Short title: Role of AtCCC1 in plant immunity

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CATION-CHLORIDE CO-TRANSPORTER 1 (CCC1) mediates plant resistance against Pseudomonas syringae

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One-sentence summary: Arabidopsis CCC1 functions in strengthening plant structural and chemical barriers and mediates plant resistance against *Pseudomonas syringae*.

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Abstract

Plasma membrane (PM) depolarization functions as an initial step in plant defense signaling pathways. However, only a few ion channels/transporters have been characterized in the context of plant immunity. Here, we show that the Arabidopsis (Arabidopsis thaliana) Na⁺:K⁺:2Cl⁻ (NKCC) cotransporter CCC1 has a dual function in plant immunity. CCC1 functions independently of PM depolarization and negatively regulates pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI). However, CCC1 positively regulates plant basal and effector-triggered resistance to Pseudomonas syringae pv. tomato (Pst) DC3000. In line with the compromised immunity to Pst DC3000, ccc1 mutants show reduced expression of genes encoding enzymes involved in the biosynthesis of antimicrobial peptides, camalexin, and 4-OH-ICN, as well as Pathogenesis-Related (PR) proteins. Moreover, genes involved in cell wall and cuticle biosynthesis are constitutively downregulated in ccc1 mutants, and the cell walls of these mutants exhibit major changes in monosaccharide composition. The role of CCC1 ion transporter activity in the regulation of plant immunity is corroborated by experiments using the specific NKCC inhibitor bumetanide. These results reveal a function for ion transporters in immunity-related cell wall fortification and antimicrobial biosynthesis.

Introduction

Under natural conditions, plants are invariably challenged by harmful pathogens. However, plants possess several lines of defense mechanisms to protect themselves from disease (Hammond-Kosack and Jones, 1996; Hentschel, 2013). First, plants have
unique cuticle and cell wall structural barriers, and they secrete antimicrobial peptides and chemical compounds to deter microbial invaders (Lamb et al., 1989; Hématy et al., 2009; Bacete et al., 2017; Hammond-Kosack and Jones, 1996; Ahuja et al., 2012). After escaping from this first line of plant defense, pathogens might reach the cell membrane, where a second layer of the plant immune system can be triggered (Jones and Dangl, 2006; Dodds and Rathjen, 2010). Here, plant cell-surface pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) to initiate PAMP-triggered immunity (PTI) (Schwessinger and Zipfel, 2008; Bigeard et al., 2015). PTI is generally believed to be sufficient to halt nonpathogenic microbial growth (Xin and He, 2013). However, successful pathogens can breach PTI by deploying effectors to dampen PTI responses (Block and Alfano, 2011; Macho and Zipfel, 2015). To counter effector-induced suppression of PTI, a large family of polymorphic nucleotide-binding/leucine-rich-repeat receptors (NLRs) can directly or indirectly detect the presence of specific effectors. Recognition of effectors triggers a third robust layer of resistance responses called effector-triggered immunity (ETI) (Dou and Zhou, 2012; Feng and Zhou, 2012; Cui et al., 2015; Tang et al., 2017), which is essential to stop virulent pathogens from causing disease in certain plant genotypes (Lacombe et al., 2010; Xin and He, 2013).

Twenty years ago, it was shown that in cultured parsley cells, perception of PAMPs or effectors results in ion fluxes across the plasma membrane (PM) as one of the earliest defense responses (Jabs et al., 1997). Importantly, blocking ion fluxes by ion channel inhibitors prevented defense reactions, suggesting an important role of ion channels or transporters in plant defense against pathogens (Jabs et al., 1997).
Thereafter, a large number of studies has been conducted on the molecular identity and function of ion channels/transporters in plant immunity. In mesophyll cells of Arabidopsis (Arabidopsis thaliana), Jeworutzki and colleagues described flg22- and elf18-induced PM depolarization functions as an initial step in the early signaling pathway of defense response (Jeworutzki et al., 2010). CYCLIC NUCLEOTIDE-GATED CHANNEL 2 (CNGC2) regulates Ca\textsuperscript{2+} influx, which activates calcium-dependent protein kinases (CDPKs). These CDPKs regulate downstream PTI responses, linking cytosolic Ca\textsuperscript{2+} elevation to downstream defense reactions (Clough et al., 2000; Jurkowski et al., 2004; Ali et al., 2007; Boudsocq et al., 2010; Qi et al., 2010). Ca\textsuperscript{2+} signals also regulate the biosynthesis of salicylic acid (SA) by Calmodulin binding transcription factor CBP60g (Wang et al., 2009). Anion channels are also required for defense responses, especially for PTI, as inhibition of rapid-type anion channels suppresses flg22-induced ROS production in Arabidopsis suspension cells (Colcombet et al., 2009). Arabidopsis guard cell SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) is necessary to initiate stomatal closure upon pathogen treatment (Vahisalu et al., 2008; Koers et al., 2011; Montillet et al., 2013; Guzel Deger et al., 2015). Guo and colleagues reported that CHLORIDE CHANNEL D (CLC-D), which has both channel and transporter activities, is a negative regulator of PTI in Arabidopsis, demonstrating the importance of chloride channels in plant immunity (Guo et al., 2014). However, compared to the extensive knowledge on Ca\textsuperscript{2+} signaling, the role and mechanistic basis of anions in plant immunity has been poorly characterized.

In animals, CCC family members mediate movement of Cl\textsuperscript{−} coupled with that of K\textsuperscript{+} and/or Na\textsuperscript{+} cations across the PMs (Hübner and Rust, 2006). There is only one CCC
gene in Arabidopsis, referred to here as CCC1. Arabidopsis CCC1 encodes a plant homologue of the animal Na\(^+\):K\(^+\):2Cl\(^-\) cotransporter (NKCC), which was localized to the Golgi and trans-Golgi network (Henderson et al., 2015). Here, we investigated the role of CCC1 in the antibacterial defense response of Arabidopsis to three *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 strains. Pst DC3000 carries about 30 effectors (Cunnac et al., 2011), which are delivered into plants via the type III secretion system (T3SS) and suppress plant defenses (Ishiga et al., 2011; Zhang et al., 2012; Johansson et al., 2015). Pst DC3000 *avrRpm1*, however, carries the *avrRpm1* gene that is recognized by Arabidopsis RPM1 thereby rapidly inducing ETI responses that are mostly accompanied by a hypersensitive response at the infection sites (Xin and He, 2013). The third strain is Pst DC3000 *hrcC*\(^-\), in which the effectors cannot be delivered into the plant cell as the T3SS is defective. Hence, Pst DC3000 *hrcC*\(^-\) only induces PTI and allows only very low proliferation of the Pst DC3000 *hrcC*\(^-\) strain in plants (Jin and He, 2001).

We report here that CCC1 is not necessary for PM depolarization. However, CCC1 regulates both PTI and basal plant resistance to Pst DC3000. Surprisingly, although ccc1 mutant plants are compromised in defense against virulent and avirulent *Pseudomonas syringae* pathogens, they show increased PTI responses and flg22-induced resistance to Pst DC3000. To elucidate the molecular mechanism of CCC1-mediated resistance, we performed global transcriptomic analysis of wild-type plants and ccc1 mutants. Genes involved in the biosynthesis of antimicrobial peptides, defense chemical compounds, and PR proteins are repressed in ccc1 mutants. Altered gene expression for cell wall functions in ccc1 mutants corroborated changes in the
composition of cell wall monosaccharides. Experiments using the NKCC-specific
inhibitor bumetanide corroborate our findings, suggesting that ion channels play an
important role in pathogen-related cell wall fortification and the synthesis of various
antimicrobial compounds.
Results

**CCC1 is critical for resistance to virulent hemi-biotrophic bacteria Pst DC3000**

CCC1 was shown to encode the Arabidopsis homologue of the animal NKCC cotransporter, which is sensitive to the NKCC-specific inhibitor bumetanide (Colmenero-Flores et al., 2007). To investigate whether NKCC activity is required for antibacterial defense in Arabidopsis, 4-week-old wild-type plants were syringe-infiltrated with *Pst* DC3000 concomitantly with the application of ethanol (mock) or bumetanide. Bacterial growth was quantified at 72 hours post inoculation (hpi). As shown in Figure 1A, bumetanide-treated plants showed considerably higher proliferation levels of *Pst* Dc3000 when compared to wild-type plants upon ethanol treatment. The higher bacterial titer in bumetanide-treated plants was associated with more severe chlorosis and necrosis formation (Figure 1B), supporting that NKCC activity is involved in basal plant immunity.

To further confirm the role of CCC1 in antibacterial immunity, we assessed two independent CCC1 T-DNA insertion lines, SALK-048175 (*ccc1-1*) and SALK-145300 (*ccc1-2*), which have T-DNA insertions in the first exon and first intron, respectively (Supplemental Figure S1A). The mutant lines showed a stunted growth phenotype with a smaller rosette diameter compared to wild-type plants (Colmenero-Flores et al., 2007; Henderson et al., 2015) (Supplemental Figure S1B). Results from RT-qPCR confirmed that *ccc1-1* and *ccc1-2* are knockout mutants and have no detectable CCC1 transcripts (Supplemental Figure S1C). We spray-inoculated wild-type and *ccc1* mutant plants with *Pst* DC3000 and counted viable bacteria at 3 and 72 hpi. As shown in Figure 1C, bacterial growth in *ccc1* mutant plants was similar to wild type at 3 hpi, indicating that
stomatal immunity is not affected in ccc1 mutants (Figure 1C). However, at 72 hpi, ccc1
mutant plants showed significantly higher cfu than wild-type plants (Figure 1C). To ascertain that mutations in the CCC1 gene are responsible for the observed susceptibility phenotype to *Pst* DC3000, *ccc1-2* was complemented with CCC1-GFP under the control of the ubiquitin promoter. Two representative complementation lines, CL-5 and CL-7, exhibited restored resistance to *Pst* DC3000 infection (Figure 1C) and rescued the stunted growth phenotype of the *ccc1-2* mutant to the wild-type levels (Supplemental Figure S1B). The CL-5 and CL-7 complementation lines also reverted the pathogen-induced necrosis phenotype observed in *ccc1* mutants to the wild-type level (Figure 1D). Altogether, the pharmacological and genetic evidence suggests that CCC1 positively regulates basal disease resistance.

**ccc1 mutants show reduced levels of ETI against *Pst* DC3000 *avrRpm1***

Avirulent *Pst* DC3000 strains expressing pathogen effectors rapidly induce ETI responses in Arabidopsis plants carrying a functional copy of the corresponding *R* gene, and thus are unable to multiply aggressively or cause disease in these plants (Hammond-Kosack and Jones, 1996). To test whether CCC1 is involved in R protein-induced ETI, we assessed whether resistance to avirulent *Pst* DC3000 carrying the *avrRpm1* gene was affected in *ccc1* mutants. Therefore, wild type, *ccc1* mutants, and complementation lines were spray-inoculated with *Pst* DC3000 *avrRpm1* and bacterial titers were determined at 3 and 72 hpi. While *ccc1* mutant plants were more susceptible to avirulent *Pst* DC3000 *avrRpm1* (Figure 2A), the CL-5 and CL-7 lines exhibited wild-type levels of bacteria. This result indicates that CCC1 is involved in RPM1-mediated ETI.

ETI often culminates in the development of hypersensitive cell death
corresponding with bacterial growth inhibition (Cui et al., 2015). To determine whether
hypersensitive cell death was impaired in ccc1 mutants, we quantified cell death in wild-type and ccc1 mutant plants by ion leakage assays. As shown in Figure 2B, the extent of ion leakage was clearly lower in ccc1-2 mutant leaf discs when compared with those of wild-type plants. Taken together, we conclude that CCC1 acts as a positive regulator of ETI against Pst DC3000 avrRpm1.

**CCC1 modulates PTI responses and disease resistance to Pst DC3000 hrcC<sup>-</sup>**

Since PTI plays an important role in host defense against infections by a broad spectrum of pathogens (Wu et al., 2014; Yu et al., 2017), we tested whether CCC1 is involved in PTI responses. We first measured flg22-triggered ROS production in wild type, ccc1 mutants, and complementation lines. As shown in Figure 3A, in contrast to wild type and complementation lines, leaf discs from ccc1 mutant plants produced more ROS than those from wild-type plants in response to flg22 (Figure 3A). Next, we measured the expression levels of PTI marker genes NHL10 and FRK1 at 1 h after flg22 or WATER (mock) treatment. As shown in Figure 3B, the induction of NHL10 and FRK1 was moderately enhanced in ccc1-2 mutant compared with wild-type plants (Figure 3B). Since SA is an important hormone in resistance against hemi-biotrophic pathogens (Glazebrook, 2005), we quantified free SA accumulation in mock- or flg22-challenged Col-0 and ccc1-2 seedlings. Relative to wild-type plants, ccc1-2 showed an almost two-fold increase in flg22-induced SA amounts 24 h after treatment (Figure 3C).

In summary, these results demonstrate that CCC1 mutation leads to enhanced flg22-induced responses, suggesting that CCC1 is a negative regulator of PTI responses.

To further investigate the role of CCC1 in PTI, 2-week-old Col-0, ccc1-1, ccc1-2, CL-5, and CL-7 seedlings were flood-inoculated with nonpathogenic Pst DC3000 hrcC<sup>-</sup>,...
which lacks the T3SS and only triggers PTI (Yuan and He, 1996). In line with higher PTI
responses in ccc1 mutant plants, less bacterial growth was observed in ccc1 mutant plants at 72 hpi, whereas CL-5 and CL-7 showed wild-type levels of bacterial growth (Figure 3D). We next tested whether the NKCC cotransporter activity of CCC1 is also essential for resistance to Pst DC3000 hrcC−. Wild-type and ccc1-2 seedlings grown on half-strength MS plates with ethanol (mock) or bumetanide were challenged with Pst DC3000 hrcC−, and the bacterial titer was measured at 72 hpi. As shown in Figure 3E, bacterial growth was strongly suppressed in wild-type seedlings treated with bumetanide (Figure 3E). However, bumetanide- or ethanol- (mock) treated ccc1-2 seedings showed similar bacterial cfu (Figure 3E), indicating that bumetanide acts specifically on CCC1, and the NKCC cotransporter activity of CCC1 is involved in resistance to Pst DC3000 hrcC−. Taken together, these data demonstrate a negative regulatory role of CCC1 in PTI.

**CCC1 suppresses flg22-induced resistance to Pst DC3000**

To determine whether CCC1 regulates flg22-induced resistance to Pst DC3000, we carried out flg22-induced protection assays. Wild-type and ccc1-2 mutant plants were pretreated either with flg22 or WATER (mock) for 24 h before inoculation with Pst DC3000 (Zipfel et al., 2004). As shown in Figure 3F, bacterial growth was strongly suppressed in both wild-type and ccc1-2 mutant plants pretreated with flg22. While the ccc1-2 mutant plants allowed more bacterial growth than wild-type plants in the absence of flg22 treatment, ccc1-2 mutant plants were indistinguishable from wild type that received flg22 pretreatment. This result supports the negative regulatory role of CCC1 in flg22-induced resistance to Pst DC3000.

**CCC1 is not required for PM depolarization**
Changes in cellular ion concentration by ion fluxes across the PM are important mediators in inducing plant defense responses (Jabs et al., 1997; Jeworutzki et al., 2010). Since CCC1 is an ion transporter, the altered resistance to Pst DC3000 infection of ccc1 mutant plants prompted us to investigate whether CCC1 is involved in flg22-induced PM depolarization (Jeworutzki et al., 2010). As shown in Figure 4, 10 nM flg22 triggered a strong PM depolarization after a delay of approximately 2 min in both wild-type and ccc1 mesophyll cells. However, no major differences in the magnitude and velocity of the PM depolarization was observed between wild-type and ccc1-1 mutant plants, demonstrating that CCC1 is not required for PM depolarization.

**ccc1 mutants show reduced expression levels of cell wall modification and cuticle biosynthesis genes**

To elucidate the mechanism underlying the role of CCC1 in plant immunity, we performed global transcriptome analysis by RNA-Seq to identify CCC1-regulated genes in 14-day-old Col-0 and ccc1-2 seedlings. Samples from three independent biological repeats were collected for RNA-Seq. Using a 2-fold change threshold (P value < 0.05), 220 genes were shown to be differentially expressed (29 genes upregulated and 191 downregulated) in ccc1-2 in comparison to wild type (Supplemental Table S2). GO enrichment analysis of the 220 genes differentially expressed in the ccc1-2 mutant showed striking enrichment in categories of plant cell wall functions. These genes include 10 cell wall structural proteins (5 extensins, 3 Pro-rich proteins, and 2 arabinogalactans), 7 peroxidases, 2 pectin methylesterases, and 4 xyloglucan endotransglucosylases/hydrolases (Figure 5A). Moreover, we found 4 downregulated genes to be involved in cuticle deposition (Figure 5B), including KCS21 (Costaglioli et
Figure 4. CCC1 is not required for PM depolarization. 10 nM flg22-triggered PM depolarization in wild-type (n = 6) and ccc1-1 mutant (n = 10) mesophyll cells. The data represent the mean ± standard error of indicated number (n) of biological replicates. These experiments were repeated at least two times with similar results.

al., 2005), which is involved in the biosynthesis of long chain fatty acids, CUS2 (Hong et al., 2017), which is crucial for maintenance of cuticular ridges, and CER4 (Rowland et al., 2006) and WSD1 (Li et al., 2008), which are involved in cuticular wax biosynthesis.
Some genes were chosen and their expression patterns assessed by RT-qPCR analysis in wild-type and ccc1-2 mutant plants. The results, shown in Figure 5C, support the reliability of our RNA-Seq data. Overall, these data demonstrate that loss-of-function
of CCC1 causes the repression of genes associated with the biosynthesis and modification of cell walls and cuticles.

**ccc1 mutants exhibit altered cell wall composition**
Considering the important role of the cell wall in defense responses and the over-representation of cell wall function-related genes among the downregulated genes in the ccc1-2 mutant (Figure 5A, Supplemental Tables S2), we decided to analyze the cell wall composition of ccc1 mutant. As shown in Figure 5D, we determined cellulose content and the levels of non-cellulosic neutral monosaccharides and GalA of extractable homogalacturonan of leaves from non-treated wild type, the ccc1-1 mutant, and the CL-5 complementation line. The results show that the relative cellulose content was much lower in the ccc1-1 mutant than in wild-type or complemented plants. All neutral sugars except glucose were higher in the cellulosic fraction in ccc1-1 compared with wild-type and complemented cell walls, probably due to the significant loss of cellulose in ccc1-1 cell walls (Figure 5D). Taken together, these results demonstrate a function of CCC1 in regulating cell wall composition.

**Defense-related genes and antimicrobial molecule biosynthesis genes are constitutively suppressed in ccc1-2 mutant plants**

Our RNA-Seq data identified several constitutively downregulated genes in ccc1-2 mutants, which had previously been implicated in plant defense (Table 1). Among these, the three PR genes PR1, PR2, and CHI-B are rapidly induced and their products secreted to the plant apoplast as part of the host immunity to combat extracellular pathogens such as *P. syringae* (van Loon et al., 2006; Kalde et al., 2007). Besides PR genes, PBS3, WRKY70, FMO1, and RLP23 function in plant defense against *P. syringae* strains (Nobuta et al., 2007; Albert et al., 2015; Berndorff et al., 2016; Jiang et al., 2016). Expression levels of several defense-related and PR genes were validated by RT-qPCR (Figure 6A and B). The reduced expression of these crucial defense genes
20 corroborates the differential susceptibility of ccc1 mutant plants to bacterial pathogens.

294 Figure 6. Defense-related genes are constitutively suppressed in ccc1-2 mutant.
(A) and (B) RT-qPCR validation of CCC1-regulated PR genes and other defense genes. Gene expression was normalized to UBQ10. The data are the means ± SE, n = 3. Statistical differences were detected based on a two-tailed Student’s t-test. *, P value < 0.05, **, P value < 0.01, ***, P value < 0.001 when compared with the wild type.
The RNA-Seq data also showed repression of essential genes for the biosynthesis of antimicrobial compounds including camalexin, 4-OH-ICN, marneral, and thalianol in ccc1 mutant plants (Figure 7A and 7B). RT-qPCR results confirmed that these genes were downregulated in ccc1-2 mutant plants (Figure 7C). In addition, a large number of genes involved in the production of several main families of antimicrobial peptides (AMPs) were constitutively downregulated in ccc1-2 mutant compared to wild-type plants. These AMPs include 3 thionins, 7 defensins, 1 hevein, 1 knottin, and 22 lipid transfer proteins (Table 2). Taken together, mutation of CCC1 affects a large number of biosynthetic pathways for the production of antimicrobial peptides and compounds, highlighting an important role of CCC1 as a positive regulator of defense signaling.
Discussion

Ion fluxes are one of the earliest physiological responses of plant cells to PAMP recognition and play an important role in defense response induction. However, only a scant number of channels/transporters have been analyzed for their contribution to plant
immunity. Using pharmacological, genetic, and biochemical analyses, we show here that the Golgi- and TGN-localized cation-chloride cotransporter CCC1 positively regulates basal resistance and ETI towards *Pst* DC3000 in Arabidopsis. However, CCC1 deficiency increases PTI responses and flg22-induced resistance to *Pst* DC3000. These results demonstrate a complex dual regulatory role of intracellular ion transporters in plant immunity.

Plant cuticles and cell walls provide a physical barrier to protect plants from desiccation (Cantu et al., 2008; Serrano et al., 2014). The increased availability of water supports apoplastic pathogen growth and pathogenicity (Schreiber et al., 2005; Xin et al., 2016) and some cuticle-defective mutants of Arabidopsis increase leaf cuticle permeability and susceptibility to *P. syringae* infection (Xiao et al., 2004; Tang et al., 2007). Our comparative transcriptomic analysis of *ccc1* and wild-type plants revealed that genes involved in cuticle biosynthesis, and cell wall remodeling and modification were suppressed in *ccc1* mutant plants (Figure 5A-C), indicating that CCC1 may regulate cuticle and cell wall biosynthesis and/or modification. Interestingly, rates of water diffusion across the cuticle and cell walls of *ccc1* mutants were significantly higher than those of wild-type plants, as indicated by faster water loss of detached leaves and a severe water soaking phenotype under high humidity, although further experiments are necessary to determine the epidermal cell wall and/or cuticle thickness in *ccc1* mutant plants.

Quantification of the cell wall composition from wild-type plants and *ccc1* mutants showed that the composition of monosaccharides was significantly changed in *ccc1* mutants (Figure 5D). Interestingly, CCC1 has been shown to be an endomembrane
protein, which is localized to the Golgi and TGN (Henderson et al., 2015). The possibility that CCC1 regulates ion levels in the Golgi and consequently cell wall biosynthesis in the Golgi apparatus (Sandhu et al., 2009) needs to be tested in future studies.

Our RNA-Seq data showed the downregulation of essential genes involved in the biosynthesis of the phytoalexins camalexin and 4-OH-ICN in ccc1 mutant plants (Figure 7A). Camalexin, one of the main phytoalexins accumulating in Arabidopsis, not only shows antifungal activity but also functions in the antibacterial defense response (Tsuji et al., 1992; Rogers et al., 1996; Zhang et al., 2014; Rajniak et al., 2015). 4-OH-ICN, which is a recently recognized cyanogenic metabolite in Arabidopsis, is required for resistance against Pst DC3000 (Rajniak et al., 2015). T-DNA insertion mutants of four biosynthesis genes of camalexin and 4-OH-ICN, which are CYP71A12, CYP82C2, CYP71A13 and PAD3, indicate that these compounds contribute non-redundantly to plant resistance against Pst DC3000 (Rajniak et al., 2015). However, production of tryptophan-derived glucosinolates does not seem to be affected by mutation of CCC1 (Figure 7A). Upon attack by pathogens, land plants also produce a heterogeneous group of phytoantipicins, which show inhibitory effects to a variety of invading microbes (González-Lamothe et al., 2009). Two gene clusters, which are required for the synthesis of the phytoantipicins marneral and thalianol, were also suppressed in ccc1 mutants (Figure 7B). Marneral and thalianol have been proposed to function in plant defense in Arabidopsis by analogy with well characterized phytoantipicins from cereals (Supplemental Figure S2), although genetic evidence for their involvement in plant immunity is still lacking (Field and Osbourn, 2008; Chu et al., 2011; Field et al., 2011).
AMPs are secreted peptides that kill a variety of invading pathogens by disrupting the membranes or inactivating their ribosomes (García-Olmedo et al., 1998; Chen et al., 2002; Shai, 2002; Marmiroli and Maestri, 2014; Goyal and Mattoo, 2016). The RNA-Seq data showed that a large number of genes involved in the production of AMPs are constitutively downregulated in ccc1 mutant compared to wild-type plants. Transgenic plants which constitutively express some AMPs such as thionin (Thi2.1) and lipid transfer proteins have been shown to have enhanced resistance to *P. syringae* in several plant species (Molina and Garcia-Olmedo, 1997; Chan et al., 2005; Sarowar et al., 2009). Defensins are best known for their antifungal activities, but purified defensins from spinach also showed *in vitro* antibacterial activity (Segura et al., 1998).

Taken together, CCC1 affects both the biosynthesis and modification of plant cell walls and cuticles as well as many antimicrobial compounds, all of which might explain the compromised pathogen resistance phenotype of ccc1 mutants. A current denominator for these diverse compounds is their necessary transit through the Golgi and TGN to be secreted into the extracellular space. Most immune signaling processes related to ion fluxes have focused on the PM, where PAMP-PRR interaction takes place to trigger intracellular defense signaling pathways (Roelfsema et al., 2012). At present, it is not clear whether ion fluxes across the Golgi and TGN membranes are regulated in response to pathogen signals. However, the Golgi-TGN network clearly serves as a hub for protein trafficking and secretion of a number of substances, including cell wall and antimicrobial components (LaMontagne and Heese, 2017; Uemura et al., 2019) and further studies are necessary to clarify whether CCC1 is involved in the sorting of immune-related products in the Golgi-TGN network.
CONCLUSIONS

Plant cuticles, cell walls, and secreted antimicrobial compounds constitute the first line of plant defense against harmful pathogens. We reported here that CCC1 mutation caused the suppression of cuticle and cell wall related genes and genes for the biosynthesis of antimicrobial peptides and defense chemicals. Chemical analyses of cell walls from wild-type and ccc1 mutant plants showed that cell wall composition was strongly changed in ccc1 mutants compared with that in wild-type plants. Consistently, ccc1 mutant plants were more susceptible to the virulent bacterial pathogen *P. syringae* (Pst) DC3000. In addition, using a pharmacological approach, the NKCC cotransporter activity of CCC1 was demonstrated to be essential for the functionality of CCC1 in the regulation of PTI and resistance to *Pst* DC3000. These results revealed the function of the Golgi-localized ion transporter CCC1 in the reinforcement of plant structural and chemical barriers in plant immunity.

Materials and Methods

Plant materials and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Col-0 was used as wild type in this study. *ccc1-1* (SALK_048175) and *ccc1-2* (SALK_145300) T-DNA insertion lines were obtained from Nottingham Arabidopsis Stock Centre (NASC). Homozygous T-DNA insertion lines were screened by PCR-based genotyping using primers listed in Supplemental Table S1. Transgenic plants expressing pUBQ10:CCC1-GFP in the background of *ccc1-2* mutant were generated in this study. Arabidopsis plants were
grown individually in Jiffy-7 pellet in a controlled cultivation chamber with a 12-h-day
(110 μmol m⁻² s⁻¹) / 12-h-night cycle and a relative humidity of 60%. Day and night
temperatures were set to 22 °C and 20 °C, respectively. 4-week-old plants were used
for bacterial disease and ROS burst assays. For microelectrode studies, plants were
grown for 5 to 6 weeks in a growth chamber, with 60% relative humidity, a day/night
cycle of 12/12 h, temperatures of 21/18 °C, and a photon flux density of 100 μmol m⁻² s⁻¹. For MAPK, RT-qPCR assay, RNA-Seq, SA quantification, surface-sterilized seeds
were vernalized at 4 °C in the dark for 3 days and then germinated on half-strength
Murashige and Skoog (MS, Sigma M6899) plates, containing 0.5% (w/v) sucrose
(Sigma S5016), 1% (w/v) agar (Sigma A1296), and 0.05% (w/v) MES (Sigma M8250),
grown at 23 °C, 60% humidity, and 125 μmol m⁻² s⁻¹ light with a 16-h-light / 8-h-dark
photoperiod. The plants were transferred to a new 60 mm x 60 mm petri dish with 10
mL half-strength MS medium overnight before treatment. Then the plants were treated
with flg22 for the indicated amounts of time. For MAPK assays, 10-day-old seedlings
were used. For RT-qPCR assay, RNA-Seq, SA quantification, 14-day-old seedlings
were used.

Plasmid construction

CCC1 CDS was amplified from Col-0 cDNA using Phusion® High-Fidelity DNA
Polymerase (NEB), the entry clone CCC1-pDONR207 was generated using Gateway
BP Clonase II Enzyme mix (Invitrogen). pUBC-GFP-DEST (Grefen et al., 2010) was
used to generate complementation construct pUBQ10:CCC1-GFP using Gateway LR
Clonase II Enzyme Mix (Invitrogen). All the constructs were verified by Sanger
sequencing and primers are listed in Supplemental Table S1.
**Bacterial disease assays**

Spray-inoculation, syringe-infiltrated inoculation and flooding-inoculation were used in this study (Jiang et al., 2019). Briefly, different *Pst* DC3000 strains were cultivated at 28 °C for 24 hours on King Agar B (Sigma 60786) plates containing appropriate antibiotics. Bacteria were suspended and adjusted to different final densities with 10 mM MgSO$_4$. For spray-inoculation, 4-week-old plants were sprayed with bacterial suspensions of OD$_{600} = 0.2$ (approximately $1 \times 10^8$ cfu/mL), with 0.04% (w/v) Silwet L-77 (LEHLE SEEDS) added, and then covered with a clear plastic dome immediately for disease to develop. For syringe-infiltration, bacteria were further diluted to cell densities of $1 \times 10^5$ cfu/mL (OD$_{600} = 0.0002$). Leaves of 4-week-old plants were hand-infiltrated with the bacterial suspension using a needleless syringe. Infiltrated plants were first kept under low humidity for 2 h to help water to evaporate, and then covered with the plastic dome. For flg22 protection assay, 4-week-old plants were spray-inoculated with 2 mM flg22, or WATER as a control, 24 h before being sprayed with *Pst* DC3000 ($1 \times 10^8$ cfu/mL, OD = 0.2). In planta bacterial growth was determined at 3 and 72 hpi. Three leaves were detached from each plant, sterilized in 75% (v/v) ethanol for 10 s, and rinsed in sterile distilled water twice. One leaf disc was taken using a cork borer (4/16 inch in diameter) from each leaf and pooled together, and ground in 10 mM MgSO$_4$ using a TissueLyser (Qiagen) with three grinding beads in each tube at a frequency of 27 times/s for 3 min. Serial dilutions were plated on LB agar plates with appropriate antibiotics, which were kept at 28 °C for 24 h before colonies were counted. For flood inoculation, 14-day-old seedlings on half-strength MS agar plates were flood-inoculated according to the protocol published by Ishiga and colleagues with $1 \times 10^7$ cfu/mL of *Pst*.
DC3000 hrcC⁻ (Ishiga et al., 2011).

**Ion leakage assay**

Ion leakage from leaf discs of wild type and ccc1-2 mutants was monitored as described previously (Hatsugai et al., 2016). *Pst* DC3000 *avrRpm1* were cultivated at 28 °C for 24 h on King Agar B (Sigma 60786) plates containing 50 mg/L rifampicin and 30 mg/L kanamycin before plant infection. Bacteria was resuspended at 5×10⁷ cfu/mL in WATER and infiltrated into leaves of 4-week-old wild-type and ccc1-2 plants. 15 leaf discs were collected from leaves immediately after bacterial infiltration and washed in 10 mL of WATER for 30 min. Then the leaf discs were transferred into 10 mL fresh WATER and incubated in a shaker at 22°C. The conductivity of WATER was measured at the indicated time points using an VWR conductivity meter (PC 5000L).

**Reactive oxygen species (ROS) burst assay**

ROS burst was measured using a luminol-based assay. Leaf discs (4/16 inch in diameter) were incubated overnight in a white 96-well plate (Costar, Fisher Scientific) containing sterile WATER to reduce wounding response. The next day, WATER was carefully removed and leaf discs were floated on 100 μL of Elicitation Solution (34 ug/mL luminol, 20 μg/mL horseradish peroxidase, and 1 μM flg22). Luminescence was detected with a TECAN Infinite 200 PRO microplate reader using integration intervals of 1-1.5 s. Each treatment had a minimum of eight samples. For bumetanide treatment, wild-type leaf discs were floated on 100 μL of the Elicitation Solution with application of ethanol or 100 nM bumetanide. The leaf discs were vacuumed for 5 min and luminescence was detected with a TECAN Infinite 200 PRO microplate reader as mentioned above.
Quantification of SA

SA quantification was done according to a method described previously (Forcat et al., 2008). 14-day-old Arabidopsis Col-0 and ccc1-2 seedlings were lyophilized and ground into powder. About 5 mg dry weight of powdered tissues were extracted with 400 μL of 10% (v/v) methanol containing 1% (v/v) acetic acid twice. 11.1 ng of \(^{2}\text{H}_{4}\)-SA (OlchemIm Ltd., Olomouc) was added as an internal standard. The supernatants were filtered through 0.22 μm PTFE filters before LC-MS/MS analysis. Analysis of SA was performed by using an Agilent 1200 HPLC (Agilent Technologies) coupled to a Q-TRAP 5500 MS (AB SCIEX) with an electrospray source. Chromatographic separation was carried out on a Phenomenex (Torrance) Gemini C18 (150×2.0 mm, 5 μm) column with mobile phases of water/acetonitrile (95/5, v/v; A) and acetonitrile/water (5/95, v/v; B) at 35 °C. The gradient used was 0-20 min, 0%-100% B; 20-25 min, 100%B; 25-26 min, 100%-0% B; 26-36 min, 0% B. MS was operated in negative ionization mode. The MS conditions were as follows: temperature, 500 °C; ion source gas 1, 50 psi; ion source gas 2, 60 psi; ion spray voltage, -4500 V; curtain gas, 40 psi; collision gas, medium; DP, -25 V; EP, -9; CXP, -2; and CE, -38. Multiple Reaction Monitoring (MRM) of ion pairs for labeled and endogenous SA using following mass transitions: \([^{2}\text{H}_{4}]\text{SA}\ 141>97, \text{SA}\ 137>93\). Data were acquired and analyzed using Analyst 1.4 software (Applied Biosystems).

Plasma membrane potential measurements

The central vein of Arabidopsis leaves was removed with a razor blade and the adaxial epidermis was peeled, while the remainder of the leaf was cut into a 1 cm\(^2\) square that was attached to the bottom of a Petri dish with double sided adhesive tape. The petri dish was filled with a solution containing 0.1 mM KCl, 1 mM CaCl\(_2\), and 1 mM Mes/BTP.
pH 6.0. Mesophyll cells were impaled with single barreled microelectrodes, pulled from glass capillaries (outer diameter 1 mm, inner diameter 0.58 mm, Hilgenberg GmbH, https://www.hilgenberg-gmbh.de), using a laser puller (P2000, Sutter Instruments, https://www.sutter.com). The electrodes were filled with 300 mM KCl and had a tip resistance of approximately 60 MΩ and a capillary filled with 300 mM KCl and plugged with 2% (w/v) agarose in 300 mM KCl, which served as a reference electrode. The electrode and reference were coupled to Ag/AgCl half cells and a Bio-Logic micro electrode amplifier (CA 100, https://www.bio-logic.net/), equipped with a HS-180 headstage. The microelectrode was impaled into mesophyll cells with use of a MM3A micromanipulator (Kleindiek, https://www.kleindiek.com). The voltage data were filtered with a Bessel filter (LPF 202A; Warner Instruments Corp., www.warneronline.com) and sampled at 0.1 Hz with WinEDR software (Dempster, 1997) (University of Strathclyde, https://www.strath.ac.uk), using an ITC-18 Interface (Instrutech Corp., USA).

**RNA extraction and RT-qPCR analysis**

2-week-old seedlings were treated with 1 μM flg22 for 1 h. Total RNA was isolated from the seedling samples using NucleoSpin RNA Plant kit (Macherey Nagel) following the manufacturer’s instructions. 2 μg total RNA were then used for first-strand cDNA synthesis using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) with oligo (dT) primer. RT-qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems) following standard protocol. The expression levels of interest genes were normalized to At4g05320 (UBQ10). Primers for RT-qPCR were listed in Supplemental Table S1.

**RNA-Seq and transcriptome analysis**
2-week-old Col-0 and ccc1-2 seedlings were collected for RNA-Seq. 100 ng total RNA were used for RNA-Seq library preparation, which was performed in KAUST Bioscience Core Lab using TruSeq Stranded mRNA LT Sample Prep Kit following manufacturer’s instructions (Illumina). Sequencing was performed on an Illumina Hi-Seq 4000 platform with 150-nucleotide paired-end reads. Prior to the analysis of RNA-Seq data, the sequenced reads were quality-checked using FastQC v0.11.3 (Andrews, 2018). Because of the good quality of the sequencing data, we skipped the trimming process to avoid any potential biases (Williams et al., 2016). To quantify the expression level of genes, a total of 392 million reads were pseudo-aligned to the publicly available TAIR10 A. thaliana transcriptome (release 34) using kallisto v0.43.0 (Bray et al., 2016). The estimated read counts and calculated transcripts per million (TPM) were subsequently passed to sleuth v0.28.1 (Pimentel et al., 2017) for differential expression analysis. Significantly differentially expressed genes were identified based on a cutoff of fold-change > 2 and q-value < 0.05. Gene ontology enrichment analysis was conducted using clusterProfiler (Yu et al., 2012) and Pathview (Luo and Brouwer, 2013).

Cell wall analysis

Analyses were performed on 4-day-old dark-grown hypocotyls using an alcohol-insoluble residue (AIR) prepared as follows. Freshly collected samples were submerged into 96% (v/v) ethanol, grinded, and incubated for 30 min at 70°C. The pellet was then washed twice with 96% (v/v) ethanol and once with acetone. The remaining pellet of alcohol insoluble residues (AIR) was dried in a fume hood overnight at room temperature. The monosaccharide composition of the non-cellulosic fraction was determined by hydrolysis of AIR with 2 M TFA for 1 h at 120 °C. After cooling and
centrifugation, the supernatant was dried under a vacuum, resuspended in 200 μL of water, and retained for analysis. To obtain the glucose content of the crystalline cellulose fraction, the TFA-insoluble pellet was further hydrolyzed with 72% (v/v) sulfuric acid for 1 h at room temperature. The sulfuric acid was then diluted to 1 M with water and the samples incubated at 100 °C for 3 h. All samples were filtered using a 20-μm filter caps, and quantified by HPAEC-PAD on a Dionex ICS-5000 instrument (ThermoFisher Scientific) as described (Fang et al., 2016).

Accession numbers

Sequencing data of RNA-seq were deposited in the National Center for Biotechnology Information Sequence Read Archive under BioProject code PRJNA589953.

Supplemental Data

Supplemental Figure S1. Characterization of CCC1 T-DNA knockout lines and complementation transgenic lines.

Supplemental Figure S2. Triterpene biosynthetic pathways in Arabidopsis and Oat.

Supplemental Table S1. Primers used in this study.

Supplemental Table S2. Differential expressed genes in WT and ccc1-2.

Acknowledgements

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**Tables**

Table 1. Defense-related genes were constitutively downregulated in the ccc1-2 mutants.

<table>
<thead>
<tr>
<th>Gene Description</th>
<th>Locus</th>
<th>Fold change (WT/ccc1-2)</th>
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<td>PR1</td>
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<td>PR3 (CHI-B)</td>
<td>AT3G12500</td>
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<td>(Legrand et al., 1987)</td>
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<td>PBS3</td>
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<td>FMO1</td>
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<td>AT1G04370</td>
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<td>AtRLP23</td>
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Table 2. Genes involved in antimicrobial peptides production are repressed in ccc1-2 mutant plants compared to wild-type plants.

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**Figure legends**

**Figure 1. CCC1 is critical for resistance to virulent hemi-biotrophic bacteria *Pst* DC3000**

(A) Growth of *Pst* DC3000 in wild-type plants treated with ethanol (mock) orbumetanide (100 nM) at 72 hours post-infection (hpi). 4-week-old wild-type plants were inoculated by syringe-infiltration with *Pst* DC3000 suspension in 10 mM MgSO\(_4\) at a density of 1×10\(^5\) cfu/mL concomitantly with application of ethanol or bumetanide. Bacterial growth was quantified at 72 hpi. The data represent the mean ± standard error, \(n = 8\), P value < 0.01. (B) Disease symptoms of wild type treated with ethanol or bumetanide at 72 hpi. (C) Bacterial growth of *Pst* DC3000 in Col-0, *ccc1-1*, *ccc1-2*, CL-5, and CL-7 at 3 and 72 hpi. 4-week-old wild-type, *ccc1-1*, *ccc1-2*, CL-5, and CL-7 plants were spray-inoculated with bacterial suspensions in 10 mM MgSO\(_4\) at a density of 1×10\(^8\) cfu/mL. Bacterial growth was quantified at 3 and 72 hpi. The data represent the mean ± standard error of eight biological replicates. Different letters denote statistically
significant differences (p < 0.05, two-tailed t test) compared with wild-type plants at 3 hpi. (D) Disease symptoms of the genotype mentioned in (C) at 96 hpi. All experiments were repeated at least two times with similar results.

Figure 2. ccc1 mutants showed reduced levels of ETI against Pst DC3000

avrRpm1

(A) Bacterial growth of avirulent Pst DC3000 carrying the avrRpm1 gene in Col-0, ccc1-1, ccc1-2, CL-5, and CL-7 at 3 hpi and 72 hpi. 4-week-old Col-0, ccc1-1, ccc1-2, CL-5, and CL-7 plants were spray-inoculated with bacterial suspensions in 10 mM MgSO4 at a density of 1×10⁸ cfu/mL. Bacterial growth was quantified at 3 and 72 hpi. The data represent the mean ± standard error of eight biological replicates. Different letters denote statistically significant differences (p < 0.05, two-tailed t test) compared with wild-type plants at 3 hpi. (B) Ion leakage from leaf discs of wild-type and ccc1-2 plants upon water or Pst DC3000 avrRpm1 (OD₆₀₀ = 0.1) treatment. Water and Pst DC3000 avrRpm1 were infiltrated into leaves of wild type and ccc1-2, and ion leakage from leaf discs of wild type and ccc1-2 was measured at the indicated time points. Error bars indicate standard errors of two biological replicates. All experiments were repeated at least two times with similar results.

Figure 3. CCC1 suppresses PTI responses, disease resistance to Pst DC3000

hrcC⁻, and flg22-induced resistance to Pst DC3000

(A) Flg22-induced early reactive oxygen species (ROS) bursts in Col-0, ccc1-1, ccc1-2, CL-5, and CL-7 were measured using a luminol-based assay (relative light units, RLU). Total ROS production is shown and refers to Col-0 treated with flg22 as 100%. Leaf discs from 4-week-old plants were treated with 1 μM flg22 for 40 min. n = 9. (B)
Induction of two PTI marker genes *NHL10* and *FRK1* after 1 μM flg22 treatment for 1 h in wild-type and *ccc1-2* plants. *n* = 3. (C) Free salicylic acid (SA) accumulation was measured 24 h after treatment with WATER or 1 μM flg22 in 2-week-old Col-0 and *ccc1-2* seedlings. *n* = 3. (D) Bacterial growth of nonpathogenic strain *Pst* DC3000 *hrcC* in Col-0, *ccc1-1, ccc1-2, CL-5, and CL-7*. 2-week-old Col-0, *ccc1-1, ccc1-2, CL-5, and CL-7* plants were flooded with bacterial suspensions in 10 mM MgSO₄ at a density of 1×10⁷ cfu/mL, and bacterial growth was quantified at 3 and 72 hpi. *n* = 8. (E) Growth of *Pst* DC3000 *hrcC* in wild-type and *ccc1-2* seedlings with ethanol (mock) or bumetanide (100 nM) treatment at 72 hpi. 2-week-old wild-type and *ccc1-2* seedlings grown on half-strength MS plate with ethanol or 100 nM bumetanide were flooded with *Pst* DC3000 suspension in 10 mM MgSO₄ at a density of 1×10⁷ cfu/mL. Bacterial growth was quantified at 72 hpi. *n* = 8. (F) CCC1 suppresses flg22-induced resistance to *Pst* DC3000. Col-0 and *ccc1-2* were pretreated with WATER or flg22 for 24 h, and then sprayed with *Pst* DC3000. Bacterial growth was determined at 72 hpi. The data represent the mean ± standard error of indicated number (*n*) of biological replicates. Different letters indicate significant differences (*p* < 0.05, two-tailed t test) compared with wild-type plants or mock treatment. All experiments were repeated at least two times with similar results.

**Figure 4. CCC1 is not required for PM depolarization**

10 nM flg22-triggered PM depolarization in wild-type (*n* = 6) and *ccc1-1* mutant (*n* = 10) mesophyll cells. The data represent the mean ± standard error of indicated number (*n*) of biological replicates. These experiments were repeated at least two times with similar results.
Figure 5. *ccc1* mutants show lower expression level of cell wall modification and cuticle biosynthesis genes and altered cell wall composition.

(A) and (B) Heat maps of cell wall-related genes (A) and cuticle biosynthesis genes (B) in Col-0 and *ccc1*-2 mutant. The original transcript per million (TPM) values were subjected to generate the heat map. Red color indicates higher and blue color is for lower expression. CWPs, cell wall structural proteins; PERs, peroxidases; PMEs, pectin methyl esterases; XTHs, xyloglucan endo-transglucosylase/hydrolases. (C) RT-qPCR validation of CCC1-regulated cuticle and cell wall related genes. Gene expression was normalized to *UBQ10*. (D) Monosaccharides composition of the cell wall in AIR extracted from wild-type plants, *ccc1*-1 mutant, and complementation line CL-5. Bars in (C) and (D) represent means ± SE of three biological replicates. Statistical differences in (C) and (D) were detected based on a two-tailed Student’s t-test. *, P value < 0.05, **, P value < 0.01, ***, P value < 0.001 when compared with the wild type.

Figure 6. Defense-related genes are constitutively suppressed in *ccc1*-2 mutant.

(A) and (B) RT-qPCR validation of CCC1-regulated *PR* genes and other defense genes. Gene expression was normalized to *UBQ10*. The data are the means ± SE, *n* = 3. Statistical differences were detected based on a two-tailed Student’s t-test. *, P value < 0.05, **, P value < 0.01, ***, P value < 0.001 when compared with the wild type.

Figure 7. Antimicrobial chemical compounds biosynthesis genes are constitutively downregulated in *ccc1*-2 mutant.

(A) Biosynthetic pathways of tryptophan-derived antimicrobials camalexin, 4-OH-ICN, and indole glucosinolates in Arabidopsis (Rajniak et al., 2015; Xu et al., 2016). Key genes which are downregulated in *ccc1*-2 mutant plants are in bold and cyan color. (B)
Heat maps of marneral and thalianol gene cluster in Col-0 and ccc1-2 mutant. The original TPM values were used to generate the heat map. Red color indicates higher and blue color is for lower expression. (C) RT-qPCR validation of CCC1-regulated chemical compound synthesis genes. Gene expression was normalized to *UBQ10*. The data are the means ± SE, n = 3. Statistical differences were detected based on a two-tailed Student’s t-test. *, P value < 0.05, and ***, P value < 0.001 when compared with the wild type.
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