The chloride channel family gene CLCd negatively regulates pathogen-associated molecular pattern (PAMP)-triggered immunity in Arabidopsis

Wei Guo¹, Zhangli Zuo¹, Xi Cheng¹, Juan Sun¹, Huali Li¹, Legong Li² and Jin-Long Qiu¹,*

¹ State Key Laboratory of Plant Genomics, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
² School of Life Sciences, Capital Normal University, Beijing 100048, China

* To whom correspondence should be addressed. E-mail: qiujl@im.ac.cn

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Abstract

Chloride channel (CLC) family genes are ubiquitous from prokaryotes to eukaryotes and encode proteins with both channel and transporter activities. The Arabidopsis thaliana genome encodes seven CLC genes, and their products are found in a variety of cellular compartments and have various physiological functions. However, a role for AtCLCs in plant innate immunity has not previously been demonstrated. Here it is reported that AtCLCd is a negative regulator of pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). T-DNA insertion mutants of AtCLCd exhibited enhanced responses to the elicitor, flg22. The PTI phenotypes of the cld mutants were rescued by expression of AtCLCd. Overexpression of AtCLCd led to impaired flg22-induced responses. In line with a role for AtCLCd in PTI, the clcd mutants were more resistant to a virulent strain of the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 when spray inoculated, while AtCLCd-overexpressing lines displayed increased susceptibility to this pathogen. Interestingly, flg22 treatment was found to repress the expression of AtCLCd. In addition, its expression was elevated in mutants of the flg22 pattern recognition receptor (PRR) FLS2 and the PRR regulatory proteins BAK1 and BKK1, and reduced in an FLS2-overexpressing line. These latter findings indicate that FLS2 complexes regulate the expression of AtCLCd, further supporting a role for AtCLCd in PTI.

Key words: Arabidopsis thaliana, AtCLCd, chloride channel, PAMP-triggered immunity.

Introduction

Under natural conditions, plants are constantly exposed to harmful pathogens. Plants, being sessile, cannot simply escape from biotic stresses. However, they have evolved a complicated innate immune system to fight pathogen attacks. Their innate immune system generally consists of two layers of defence (Jones and Dangl, 2006). The first, named PAMP-triggered immunity (PTI), is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) by plant cell surface pattern recognition receptors (PRRs). Adapted pathogens can secrete effector proteins into host cells to suppress PTI. The second layer of defence, termed effector-triggered immunity (ETI), originates in the cytoplasm and is triggered directly or indirectly by the recognition of secreted microbial effectors by plant resistance (R) proteins. Thus, activation of plant innate immunity is largely dependent on recognition of ‘non-self’ signals (Robatzek and Saijo, 2008; Boller and He, 2009; Tsuda and Katagiri, 2010).

After perception of PAMPs or effectors, host cells initiate a series of physiological processes, of which the oxidative burst, extracellular alkalinization, and protein phosphorylation are the earliest (Felix et al., 1999; Bauer et al., 2001; Kunze et al., 2004). Extracellular alkalinization is caused by ion fluxes across the plasma membrane (Nürnberg et al., 1994; Jabs et al., 1997), indicating that ion channels are activated in response to pathogen attacks. A number of channels have been shown to play a role in plant defence responses (Jeworutzki et al., 2010; Qi et al., 2010; Koers et al., 2011). Anion channels have been shown to be required, especially...
for PTI. Elicitors or PAMPs such as cryptogein and flagellin can induce massive anion efflux (Wendehenne et al., 2002; Jeworutzki et al., 2010). The production of reactive oxygen species (ROS) is a common early response to PAMPs. Using a pharmacological approach, Colcombet et al. (2009) showed that rapid-type (R-type) anion channels are important in flagellin-induced ROS production in Arabidopsis suspension cells, and Jeworutzki et al. (2010) recorded strong anion currents in mesophyll and root hair cells of Arabidopsis upon PAMP treatment. In addition, inhibition of anion channels impaired the cryptogein-induced cell death in the hypersensitive response (HR) of tobacco suspension cells (Wendehenne et al., 2002). PAMP-triggered plasma membrane anion channel opening was found to be dependent on PRRs and BAK1, suggesting that the anion channels are downstream of PRRs (Jeworutzki et al., 2010).

Several gene families encoding anion channels/transporters have been identified in plants. Of these, three families, SLAC1 (slow anion channel 1), ALMT1 (aluminium-activated malate transporter 1), and CLC (chloride channel), have been the most studied (Barbir-Brygoo et al., 2011). Patch-clamp studies on Vicia faba guard cells revealed the presence of R-type and slow-type (S-type) anion channels (Schroeder and Keller, 1992). The S-type channel is encoded by SLAC1 (Vahisalu et al., 2008), and the R-type channel by members of the ALMT transporter family (Meyer et al., 2010). Interestingly, barley powdery mildew was shown to activate host cell S-type anion channels and thereby inhibit light-induced stomatal opening (Koers et al., 2011). Similarly, the Arabidopsis guard cell SLAC1 was found to be necessary for stomatal closure in response to biotic stress (Negi et al., 2008; Saji et al., 2008; Vahisalu et al., 2008; Montillet et al., 2013). Despite the evidence of the involvement of plant anion channels in defense responses, there is so far little direct evidence of the participation of anion channels in innate immunity, and how these channels regulate plant defense responses also remains elusive.

CLC family proteins are present in prokaryotes and eukaryotes and have both channel and transporter activities (Jentsch, 2008; Barbier-Brygoo et al., 2011). The Arabidopsis genome encodes seven AtCLC genes (AtCLCa–AtCLCg) (Hechenberger et al., 1996; Lv et al., 2009), and their products are found in several cellular compartments including the vacuole membrane (AtCLCa, AtCLCb, and AtCLCc) (De Angeli et al., 2007; von der Fecht-Bartenbach et al., 2010), the Golgi apparatus (AtCLCd and AtCLCe), and chloroplast membranes (AtCLCe) (Marmagne et al., 2007; von der Fecht-Bartenbach et al., 2007). AtCLCa, AtCLCb, and AtCLCe are required to maintain normal cellular nitrate levels (De Angeli et al., 2007; von der Fecht-Bartenbach et al., 2010), and AtCLCc participates in both nitrate and chloride homeostasis and regulates stomatal movement and salt tolerance (Jossier et al., 2010). AtCLCd has been proposed to regulate luminal pH in the trans-Golgi network (TGN) (von der Fecht-Bartenbach et al., 2007). However, a role for AtCLCs in plant innate immunity has not previously been demonstrated. In this work, the defence phenotypes of all available AtCLC family mutants were examined and it was found that AtCLCd negatively regulates PTI.

Materials and methods

Plant materials and growth conditions

The T-DNA insertion mutants clcd-1 (SALK_042895), clcd-2 (SALK_052368C), clca (CS857712), clcb (SALK_027349C), clcc (SALK_115644C), clce (SALK_010237), and clec (SALK_087699) were obtained from the Arabidopsis Biological Resource Center. Homozygous T-DNA insertion lines were screened by PCR and confirmed by reverse transcription–PCR (RT–PCR) using gene-specific primers (see Supplementary Table S1 available at JXB online). 

Gene constructs and plant transformation

To complement the clcd mutant, the 1.6 kb promoter region of AtCLCd and the full-length open reading frame were separately PCR amplified from Arabidopsis Col-0 genomic DNA and cDNA, respectively, and the NOS terminator was then cloned into the binary vector pCB302 (Xiang et al., 1999) to create construct pAtCLCd:AtCLCd. To overexpress AtCLCd, the full-length coding sequence was amplified and cloned downstream of the Cauliflower mosaic virus (CaMV) 35S promoter in the binary vector pCB302-3 (Xiang et al., 1999) to obtain p35S:AtCLCd. To overexpress FLS2, the full-length coding sequence was amplified from cDNA and cloned into binary vector pCAMBIA1300 (Cambia) between the Bar promoter and SpeI multiple cloning sites to form p35S:FLS2. The primers used for making the constructs are listed in Supplementary Table S1 available at JXB online. All constructs were verified by sequencing and introduced into Agrobacterium tumefaciens strain GV3101 by electroporation. Agrobacterium tumefaciens carrying the constructs was used to transform Arabidopsis Col-0 by floral dip (Clough and Bent, 1998). Transformed Arabidopsis lines were selected on soil by spraying with a 1:1000 dilution of Basta (Bayer CropScience) or on MS (Murashige and Skoog) agar plates supplied with 20 μg ml−1 hygromycin B.

Semi-quantitative and quantitative real-time RT–PCR

Total RNA was extracted from Arabidopsis leaves with TRIzol reagent (Invitrogen). A sample containing 2 μg of total RNA was treated with DNase I (Invitrogen) and reverse transcribed with M-MLV Reverse Transcriptase (Promega). For semi-quantitative PCR, 25 μl reaction mixtures contained 0.5 U of Taq DNA polymerase (MBI, Fermentas), 80 ng of cDNA, 200 μM of each dNTP, and 0.2 μM of each primer. PCR parameters were: 3 min at 95 °C followed by 28 cycles of 95°C for 30 s, 60 °C for 30 s, and 72 °C for 40 s.

Real-time PCR was performed using Takara SYBR Premix Ex Taq following the manufacturer's instructions, and run in a Bio-Rad CFX96 (Bio-Rad Laboratories). The reaction volume was 20 μl containing 10 μl of SYBR pre-mix, 0.5 μM of each primer, and 20 ng of cDNA. A three-step protocol was used: a denaturation program (95 °C for 30 s), an amplification and quantification program repeated 40 times (95 °C for 5 s, 60 °C for 30 s and 72°C for 30 s with the fluorescence measurement), and a melting curve program (55–95 °C, with a 0.5 °C increment each cycle). Each sample was replicated three times. ACTIN2 was used as an internal reference gene, and normalized fold expression was calculated employing CFX Manager Software (Bio-Rad) and the ΔΔC(t) method. Unless otherwise indicated, result values displayed are relative to wild-type (Col-0) untreated plants, which are set to a relative value of 1. The primers used in semi-quantitative and real-time RT–PCR are listed
Bacterial growth assays

Five-week-old plants were spray inoculated with *Pseudomonas syringae pv. tomato* DC3000 (*Pst. DC3000*), and bacterial growth in planta was analysed as described by Zipfel et al. (2004). The bacterial suspension contained 2.5 \times 10^8 (Fig. 4A) or 2.5 \times 10^6 (Fig. 4B) colony-forming units (cfu) ml^{-1} in 10 mM MgCl₂ with 0.01% Silwet L-77. Eight plants of each genotype were used per experiment, and the experiments were repeated at least three times. Bacterial numbers in mutant and transgenic lines were compared with those in Col-0 using a two-tailed Student’s t-test.

ROS burst assays

Flg22 peptide (QRLSTGSRINSAKDDAAGLQIA) and elf18 peptide (acetyl-MSKEKFERTKPHVNVGTI) were synthesized by GenScript Corp. Chitin (Seikagaku) was kindly provided by Dr Morten Petersen. All the above PAMPs were dissolved in sterile water. PAMP-induced ROS production was measured as previously described (Roux et al., 2011; Schuessinger et al., 2011). Briefly, leaf discs (0.125 cm²) of 5-week-old plants were incubated overnight in 96-well plates in a water. The water was replaced with 200 μl of a solution containing 10 μg ml⁻¹ peroxidase (Sigma-Aldrich) and 20 μM luminol in the presence of 100 nM flg22, 100 nM elf18, or 100 μg ml⁻¹ chitin. Luminescence is shown as relative light units (RLUs), measured and calculated using a Berthold Centro LB960 luminometer (Berthhold Technologies).

Callose deposition assays

Leaves of 5-week-old *Arabidopsis* plants were infiltrated with 1 μM flg22 with a needleless syringe. After 16 h, the leaves were cleared, stained with aniline blue as previously described (Hann and Rathjen, 2007), mounted in 50% glycerol, and examined with a UV epifluorescence microscope. The numbers of bright spots (corresponding to callose deposits) per microscopic field of 1 mm² were counted using Image J software (http://imagej.nih.gov/ij/), and 12 microscopic fields were counted per sample.

Root growth assays

*Arabidopsis* seeds were surface-sterilized and sown on 1/2 MS agar medium. Seeds were stratified at 4 °C for 2 d and grown vertically for 5 d in short-day conditions (8 h light/16 h dark). Seedlings were then transferred to a new square Petri dish with 1/2 MS agar medium supplemented with different amounts of flg22 peptide. The lengths of the main roots after growth under long-day conditions (16 h light/8 h dark) were measured with Image J software.

Seedling growth assays

The seedling growth assays were performed as described (Pfund et al., 2004). The fresh weight of seedlings was measured 8 d after flg22 treatment.

Accession numbers

Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank/EMBL databases under the following accession numbers: *Arabidopsis AtCLCa* (At1g50980), *AtCLCb* (At3g27170), *AtCLCc* (At5g49890), *AtCLCd* (At1g51890), *AtCLCe* (At2g17740), *AtCL Cf* (At1g55620), *AtCL Mg* (At5g33280), *FRK1* (At2g19190), *FLS2* (At5g46330), *PRI* (At2g14610), *ACTIN7* (At5g09810), and *ACTIN2* (At3g18780).

**Results**

Arabidopsis clcd mutant exhibits enhanced flg22-induced responses

In a search for the anion channel(s) involved in plant defence responses, especially PTI, all available mutants of the *Arabidopsis CLC* gene family were screened using flg22-induced ROS as an indicator. flg22, a conserved 22 amino acid sequence at the N-terminus of flagellin, is a well-studied PAMP in plant innate immunity (Felix et al., 1999; Bauer et al., 2001). ROS production triggered by flg22 was measured in *Arabidopsis* T-DNA insertion lines of the AtCLCa, b, c, d, e, and g genes (Supplementary Fig. S1 available at *JXB* online). Leaf discs from all these lines except the clcd mutant produced similar ROS bursts to those produced by Col-0 (wild-type) *Arabidopsis* plants (Fig. 1A). However, the flg22-triggered ROS burst was significantly larger in the clcd-2 mutant (Fig. 1A). This result was confirmed with another clcd mutant, clcd-1 (Fig. 1B). The impact of *AtCLCd* mutations on other temporal responses triggered by flg22 was then examined (Roux et al., 2011). The ROS burst is an early response to PAMPs, whereas callose deposition is a late response, detected in *Arabidopsis* by aniline blue staining ~16 h after flg22 treatment (Boller and Felix, 2009). The flg22-induced callose deposition was also larger in clcd-1 and clcd-2 than in Col-0 (Fig. 1C). These findings indicate that AtCLCd is involved in PTI. Since the clcd-1 and clcd-2 mutants had almost identical phenotypes, to simplify the work, further analyses concentrated on the clcd-1 mutant.

The expression of PAMP-inducible genes in the clcd mutants was next assessed. The expression levels of three different PTI marker genes FRK1, At1g51890, and At2g17740 (He et al., 2006) were measured 1 h after infiltration with 1 μM flg22. As shown in Fig. 1D, the induction of all three PTI marker genes was enhanced in the clcd-1 mutant. Interestingly, the late response gene PRI was also induced to a strikingly higher level in clcd-1 than in Col-0 (Fig. 1E).

In order to confirm that the phenotype of the clcd mutants is actually caused by mutation in the *AtCLCd* gene, clcd-1 plants were transformed via *A. tumefaciens* pAtCLCd:AtCLCd, a construct carrying the full-length open reading frame of *AtCLCd* driven by its own promoter (1571 bp upstream of the start codon). A number of transgenic plants were obtained, most of which had the wild-type level of *AtCLCd* expression as checked by semi-quantitative RT–PCR (Fig. 1F). Representative lines homozygous for the rescue construct were used for further phenotypic analysis. The changes in the flg22-triggered ROS burst (Fig. 1G) and in the expression of FRK1 (Fig. 1H) were also rescued by expressing *AtCLCd*.

In summary, it was shown that mutations in *AtCLCd* lead to enhanced early and late responses to flg22, suggesting that AtCLCd negatively regulates PTI.

**Overexpression of AtCLCd impairs limited flg22-induced responses**

To establish further the role of AtCLCd in PTI, transgenic plants constitutively overexpressing *AtCLCd* were created.

In Supplementary Table S1 available at *JXB* online. Data are representative of two to three independent biological experiments.
Fig. 1. PAMP-triggered immunity is enhanced in Arabidopsis clcd mutants. (A) Total ROS production triggered by 100 nM flg22 in Arabidopsis leaf discs in relative light units (RLU). Results are expressed as percentages of flg22-treated Col-0. (B) flg22-induced ROS bursts in Col-0, clcd-1, and clcd-2 leaf discs. (C) Callose deposition in Col-0, clcd-1, and clcd-2 leaves after infiltration with 1 μM flg22 (n=22). (D and E) Quantitative real-time PCR analysis of the expression of PTI marker genes (D) and PR1 (E) in Arabidopsis leaves 1 h (D) and 24 h (E) after infiltration with 1 μM flg22 or water. The samples were measured in triplicate and normalized to ACTIN2. (F) Expression of AtCLCd determined by semi-quantitative RT–PCR in the wild-type (Col-0), AtCLCd complementation lines (NP-d4 and NP-d9), and clcd-1. Levels of ACTIN7 transcripts were used as loading controls. (G) Flg22-induced ROS bursts in Col-0, clcd-1, NP-d4, and NP-d9 in RLU. (H) Quantitative real-time PCR analysis of the expression of the PTI marker gene FRK1 in Col-0, clcd-1, and NP-d4 plants 1 h after infiltration with 1 μM flg22 or water. Samples were assayed in triplicate and normalized to ACTIN2. Values are means ±SD. NT, no treatment. **P<0.01 (t-test).
Altered bacterial disease resistance in Arabidopsis clcd mutants and AtCLCd-overexpressing plants

PTI plays an important role in basal resistance to bacterial pathogens (Jones and Dangl, 2006), and defects in PTI, due to mutations in the PRR FLS2 and its positive regulator BAK1, result in enhanced susceptibility to the virulent bacterial pathogen Pst. DC3000 upon spray inoculation (Zipfel et al., 2000). The most striking difference between the AtCLCd-misexpressing plants and the wild type in their responses to flg22 was observed after treating them with a lower dose (10 nM) of the peptide (Fig. 3A, B). It is possible that a saturating dose of flg22 prevents detection of a partial effect of the mutation or of overexpression of AtCLCd. The effect of AtCLCd misexpression on flg22-triggered responses was further revealed by seedling growth inhibition assays (Supplementary Fig. S3 available at JXB online).

Responses of the AtCLCd-misexpressing plants to different PAMPs were next examined. Production of ROS elicited by both chitin and elf18 was significantly enhanced in the clcd mutant, but reduced in the AtCLCd-overexpressing lines (Fig. 3C, D). Accordingly, expression of the PTI marker genes At1g51890 and At2g17740 was also increased in the mutant, but decreased in the overexpressing lines (Fig. 3E, F). In addition, the morphological phenotypes and expression levels of FLS2 (Supplementary Fig. S4 available at JXB online) were not changed in the AtCLCd-misexpressing plants. These results support a general role for AtCLCd in PTI.

Misexpression of AtCLCd affects PTI responses

To confirm the regulatory role of AtCLCd in PTI, the phenotypes of AtCLCd-misexpressing plants were investigated in more detail. The responses of the clcd mutants and the AtCLCd-overexpressing lines to various doses of flg22 were first analysed. As shown in Fig. 3A, production of ROS induced by flg22 was increased in the clcd mutant and reduced in the AtCLCd-overexpressing lines compared with the Col-0 wild type. Root growth inhibition is another characteristic effect of flg22 treatment (Boller and Felix, 2009). The clcd-1 seedlings exhibited increased root growth inhibition in the presence of flg22, while root growth was less inhibited by flg22 in the AtCLCd-overexpressing lines (Fig. 3B). However, the most
**Fig. 3.** PAMP-triggered immunity is affected by misexpression of AtCLCd. (A) Total ROS production elicited by different amounts of flg22 in Arabidopsis leaf discs is represented as relative light units (RLU). Results are expressed as percentages of flg22-treated Col-0, and are means ±SD (n=8). (B) Inhibition of primary root growth by different doses of flg22. Results are expressed as percentages of inhibition relative to the untreated control; means ±SD of three independent experiments (n>20). (C and D) Total ROS production induced by chitin or elf18 in Col-0, clcd-1, and OE-d2, measured in RLU. Values are means ±SD (n=8). (E and F) Quantitative RT–PCR analysis of the expression of the PTI marker genes 1 h after treatment with chitin and elf18. NT, no treatment. *P<0.05; **P<0.01 (t-test).

**Fig. 4.** Responses of the clcd mutants and AtCLCd-overexpressing lines to the pathogen Pseudomonas syringae. (A and B) Bacterial growth of Pst. DC3000 was measured in Col-0, clcd-1, and clcd-2 (A) or Col-0 and AtCLCd-overexpressing lines OE-d2 and OE-d4 (B) on 0 d and 3 d post spray inoculation (dpi). Bacterial suspensions containing 2.5 × 10⁶ (A) and 2.5 × 10⁵ (B) cfu ml⁻¹ were used. Values are mean ±SD (n=8). *P<0.05; **P<0.01 (t-test). cfu, colony-forming units.
Tests were carried out to determine whether AtCLCd controls disease resistance. Four-week-old clcd-1 and clcd-2 plants were spray inoculated with Pst. DC3000, and growth of the bacterial pathogen in the leaves was assessed. As shown in Fig. 4A, growth of Pst. DC3000 in the leaves of the clcd mutants was reduced, whereas in the AtCLCd-overexpressing lines it was increased (Fig. 4B). These data further support the inhibitory role of AtCLCd in PTI.

**Treatment with the PAMP, flg22, represses the expression of AtCLCd**

Since AtCLCd negatively regulates PTI, it was of interest to see whether AtCLCd expression was affected by PAMPs. To this end, 4-week-old Col-0 plants grown under short-day conditions were infiltrated with 1 μM flg22 or water (mock). The expression of AtCLCd was reduced by the flg22 treatment (Fig. 5A). Expression of AtCLCd was then examined in more detail (Fig. 5B). Treatment with water (as a control) stimulated the expression of AtCLCd (Fig. 5B), probably due to damage introduced by the infiltration. However, compared with the water-treated plants, the accumulation of AtCLCd mRNA was significantly reduced in the flg22-treated plants at 5 h post-treatment (Fig. 5B), further showing that flg22 negatively regulates the expression of AtCLCd. By 10 h, expression of AtCLCd was similar in the flg22-treated and water-treated plants. To see whether the repression of AtCLCd by flg22 is dependent on FLS2, expression of AtCLCd was measured in fls2 mutant plants (SALK_141277, Xiang et al., 2008) infiltrated with 1 μM flg22 or water. As shown in Fig. 5C, transcripts of AtCLCd accumulated to similar level in flg22-treated plants and mock-treated plants, indicating that flg22 does not suppress the expression of AtCLCd in the fls2 mutant. Thus, it can be concluded that recognition of flg22 by FLS2 is needed for the inhibition of AtCLCd expression.

Next, the response of all other Arabidopsis CLC family genes to flg22 was examined. Interestingly, all were affected in the same way as AtCLCd (Fig. 5D). It appears therefore that the sensitivity of expression to flg22 may be common to all Arabidopsis CLC genes.

**FLS2 signalling complexes regulate the expression of AtCLCd**

It was noticed that expression of AtCLCd was higher in the fls2 mutant than in Col-0 (Fig. 6A). To confirm this finding, transcript levels in the fls2 mutant and the Wassilewskija (Ws-0) background were compared. The ecotype Ws-0 is a natural fls2 mutant (Gómez-Gómez and Boller, 2000; Zipfel et al., 2004). As shown in Fig. 6B, AtCLCd transcript levels in the Ws-0 background were similar to those in the fls2 mutant and almost 2- to 2.5-fold higher than in Col-0. Moreover, AtCLCd transcript levels were strikingly reduced in the FLS2-overexpressing line (Fig. 6C). These results indicate that PRR FLS2 negatively regulates the expression of AtCLCd.

**Arabidopsis** somatic embryogenesis receptor-like kinases (SERKs) form complexes with PRRs in a ligand-dependent manner (Monaghan and Zipfel, 2012). BAK1/SERK3 and BKK1/SERK4 are required for FLS2-mediated PTI signaling in Arabidopsis (Roux et al., 2011). Recently, a novel bak1-5 mutant allele was identified in which only PTI was impaired, thereby avoiding the pleiotropic effects of the other bak1 mutations (Schwessinger et al., 2011). Even though the bkk1-1 mutant exhibited wild-type-like responses to flg22, loss of BKK1 further decreased the early and late responses of bak1-5 to flg22 (Roux et al., 2011). The study was thus extended to quantify expression of AtCLCd in bakk1-5, bkk1-1, and a bak1-5/bkk1-1 double mutant. As shown in Fig. 6D, transcripts of AtCLCd accumulated to higher levels in the three mutants than in Col-0. Expression of AtCLCd was higher in bkk1-1 than in bakk1-5. The combination of the two mutations had no additive effect on expression (Fig. 6D). These findings imply that the FLS2 regulatory proteins BAK1 and BKK1 play a role in the regulation of AtCLCd expression and that they function in the same pathway.

Plants are always exposed to a variety of microbes under non-sterile soil conditions; therefore, AtCLCd expression in sterile seedlings was checked. As shown in Fig. 6E, the accumulation of AtCLCd transcripts did not change significantly in Col-0, Ws-0, fls2, and FLS2-overexpressing plants under sterile conditions. Interestingly, expression of AtCLCd in
bak1-5, bkk1-1, and bak1-5/bkk1-1 plants was also significantly reduced under sterile conditions, but was still a little higher than that in wild-type plants (Fig. 6F). These results support that PAMP perception is required for FLS2 to regulate AtCLCd expression.

Taken together, the above results indicate that the FLS2 signalling complex regulates the expression of AtCLCd in Arabidopsis.

**Discussion**

Changes in cellular anion content are thought to be associated with plant defence responses (De Angeli et al., 2007; Gauthier et al., 2007; Erraki et al., 2008; Colcombet et al., 2009). Arabidopsis CLC family genes encode putative anion channels (Barbier-Brygoo et al., 2011). However, whether CLC channels participate in plant innate immunity was still unclear. Therefore, T-DNA insertion lines of AtCLCa, b, c, d, e, and g were screened for changes in flg22-induced ROS, and it was found that cled mutants were unique in displaying enhanced ROS production in response to flg22 (Fig. 1). This and other findings (Figs 2, 3) provide ample evidence that AtCLCd is a negative regulator of PTI.

It was further shown that the PAMP, flg22, represses the expression of AtCLCd (Fig. 5A, B). However, the repression was not seen in a mutant of the flg22 receptor FLS2 (Fig. 5C). This and other findings (Fig. 6) showed that the FLS2 complex...
is required for maintaining the expression of AtCLCd. Since expression of FLS2 is induced by flag22 (Zipfel et al., 2004), it is possible that the decrease in expression of AtCLCd by flag22 is due in part to an increase of FLS2. Expression of the other Arabidopsis CLC genes was also reduced upon treatment with flag22 (Fig 5D), suggesting that these genes also play a role in PTI. However, ROS production was unchanged in the corresponding mutants (Fig. 1). A possible explanation for that is that there is functional redundancy of these genes. When compared with CLC genes from the monocot, rice, AtCLCd forms its own group, whereas the other AtCLC genes cluster together (von der Fecht-Bartenbach et al., 2010). Analysis of combinations of these mutants may be necessary to elucidate their roles in plant innate immunity.

AtCLCd has been shown to localize to the TGN (von der Fecht-Bartenbach et al., 2007; Lv et al., 2009). Therefore, it is unlikely that it is directly involved in downstream signalling upon PAMP perception, which takes place at the plasma membrane. The yeast genome encodes only one CLC protein, Gfl1p, and it regulates the intra-Golgi pH (Hechenberger et al., 1996). Expression of AtCLCd fully rescues the gfl1 yeast mutant phenotype (Hechenberger et al., 1996; Marmagne et al., 2007; Lv et al., 2009), suggesting that AtCLCd may have a function similar to Gfl1p. Moreover, AtCLCd has been shown to co-localize with the V-type ATPase subunit, VHA-a1, in the TGN (von der Fecht-Bartenbach et al., 2007). Inhibition of VHA-a1 affects Golgi morphology and restricts cell expansion (Dettmer et al., 2005, 2006; Brüx et al., 2008), and this effect is enhanced in the clcd mutant (von der Fecht-Bartenbach et al., 2007), further implying that AtCLCd is involved in adjusting the luminal pH of the TGN. pH homeostasis of the TGN is essential for its functioning (Demaux et al., 1998; Dettmer et al., 2006). Therefore, AtCLCd most probably regulates the functioning of the TGN by affecting the pH within it.

The TGN is an important platform for sorting cargo proteins to the cell surface or vacuole and lysosome (Viotti et al., 2010; Beck et al., 2012a). A distinctive characteristic of the membrane trafficking system in plants is the convergence of the secretory and endocytic pathways at the TGN (Dettmer et al., 2006; Dhonukshe et al., 2007; Viotti et al., 2010). Endocytic membrane transport has been observed for several plasma membrane receptors in plants, and this seems to be a general regulatory mechanism for perception of extracellular stimuli by plasma membrane receptors (Takano et al., 2002; Robatzek et al., 2006; Beck et al., 2012a). FLS2, the PRR for flag22 (Gómez-Gómez and Boller, 2000, 2002), is localized to the plasma membrane and becomes specifically internalized into highly mobile vesicles upon addition of flag22 (Robatzek et al., 2006). The endocytic transport of FLS2 is critical for its function in PTI (Robatzek et al., 2006). Recently, it was shown that the TGN is an essential compartment for membrane trafficking of FLS2 (Beck et al., 2012b; Choi et al., 2013; Uemura and Nakano, 2013). Interestingly, AtCLCd has been previously suggested to be involved in membrane trafficking, since AtCLCd-green fluorescent protein (GFP) co-localized with endocytosed FM4-64, a dye widely used for tracing endocytic membrane traffic (von der Fecht-Bartenbach et al., 2007). It thus seems likely that AtCLCd regulates PTI via the TGN, probably by affecting FLS2 trafficking. Endocytosis is a feature of most of the PRRs in plants (Beck et al., 2012a).

In agreement with this, it was also found here that different PAMP-induced defence responses were impaired in the AtCLCd-misexpressing plants (Fig. 3). Nevertheless, further studies are needed to reveal whether FLS2 trafficking is affected in the clcd mutant and the overexpressing lines.

In summary, it has been shown here that AtCLCd negatively regulates Arabidopsis PTI, probably by interacting with the PRR signalling pathway. Its sequence indicates that AtCLCd encodes a chloride/proton exchanger (Zifarelli and Pusch, 2010). Future work involving classical and patch-clamp electrophysiology should establish whether the role of AtCLCd in PTI requires a functional channel.

Supplementary data

Supplementary data are available at JXB online.

Table S1. Sequences of primers used in this work

Figure S1. Characterization of Arabidopsis CLC T-DNA insertion lines.

Figure S2. PAMP-triggered immunity is compromised in the AtCLCd-overexpressing lines.

Figure S3. Shoot growth inhibition induced by flag22 in Col-0, clcd mutant and AtCLCd-overexpressing plants.

Figure S4. Morphological phenotypes and FLS2 expression levels in the AtCLCd-misexpressing plants.

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References


AtCLCd negatively regulates PTI


