect on $[cAMP]_{cyt}$ in *dnd1* plants (Fig.5). The AtCNGC2 mutation may impair JA signal transduction-mediated production of cAMP, but this needs to be validated by further experiments.

Effects of JA and db-cAMP on JA responsive gene expression

JAZ and *MYC2* are primary response genes, and *VSP* has been used as a marker of secondary response genes in the JA signalling pathway (Chung *et al.*, 2008). To investigate the impact of JA and cAMP on JA signalling, we examined the effects of JA and db-cAMP application on *JAZ1*, *MYC2*, and *VSP1* expression. *JAZ1* and *MYC2* expression were induced

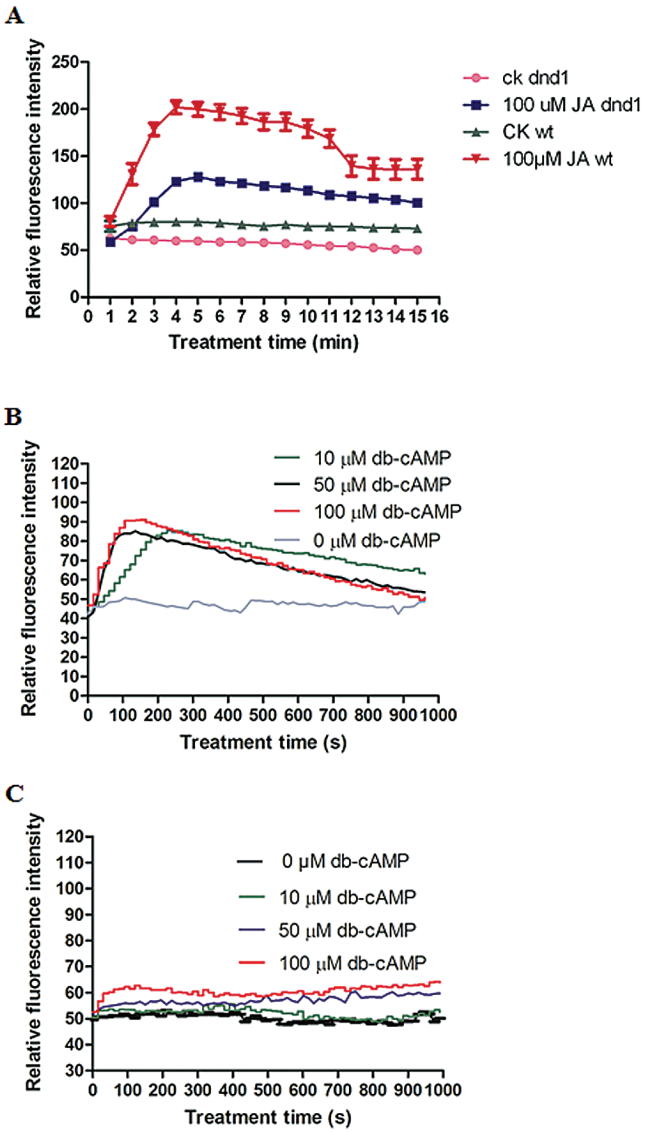

Fig. 4. Effect of JA on the membrane potential of WT and *dnd1* stem epidermal cells. The stem epidermis from 10-day-old seedlings was sliced into strips, and the strips were loaded with the lipophilic anionic membrane potential probe DiBAC4(3). Emission filters for fluorescence measurements for excitation and emission were 488 ± 10 nm and 530 ± 40 nm, respectively. The scan mode was three-dimensional (XY-T). The fluorescence intensity was recorded every minute. Data are means ± SE (n = 6). (**A**) Strips were either treated with 100 μM JA (100 μM JA wt, 100 μM JA dnd1) or distilled H_2O as the control (ck wt, ck dnd1). (**B**) Wild-type or (**C**) *dnd1* seedlings were treated with 10, 50, or 100 μM cAMP. The control was left untreated. (This figure is available in colour at *JXB* online.)

by JA treatment both in WT and dnd1 *Arabidopsis* (Fig. 6A, B, C, D). *JAZ1* and *MYC2* expression reached a maximum at 30 min and 60 min in WT, respectively, and then decreased over the remainder of the testing period (Fig. 6A, C). Both *JAZ1* and *MYC2* showed a time delay in reaching maximum expression (Fig. 6C, D). In contrast to WT, *JAZ1* expression induced by JA in WT was significantly inhibited (Fig. 6B), and *MYC2* expression showed less significant but still obvious inhibition (Fig. 6D). *VSP1* was only induced by JA in WT; no significant changes were detected in *dnd1* (Fig. 6E). Application of db-cAMP increased the expression level of *MYC2* and *VSP1* in a dose-dependent manner in WT *Arabidopsis* (Fig. 6F, H), but

not in the *dnd1* mutant (Fig. 6G, I). In contrast, db-cAMP had no evident effect on *JAZ1* expression (data not shown).

Effect of MeJA on the primary root length of A. thaliana

Primary root growth induction was observed both in *A. thaliana* and in *dnd1* under treatment with 0.1 μM MeJA (Fig. 7B).

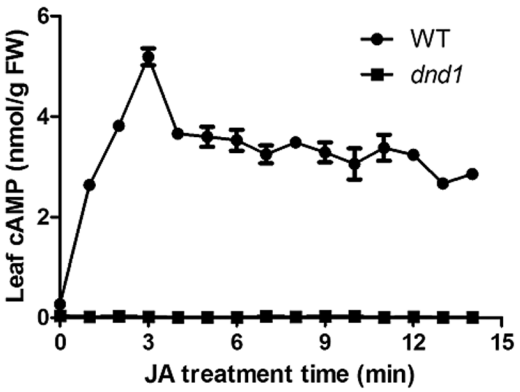

Fig. 5. Effect of JA on the cAMP concentration in WT and *dnd1* leaves. Ten-day-old WT and *dnd1* seedlings were incubated in MS medium and harvested at different times after JA (100 μM final concentration) was added to the medium.

MeJA at 0.1 μM induced a 98.15% increase in WT, but only 82.5% in *dnd1* (Fig. 7C). The roots for *dnd1* grown in MeJA-free medium showed about the same length as WT (Fig. 7A). This result is contrary to Staswick's report that primary root growth of WT *A. thaliana* seedlings was inhibited 50% when seedlings were grown on agar medium containing 0.1 μM MeJA (Staswick *et al.*, 1992). Recently, Lischweski *et al.* reported that jasmonates act as positive regulators of adventitious root formation in WT *Petunia* (Lischweski *et al.*, 2015).

Effect of JA on stomatal movement of A. thaliana

Stomatal closure of WT *A. thaliana* was induced under 10 μM JA treatment (Fig. 8, a and b). Stomata reopened fully when JA was washed out with buffer (Fig. 8, c). which is consist with Liu's results (Liu *et al.*, 2005). JA had little effect on stomatal closure of *dnd1* (Fig. 8, d, e, f).

Discussion

AtCNGC2 is involved in JA-induced apoplastic Ca^{2+} *influx*

Ca^{2+} signals are core transducers and regulators in many adaptational and developmental processes in plants (Tegg

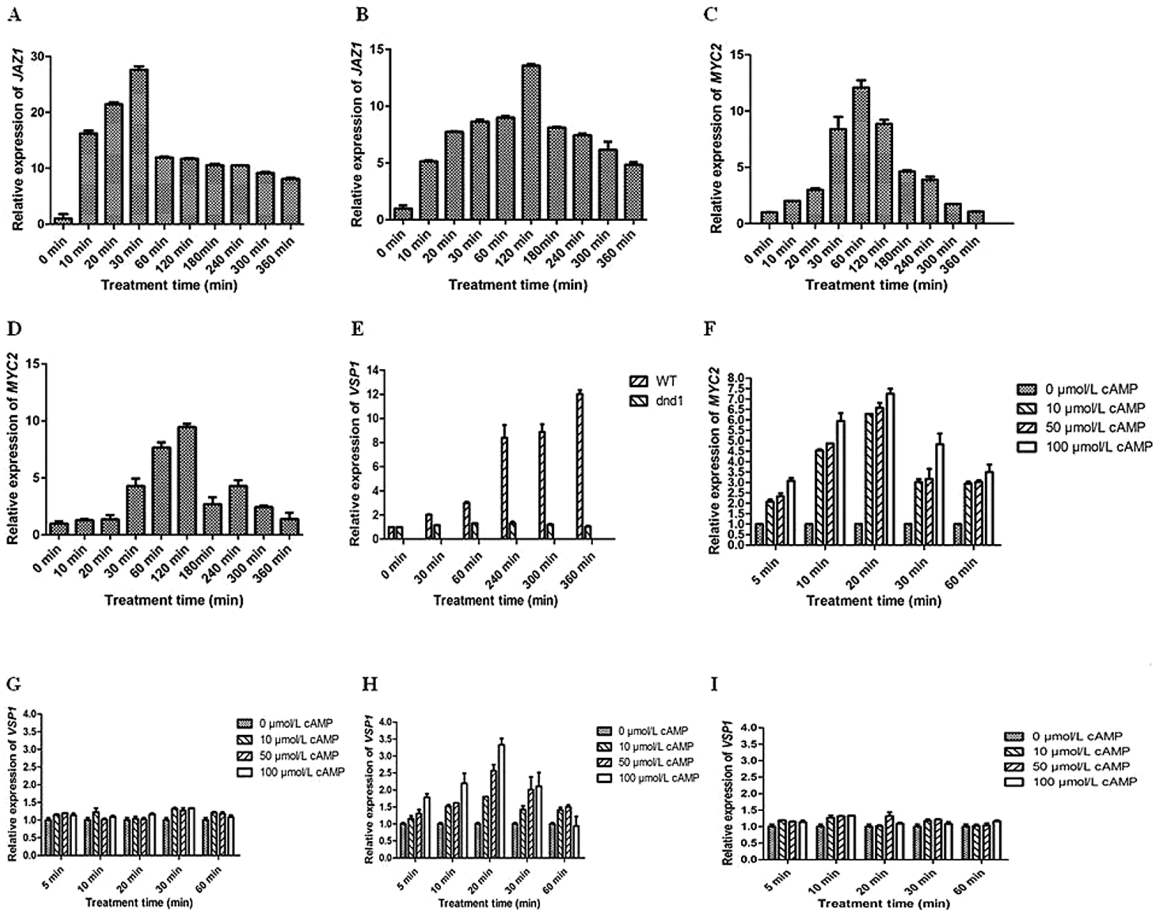

Fig. 6. Effect of JA and db-cAMP on JA responsive genes. Quantitative real-time PCR analysis of *JAZ1*, *MYC2*, and *VSP1* genes in 10-day-old WT (**A, C, E, F, H**) and *dnd1* (**B, D, E, G, I**) *Arabidopsis* seedlings treated with 100 μM JA (A, B, C, D, E) and 0, 10, 50, or 100 μM cAMP for 5, 10, 20, 30, or 60 min (F, G, H, I). The level of each gene is relative to that of *ACTIN8*. Data are means ± SE (three technical replicates).

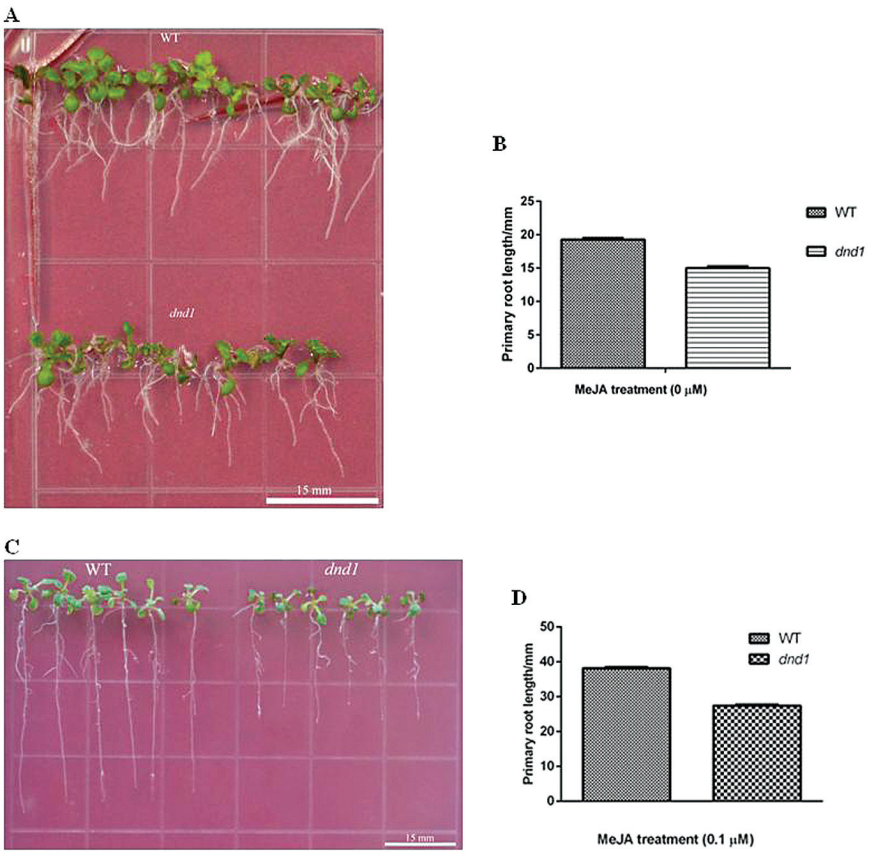

Fig. 7. Growth induction of primary roots by MeJA. Photographs were taken 9 days after sowing. Root length of WT *Arabidopsis* seedlings and *dnd1* was measured 9 days after sowing. (**A**) Seedlings and (**B**) primary root length of seedlings grown on MeJA-free agar. (**C**) Seedlings and (**D**) primary root length of seedlings grown on agar contained 0.1 μM MeJA. (This figure is available in colour at *JXB* online.)

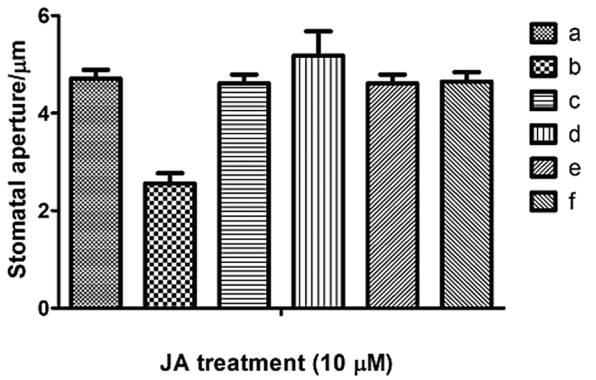

Fig. 8. Effect of MeJA on stomatal movement of WT *Arabidopsis* untreated with JA (a); treated with 10μM JA (b); and with JA washed out with Mes KOH (c). Effect of MeJA on stomatal movement of *dnd1* mutants untreated with JA (d); treated with 10 μM JA (e); and with JA washed out with Mes KOH (f).

et al., 2005). $[Ca^{2+}]_{cyt}$ elevation may serve as a crucial signal in JA-induced downstream responses, including JA response gene 1 (*JR1*) gene expression (Sun *et al.*, 2006). It is widely accepted that complex families of Ca^{2+}-binding proteins, functioning as calcium sensors, provide a toolkit for deciphering various Ca^{2+} signatures (Kudla *et al.*, 2010). CaMs, CaM-like proteins (CMLs), calcium-dependent protein kinases, and calcineurin B-like protein–calcineurin B-like interacting protein kinase complexes form intricate signalling networks for translating these signatures into downstream phosphorylation events and transcriptional responses (Tegg *et al.*, 2005). Ion channels can be classified as voltage-gated, ligand-gated, and stretch-activated Ca^{2+} channels (Cosgrove and Hedrich, 1991; White *et al.*, 2002; White and Broadley, 2003; Dutta and Robinson, 2004; Nakagawa *et al.*, 2007; Kudla *et al.*, 2010). CNGCs are considered candidates for conducting Ca^{2+} through the plant cell plasma membrane (Ma *et al.*, 2009b). CNGC1 (Ali *et al.*, 2006; Ma *et al.*, 2006), CNGC2 (Ali *et al.*, 2007; Ma *et al.*, 2010), CNGC11, CNGC12 (Urquhart *et al.*, 2007), and CNGC18 (Frietsch *et al.*, 2007) are involved in Ca^{2+} influx in *Arabidopsis*. As shown in Fig. 1, JA induced a $[Ca^{2+}]_{cyt}$ increase in WT *A. thaliana*, but not in the *dnd1* mutant (Fig. 1A, B). CNGC2 plays a pivotal role in providing a pathway for Ca^{2+} entry into plant cells and also contributes to Ca^{2+} uptake for nutrition (Ma and Berkowitz, 2011). Two major pathways for Ca^{2+} release into the cytoplasm may operate in plant cells (Blume *et al.*, 2000): direct Ca^{2+} influx through plasma membrane Ca^{2+} channels and Ca^{2+} entry by secondary messenger-induced depletion of intracellular stores, followed by store replenishment through Ca^{2+} release-activated Ca^{2+} currents across the plasma membrane (Sun *et al.*, 2009). Our previous work showed that extracellular and intracellular Ca^{2+} are

involved in JA-induced Ca^{2+} mobilization in *A. thaliana* (Sun *et al.*, 2010). The above analysis shows that extracellular Ca^{2+} influx through AtCNGC2 may be the first step in JA-induced $[Ca^{2+}]_{cyt}$ elevation.

JA-induced changes in the plasma membrane potential of A. thaliana

To investigate changes in the $[Ca^{2+}]_{cyt}$ and membrane potential in leaf epidermal cells, we recorded the kinetics of fluorescence intensity in leaf cells of WT and *dnd1* using LSCM. W-7, an antagonist of CaM, was used to evaluate the roles of AtCNGC2 and CaM in JA signalling and to investigate JA-induced $[Ca^{2+}]_{cyt}$ changes in WT and *dnd1* plants and changes in the expression of the JA responsive genes *JAZ1*, *MYC2*, and *VSP1*. Fluo-3, located in the cytoplasm, does not move to the vacuole or apoplast (Liu *et al.*, 2003). DiBAC4(3) has very low fluorescence intensity in the extracellular aqueous environment, but increases quantum yield upon binding to the hydrophobic core of the lipid membrane. Upon depolarization of the cell membrane, the negatively charged oxonol moves from the extracellular medium into the cytosol. An increased intracellular concentration of the dye results in increased binding to cellular membranes and, hence, increased fluorescence quantum yield and emission (Wolff *et al.*, 2003). Because the DiBAC4(3) fluorescence in intact protoplasts was not detectable in the vacuole, the fluorescence intensity of DiBAC4(3) accurately represents the plasma membrane voltage. Voltage-independent dye bleaching did not seem to occur (Konrad and Hedrich, 2008).

db-cAMP induced depolarization of the WT plasma membrane, but not in the *dnd1* mutant (Fig. 4B, C). Ma and Berkowitz (2011) also reported that a cAMP-activated inward Ca^{2+} current was found to be absent in *dnd1*mutant plant cells.

Application of 100 μM JA caused a rise in DiBAC4(3) fluorescence intensity both in WT and *dnd1* cells (Fig. 4A). JA induced plasma membrane depolarization in WT and *dnd1* cells, but notably the magnitude was greater in the former than the latter. $[Ca^{2+}]_{cyt}$ increases in the cytoplasm either as a result of uptake from the extracellular space through plasma membrane channels or release from internal stores, such as the endoplasmic reticulum or vacuoles. The spatial or kinetic change of $[Ca^{2+}]_{cyt}$ depends on the nature of stimulation (Sun *et al.*, 2006; Sun *et al.*, 2009). In plants, extracellular and intracellular calcium stores are believed to contribute to JA-induced increase of $[Ca^{2+}]_{cyt}$, and a nifedipine-sensitive plasma membrane Ca^{2+} channel maybe responsible for JA-induced elevation of $[Ca^{2+}]_{cyt}$ and downstream gene expression (Sun *et al.*, 2006). There is also cross-talk between the JA-induced influx of apoplast calcium and the release of IP_3-sensitive calcium stores (Sun *et al.*, 2009). Ca^{2+} released from IP_3-sensitive calcium stores may contribute to the significant depolarization in response to JA in the *dnd1* mutant.

DiBAC4(3) is reported to be sensitive to temperature and dilution (Whiteaker *et al.*, 2001). To avoid this, a final concentration of 2μM DiBAC4(3) was added to the external solution according to Konrad and Hedrich (2008).

CaM inhibited JA-induced Ca^{2+} influx through AtCNGC2

Ca^{2+} plays a vital role as a second messenger in plant cells during various developmental processes and in response to environmental stimuli (DeFalco *et al.*, 2009). Increases in $[Ca^{2+}]_{cyt}$ have been observed in response to a variety of environmental and developmental stimuli, such as heat shock (Gong *et al.*, 1998; Liu *et al.*, 2003), cold, drought, salt (Xiong *et al.*, 2002), pathogens (Blume *et al.*, 2000; Grant *et al.*, 2000), Nod factor (Shaw and Long, 2003), blue light (Baum *et al.*, 1999; Harada *et al.*, 2003), ABA treatment (Pei *et al.*, 2000), and JA treatment (Sun *et al.*, 2006; Sun *et al.*, 2009, 2010). Stimulus-induced elevations in $[Ca^{2+}]_{cyt}$ are believed to be perceived by Ca^{2+}-binding proteins and protein kinases (Anil and Rao, 2000). CaM, a Ca^{2+}-binding protein, positively regulates expression of *JR1*, a JA response gene in the JA signalling pathway (Sun *et al.*, 2006). Additionally, CPK6, a calcium-dependent protein kinase, functions as a positive regulator of MeJA signalling in *Arabidopsis* guard cells (Munemasa *et al.*, 2011). Plants have evolved a diversity of unique proteins that bind Ca^{2+} using the evolutionarily conserved EF-hand motif. CaMs (including CMLs) are EF-hand Ca^{2+} sensors in plants (DeFalco *et al.*, 2009). They facilitate the regulation of target proteins and thereby co-ordinate various signalling pathways through conformational changes in response to Ca^{2+}binding (DeFalco *et al.*, 2009). W-7, an antagonist of CaM, inhibited JA-induced $[Ca^{2+}]_{cyt}$ increases in WT *Arabidopsis* leaf cells (Fig. 2A), whereas it had no effect on *dnd1* plants (Fig. 2B). At the same concentration, W-5, an inactive structural analogue of W-7, had no significant effect on JA-induced $[Ca^{2+}]_{cyt}$ increases (Fig. 2A). These data imply that CaM is a negative regulator of AtCNGC2. CNGCs could be inactivated by elevated Ca^{2+} or CaM in the cytosol (Ma and Berkowitz, 2011). Cytosolic CaM binds to CNGCs at a region of the protein overlapping the cNMP-binding region; CaM binding prevents cNMP activation of CNGC currents (Ma *et al.*, 2009a). CaM may prevent further-JA induced $[Ca^{2+}]_{cyt}$ increases after the initial $[Ca^{2+}]_{cyt}$ increase to prevent Ca^{2+} forming insoluble complexes with proteins, membranes, and organic acids at higher concentrations.

cAMP is involved in JA-induced apoplastic Ca^{2+} influx

$[cAMP]_{cyt}$ in *A. thaliana* increased within 1 min of treatment with 100 μM JA and reached a peak at 2 min (Fig. 5). cAMP induced the extracellular Ca^{2+} influx through AtCNGC2 (Fig. 3), and $[Ca^{2+}]_{cyt}$ is required for CaM blocking of the current (Gaymard *et al.*, 1996). JA-induced $[cAMP]_{cyt}$ reached a peak within 3 min and remained elevated during the remainder of the assay period (Fig. 5), whereas JA-induced $[Ca^{2+}]_{cyt}$ increases reached a peak later, at 4 min, and remained at this level for the rest of the testing period (Fig. 1A). In the presence of an exogenous supply of cAMP, the CNGC current is constitutively activated (Clough *et al.*, 2000), and cAMP application increases $[Ca^{2+}]_{cyt}$ in a concentration-dependent manner (Fig. 3A). Analysis showed that JA induced an elevation in $[cAMP]_{cyt}$ before it induced an increase in $[Ca^{2+}]_{cyt}$. We

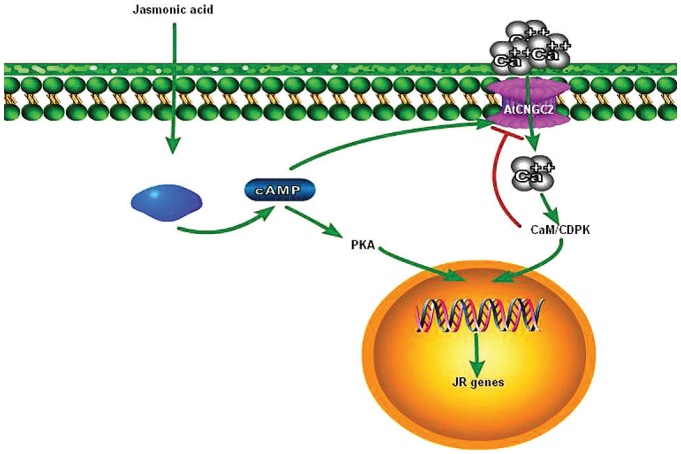

Fig. 9. Model of proposed JA-induced apoplastic Ca^{2+} mobilization. JA activates adenylate cyclase by an unknown pathway leading to an increase in $[cAMP]_{cyt}$. cAMP, as an activating ligand, activates the AtCNGC2 channel, resulting in an apoplastic Ca^{2+} influx into the cytosol and an increase in $[Ca^{2+}]_{cyt}$. This leads to mobilization of Ca^{2+} from intracellular Ca^{2+} stores, such as the endoplasmic reticulum and mitochondria, resulting in positive feed-forward regulation as a result of further increases in $[Ca^{2+}]_{cyt}$. As Ca^{2+} binds to CaM, the Ca^{2+}/CaM rise in the cytosol inhibits AtCNGC2, preventing the increase in $[Ca^{2+}]_{cyt}$, while at the same time Ca^{2+}/CaM transduces Ca^{2+} signals by modulating the activity of numerous and diverse CaM-binding proteins. (This figure is available in colour at *JXB* online.)

deduce that the elevation in $[Ca^{2+}]_{cyt}$ was due to the increase in JA-induced cAMP. With the (inside negative) membrane potentials present across the plant cell plasma membrane, a rise in cytosolic cAMP would activate the inwardly rectified cation current (Ma *et al.*, 2009a). CNGCs conduct both monovalent cations and Ca^{2+}, but when Ca^{2+} and monovalent cations coexist, CNGCs selectively gate Ca^{2+} (Leng *et al.*, 2002; White and Broadley, 2003; Ma *et al.*, 2009a). From the above data, we deduce that JA induces $[cAMP]_{cyt}$ elevation through an unknown pathway before the JA-induced $[Ca^{2+}]_{cyt}$ increase through AtCNGC2. db-cAMP is an analogue of cAMP that stimulates PKA (Yamauchi *et al.*, 2011). This agent has been shown to have no effect on cAMP levels (Bagg *et al.*, 2009). db-cAMP application results in plasma membrane depolarization, the magnitude of which increases with increasing cAMP concentration (Fig. 4B). According to the above analysis, we conclude that JA first induces an increase in $[cAMP]_{cyt}$, and this subsequently activates AtCNGC2, leading to an influx of extracellular Ca^{2+} into the cytosol (Fig. 9).

Acknowledgements

This work was supported by the National Natural Science Foundation of China (grant no.30700428), Beijing Natural Science Foundation (grant no. 5132006), and CIT&TCD (No. 201304096).

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