Mechanisms underlying plant non-host resistance to *Xanthomonas oryzae* pv. *oryzicola* (Xoc), the pathogen causing rice leaf streak disease, are largely unknown. Cyclic nucleotide-gated ion channels (CNGCs) are calcium-permeable channels that are involved in various biological processes including plant resistance. In this study, functions of two tomato CNGC genes *SlCNGC1* and *SlCNGC14* in non-host resistance to Xoc were analyzed. Silencing of *SlCNGC1* and *SlCNGC14* in tomato significantly enhanced Xoc-induced hypersensitive response (HR) and non-host resistance, demonstrating that both *SlCNGC1* and *SlCNGC14* negatively regulate non-host resistance related HR and non-host resistance to Xoc in tomato. Silencing of *SlCNGC1* and *SlCNGC14* strikingly increased Xoc-induced callose deposition and strongly promoted both Xoc-induced and flg22-elicited 
\[ \mathrm{H}_2\mathrm{O}_2 \], indicating that these two SlCNGCs repress callose deposition and ROS accumulation to attenuate non-host resistance and PAMP-triggered immunity (PTI). Importantly, silencing of *SlCNGC1* and *SlCNGC14* apparently compromised cytosolic Ca\(^{2+}\) accumulation, implying that *SlCNGC1* and *SlCNGC14* function as Ca\(^{2+}\) channels and negatively regulate non-host resistance and PTI-related responses through modulating cytosolic Ca\(^{2+}\) accumulation. *SlCNGC14* seemed to play a stronger regulatory role in the non-host resistance and PTI compared to *SlCNGC1*. Our results reveal the contribution of CNGCs and probably also Ca\(^{2+}\) signaling pathway to non-host resistance and PTI.

**Keywords:** cyclic nucleotide-gated ion channel (CNGCs), *Xanthomonas oryzae* pv. *oryzicola*, PAMP-triggered immunity, non-host resistance, Ca\(^{2+}\) signaling

**INTRODUCTION**

Each pathogen has its own host range. Non-host resistance is triggered when a non-adapted pathogen attempts to infect a plant species outside of its host range. Thus, non-host resistance is widely occurring, durable and broad-spectrum to non-adapted pathogens and is highly potential to be exploited in crop resistance engineering (Schulze-Lefert and Panstruga, 2011; Senthil-Kumar and Mysore, 2013). It has been clear that plant non-host resistance utilizes both preformed and induced defense mechanisms and frequently elicits hypersensitive response (HR).
(Mysore and Ryu, 2004; Senthil-Kumar and Mysore, 2013). The preformed mechanisms generally consist of plant physical and chemical barriers, including antibiotic compounds (Bednarek and Osbourn, 2009; Fan et al., 2011). The induced non-host resistance is elicited after the preformed defense is overcome. As observed for host resistance, pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) are often initiated in this layer of defense (Niks and Marcel, 2009; Senthil-Kumar and Mysore, 2013). Efforts to identify non-host resistance-related genes resulted in the finding that some genes, such as PLDδ, GOX, SGT1, and NHO1, contribute to both host and non-host resistance in Arabidopsis (Lu et al., 2001; Maimbo et al., 2010; Rojas et al., 2012; Pinosa et al., 2013). Generally speaking, the mechanisms underlying plant non-host resistance are far from well understood.

Xanthomonas oryzae pv. oryzae (Xoo) and X. oryzae pv. oryzaicola (Xoc) are two important pathogens of X. oryzae, causing bacterial blight and leaf streak diseases, respectively, in rice (Oryza sativa), which is a staple food in many countries and a model plant for cereal biology (Nino-Liu et al., 2006). It is notable that these two pathogens utilize distinct mechanisms to infect rice leaves. Xoo is a vascular pathogen that enters rice leaves via the hydathodes, while Xoc penetrates rice leaves mainly through stomata or wound sites and colonizes intercellular spaces in the mesophyll (Nino-Liu et al., 2006). During infection of their plant hosts, many strains secrete transcription activator-like (TAL) effectors, which enter the host cell nucleus and activate specific corresponding host genes at effector binding elements (EBEs) in the promoter (Boch et al., 2009; Moscou and Bogdanove, 2009). It has been established that host resistance to Xoo is mostly related to the action of TAL effectors, either by polymorphisms that prevent the induction of susceptibility (S) genes or by executor resistance (R) genes with EBEs embedded in their promoter, and that induce cell death and resistance (Bogdanove et al., 2010; Zhang et al., 2015). Xoc is known to suppress host resistance, and no host R gene has been identified against it (Cai et al., 2017). Compared with the host resistance (Zhang and Wang, 2013), non-host resistance to Xoo and Xoc is much less studied. We have previously identified seven genes required for non-host resistance to Xoo in Nicotiana benthamiana. Among them are a calreticulin and a peroxidase, indicating that oxidative burst and calcium-dependent signaling pathways play an important role in non-host resistance to Xoo (Li et al., 2012). However, molecular mechanisms underlying non-host resistance to Xoc remain largely unknown. Whether the oxidative burst and calcium signaling pathway contribute to the non-host resistance to Xoc as to Xoo awaits examination.

The cyclic nucleotide-gated ion channels (CNGCs) are suggested to be one of the important pathways for conducting Ca^{2+} ions in signaling transduction (Talke et al., 2003). They are ligand-gated Ca^{2+}-permeable divalent cation-selective channels that are often localized in plasma membrane, presumptively are activated by direct binding of cyclic nucleotides and are complexly regulated by binding of calmodulin (CaM) to the CaM Binding (CaMB) domain (Chin et al., 2009; Ma et al., 2009; Wang et al., 2013; Gao et al., 2014; DeFalco et al., 2016a,b; Fischer et al., 2017). The plant CNGCs are involved in numerous biological functions varying from plant development and stress tolerance to disease resistance (Kaplan et al., 2007; Qi et al., 2010; Ma and Berkowitz, 2011; Moeder et al., 2011). CNGCs are well conserved among plant species, comprising 20 members in Arabidopsis and 18 members in tomato (Saand et al., 2015a,b). Earlier studies revealed that AtCNGC2, AtCNGC4, AtCNGC11, and AtCNGC12 and their homologs play an important role in disease resistance against various pathogens (Yu et al., 1998; Clough et al., 2000; Balagú et al., 2003; Jurkowski et al., 2004; Yoshikawa et al., 2006; Ali et al., 2007; Ma and Berkowitz, 2011; Chin et al., 2013; Fortuna et al., 2015; Saand et al., 2015a). Later, other CNGC members such as SlCNGC1 and SlCNGC14 were also found to contribute to disease resistance (Saand et al., 2015a,b). We found that SlCNGC1 and SlCNGC14 play a negative role in non-host resistance to Xoo in tomato (Saand et al., 2015b). However, whether these SlCNGCs indeed encode functional CNGC channels and whether and how they function in non-host resistance to Xoc in tomato remains further study.

Our data in this study strongly indicate that SlCNGC1 and SlCNGC14 function as Ca^{2+} channels and negatively regulate tomato non-host resistance to Xoc through modulating ROS accumulation and callose deposition. Our results provide evidence for the contribution of CNGC-mediated Ca^{2+} signaling pathway to non-host resistance.

MATERIALS AND METHODS

Plant Growth and Inoculation
Tomato (cv. Money Maker) plants were grown in growth room at 21°C with 16 h light/8 h dark photoperiod. For disease resistance evaluation analyses, tomato plants were inoculated with X. oryzae pv. oryzaicola (Xoc). After single colony propagation culture in NA medium, the Xoc bacterial cells were collected and resuspended in ddH_{2}O to 1 × 10^{8} cfu mL^{-1}. The bacterial suspension was then infiltrated into leaves with sterilized needleless syringe. The infiltration zones were marked immediately after infiltration. The inoculated plants were grown in growth room at 26°C with 16 h light/8 h dark photoperiod.

Gene Silencing Analyses
The virus-induced gene silencing (VIGS) target fragments of SlCNGC1 (Solyc01g095770.2) and SlCNGC14 (Solyc03g114110.2) were amplified by RT-PCR with gene-specific primers (Supplementary Table S1) and ligated into the TRV-based VIGS vector pYL156, which was subsequently electroporated into Agrobacterium tumefaciens strain GV3101 for VIGS analyses. VIGS analyses were conducted with vacuum infiltration delivery approach as described using recombinant pYL156 with insertion of an eGFP fragment instead of an empty pYL156 as control to alleviate viral symptom (Saand et al., 2015a). At about 3 weeks post agro-infiltration, plants were inoculated with Xoc as described above.

Detection of Callose Deposition
Callose deposition were stained with aniline blue and visualized in fluorescence microscope. Briefly, the collected leaves were
washed twice with ddH₂O and ethanol, respectively, and cleared in acetic acid-ethanol (1:3) for 4 h. After washed twice with ddH₂O, the leaves were incubated in aniline blue solution [150 mM KH₂PO₄, 0.1% (w/v) aniline blue, pH 9.5] for 1 h. The stained leaves were washed with ddH₂O and observed in 30% glycerol by fluorescence microscopy.

**Bacterial Number Counting Assays**

Bacterial numbers in Xoc-infiltrated leaves of silenced plants were determined as previously reported (Li et al., 2012).

**Detection of ROS**

Xoc-inoculated leaves of tomato plants were detached and stained with 3,3-diamino benzidine hydrochloride (DAB) (1mg/mL) as described (Li et al., 2015). Quantitative determination of PAMP-elicited H₂O₂ was conducted using a luminol-based approach (Saand et al., 2015a). For each experiment, six tomato leaf disks of 3 mm at diameter from three plants were dipped in distilled water in the light over night. The leaf disks were then transferred into 50 µL of distilled water in a 96-well plate. After addition of the same volume mixture which contains 200 nM luminol (Sigma-Aldrich), 20 µg of horseradish peroxidase and 200 nM fgl22, H₂O₂ were measured for 35 min as luminescence using a Microplate Luminometer (Titertek Berthold, Germany).

**Calcium Assay**

Transient increase of cytosolic Ca²⁺ concentration was monitored in the Aequarin transgenic tomato lines. For each experiment, six leaf disks were punched from three plants and vacuum-infiltrated in 12.5 µL of coelenterazin h (Sigma) on a 96-well plate for 1 min and then set for 2 h at room temperature. Before measurement, the coelenterazin solution was gently removed and 200 µL of PAMP solution (100 nM fgl22) was added to the wells. Luminescence was measured using a Microplate Luminometer (Titertek Berthold, Germany).

**Gene Expression Analyses by qRT-PCR**

Five defense-related Ca²⁺ signaling genes SICDPK10 (Solyc11g018610.1), SICAMTA3 (Solyc04g056270.2), SICBP60G (Solyc01g100240.2), SICAM2 (Solyc10g081170.1) and SICAM6 (Solyc03g098050.2) were subjected to gene expression analyses (Zhao et al., 2013; Rahman et al., 2016b; Wang et al., 2016). Total RNA was isolated by Trizol (TaKaRa, Japan) extraction according to the manufacturer's instructions. RNAs were treated with DNase I (TaKaRa, Japan) and then reverse transcribed. Quantitative real time PCR (qRT-PCR) was performed as previously described using the StepOne Real-Time PCR system (Applied Biosystems, United States) with SYBR Green PCR Master Mix (TaKaRa, Japan) (Saand et al., 2015a). 18s rDNA was used as internal control. The accession number and the specific primers for amplification of the analyzed genes were listed (Supplementary Table S1). The expression of target genes relative to control was calculated based on a value of $2^{-\Delta\Delta C_t}$ as recommended by the manufacturer. Meanwhile, semi-qRT-PCR for these genes were conducted in parallel and obtained PCR products were analyzed by agarose gel electrophoresis.

**Statistical Analyses of Data**

All experiments were conducted three times independently. The quantitative measurement data were statistically analyzed using SPSS software and represent means ± standard error. Significant difference between mean values was determined with DMRT ($p < 0.05$).

**RESULTS**

**Silencing of SICNGC1 and SICNGC14 Enhanced Xoc-Induced Hypersensitive Response and Non-host Resistance**

In order to dissect the function of SICNGC1 and SICNGC14 in Xoc-induced HR and non-host resistance, we used TRV-based VIGS vector to perform VIGS analyses for these genes. Transcripts of SICNGC1 and SICNGC14 genes accumulated to only about 20% of the eGFP silencing control (Figure 1A), indicating that SICNGC1 and SICNGC14 genes had been efficiently silenced in these plants. Inoculation assays with Xoc in these silencing plants demonstrated that SICNGC1- and SICNGC14-silenced leaves showed more severe Xoc-induced HR than the eGFP control leaves. SICNGC1- and SICNGC14-silenced leaves showed obvious HR necrosis at 3 h post Xoc inoculation, and exhibited strong HR necrosis at 9 hpi, especially SICNGC14-silenced leaves, which had turned into desiccative, while the eGFP control plants displayed barely visible and weak HR necrosis at 3 and 9 hpi, respectively (Figure 1B). This result indicated that silencing of SICNGC1 and SICNGC14 enhanced Xoc-induced HR.

We further counted Xoc bacterial number in the infiltrated leaf areas. The result showed that compared with the eGFP controls, Xoc bacterial number in SICNGC1- and SICNGC14-silenced plants decreased significantly by 0.7 orders of magnitude at 9 hpi (Figure 1C), indicating that silencing of SICNGC1 and SICNGC14 enhanced non-host resistance to Xoc.

Collectively, these results implied that both SICNGC1 and SICNGC14 might negatively regulate non-host resistance related HR cell death and non-host resistance to Xoc in tomato.

**Silencing of SICNGC1 and SICNGC14 Increased Xoc-Induced Callose Deposition**

To probe the mechanisms underlying SICNGC1- and SICNGC14-dependent regulation of non-host resistance against Xoc, effect of these CNGC genes on callose deposition upon pathogen infection was examined. Callose deposition was visible in the aniline blue-stained leaves under the fluorescence microscopy. Microscopic observation result showed that SICNGC1- and SICNGC14-silenced leaves deposited much more callose than eGFP control leaves (Figure 2A). Further quantification revealed that callose deposits in SICNGC1- and SICNGC14-silenced leaves was 5.9- and 7.3-fold respectively, as many as in the eGFP control leaves at 4 hpi (Figure 2B). This result demonstrates that SICNGC1 and SICNGC14 repress the callose deposition upon...
pathogen infection to alleviate non-host resistance to \textit{Xoc} in tomato.

**Silencing of \textit{SlCNGC1} and \textit{SlCNGC14} Promoted ROS Accumulation**

Reactive oxygen species (ROS) is indispensable to \textit{X. oryzae} pv. \textit{oryzae} (\textit{Xoo})-induced HR and non-host resistance (Li et al., 2015). Thus, we analyzed the effect of \textit{SlCNGC1} and \textit{SlCNGC14} on the production of hydrogen peroxide (H$_2$O$_2$), one of the primary species of ROS. DAB staining result demonstrated that infiltration areas of \textit{SlCNGC1}- and \textit{SlCNGC14}-silenced leaves showed significantly stronger DAB staining than those of control (Figure 3A), implying that silencing of \textit{SlCNGC1} and \textit{SlCNGC14} increased the production of H$_2$O$_2$.

Effect of \textit{SlCNGC1} and \textit{SlCNGC14} on the PAMP (flg22)-elicited H$_2$O$_2$ accumulation was further examined using leaf disk assays and was indicated as relative luminescence (RLU). The luminol-based assay showed that flg22-induced H$_2$O$_2$ in \textit{SlCNGC1}- and \textit{SlCNGC14}-silenced leaves culminated to over 4100 RLU, while that in eGFP control leaves was peaked only to 940 RLU (Figure 3B), demonstrating that silencing of \textit{SlCNGC1} and \textit{SlCNGC14} promoted the production of flg22-elicited H$_2$O$_2$.

Together, these results suggest that \textit{SlCNGC1} and \textit{SlCNGC14} suppress the ROS accumulation to attenuate non-host resistance and PTI.

**Silencing of \textit{SlCNGC1} and \textit{SlCNGC14} Compromised Cytosol Ca$^{2+}$ Influx**

Some Arabidopsis CNGCs have been proved to be functional Ca$^{2+}$ channels (Gao et al., 2014; Wang et al., 2017). To examine whether \textit{SlCNGC1} and \textit{SlCNGC14} function as Ca$^{2+}$ channels,
effect of silencing of these genes on accumulation of cytosolic Ca\textsuperscript{2+} elicited by the PAMP flg22 was monitored through leaf disk assays using aequorin transgenic tomato lines. In eGFP control aequorin transgenic plants, flg22-triggered Ca\textsuperscript{2+} increased rapidly and peaked to 192 RLU, while those in SlCNGC1- and SlCNGC14-silenced aequorin transgenic plants strongly decreased with the peak value drop to only 97 and 39 RLU, respectively (Figure 4). This result indicates that SlCNGC1 and SlCNGC14 function as Ca\textsuperscript{2+} channels and negatively regulate non-host resistance and PTI through modulating cytosolic Ca\textsuperscript{2+} accumulation.

Silencing of SlCNGC1 and SlCNGC14 Altered Expression of Defense-Related Ca\textsuperscript{2+} Signaling Genes

To obtain a clue to understand how SlCNGC1 and SlCNGC14 genes, as Ca\textsuperscript{2+} channel genes, regulate plant disease resistance, we examined effect of silencing of these genes on expression of a set of defense-related Ca\textsuperscript{2+} signaling genes to probe the possibility of their involvement in SlCNGC1- and SlCNGC14-mediated resistance regulation. The genes under this expression analysis included two CaM genes SlCaM2 and SlCaM6, a tomato homolog (SlCDPK10) of Arabidopsis calcium-dependent protein kinase gene AtCDPK11, and the tomato homologs (SlCBP60g and SlCAMTA3) of two Arabidopsis CaM-binding transcription factors AtCBP60g and AtCAMTA3. All these genes play an important role in regulating disease resistance (Du et al., 2009; Wang et al., 2009, 2011; Boudsocq et al., 2010; Zhao et al., 2013; Sun et al., 2015; Rahman et al., 2016a,b; Wang et al., 2016). Result of qRT-PCR showed that at 4 h after inoculation with Xoc, SlCNGC1- and SlCNGC14-silenced leaves reduced expression of the negative defense regulatory gene SlCAMTA3 by 2.5-fold, and generally increased expression of the positive defense regulatory genes SlCaM6, SlCDPK10, and SlCBP60g to different extent, which was much higher in SlCNGC14-silenced leaves than SlCNGC1-silenced leaves. Expression of SlCaM6, SlCDPK10 and SlCBP60g genes in SlCNGC1-silenced leaves was 4.3-, 1.5- and 2.5-fold, respectively, as high as that in the eGFP controls, while these folds were 34.0,
AtCNGC18 have been proved to function as Ca\textsuperscript{2+} signaling pathway to this non-host resistance.

Xoc leaf streak pathogen negatively affect tomato non-host resistance to the rice bacterial CNGC two (and SlCNGC14). We previously found that two tomato CNGC DISCUSSION SlCNGC1 represents the mean ± SE of three independent experiments.

In plants, only a few CNGCs such as AtCNGC2 and AtCNGC18 have been proved to function as Ca\textsuperscript{2+} channels (Gao et al., 2014; Wang et al., 2017). Whether the tomato CNGC genes encode functional Ca\textsuperscript{2+} channels remain to be verified. In this study, we demonstrated that silencing of SlCNGC1 and SlCNGC14 significantly reduced or almost abolished cytosolic Ca\textsuperscript{2+} accumulation (Figure 4). Thus, SlCNGC1 and SlCNGC14 likely function as Ca\textsuperscript{2+} channels. Further electrophysiological studies are required to provide more evidences to verify it.

It is recently reported that Arabidopsis cngc2 mutant overaccumulates Ca\textsuperscript{2+} in apoplastic compartment and increased HR and resistance (Wang et al., 2017). Similarly, in this study, we found that silencing of SlCNGC1 and SlCNGC14 significantly reduced cytosolic Ca\textsuperscript{2+} accumulation and enhanced HR and non-host resistance to Xoc and PTI. Whether silencing of SlCNGC1 and SlCNGC14 results in overaccumulation of Ca\textsuperscript{2+} in apoplastic compartment as observed in Atengec2 mutant (Wang et al., 2017) requires further confirmation.

ROS is the well-known master signal in plant immunity and is indispensable to Xoo-induced HR and non-host resistance (Li et al., 2015). Thus, it is considerable that regulation of ROS accumulation represents one of the essential mechanisms underlying SlCNGC1- and SlCNGC14-dependent regulation of HR and non-host resistance to Xoc and PTI. How SlCNGC1 and SlCNGC14 negatively regulate ROS accumulation remains an intriguing question to be addressed. NADPH oxidase encoded by RBOH genes is the key enzyme to generate ROS during plant-pathogen interactions. Interestingly, Ca\textsuperscript{2+} affects the activity of this enzyme which contains EF-hands to bind Ca\textsuperscript{2+}. Moreover, AtCPK28, a calcium-dependent protein kinase gene, negatively regulate ROS accumulation during PTI (Monaghan et al., 2014). In this context, it is notable that silencing of SlCNGC1 and SlCNGC14 strongly represses cytosolic Ca\textsuperscript{2+} accumulation and expression of a set of defense-related Ca\textsuperscript{2+} signaling genes including SlCaM6, SlCDPK10 and SlCBP60g (Figures 4, 5). Whether and how these changes affect NADPH oxidase thereby modulate ROS accumulation deserves further investigation.

SlCNGC1 and SlCNGC14 belong to group I and group III of tomato CNGC family. They function similarly in negative modulation of tomato HR and non-host resistance to Xoc. Both of them suppress ROS accumulation and callose deposition and alter expression of a same set of defense-related Ca\textsuperscript{2+} signaling genes including SlCaM6, SlCDPK10 and SlCBP60g. However, the degree of effect in resistance responses by silencing SlCNGC1 and SlCNGC14 varies (Figures 1–5), indicating the difference of their involvement in this non-host resistance although it can be the consequence of difference of silencing degree. In animal, CNGC often forms homo- or hetero-oligomer to function (Kaupp and Seifert, 2002). Furthermore, some plant CNGC isoforms, such as AtCNGG2 and AtCNGC4, have been reported to be able to homo- or hetero-complex in planta (Chin et al., 2013). In this context, whether SlCNGC1 and SlCNGC14 form oligomer to regulate HR and plant immunity is worth further clarifying. Additionally, cellular localization of plant CNGCs other than long expected plasma membrane have been reported recently (DeFalco et al., 2016b). Whether the cellular localization and accumulation of SlCNGC1 and SlCNGC14 differ awaits further study.

FIGURE 5 | Silencing of SlCNGC1 and SlCNGC14 altered expression of defense-related Ca\textsuperscript{2+} signaling genes. Expression of defense-related Ca\textsuperscript{2+} signaling genes SlCAM2, SlCAM6, SlCDPK10, SlCAMTA3, and SlCBP60g were examined for SlCNGC1-, SlCNGC14-silenced plants relative to the eGFP non-silenced control plants after inoculation with Xoc at 4 hpi by qRT-PCR with 18s rDNA gene serving as a loading control gene. Data represent the mean ± SE of three independent experiments.

4.5, and 9.1 for that in SlCNGC14-silenced leaves (Figure 5). This result implies that SlCNGC1 and SlCNGC14 might modulate the expression of these Ca\textsuperscript{2+} signaling genes.

DISCUSSION

We previously found that two tomato CNGC genes SlCNGC1 and SlCNGC14 play an important role in tomato HR elicited by the rice bacterial blight pathogen X. oryzae pv. oryzae (Xoo) (Saand et al., 2015a). Here, we further reveal that these two CNGC genes likely encode functional Ca\textsuperscript{2+} channels and negatively affect tomato non-host resistance to the rice bacterial leaf streak pathogen Xoc, through modulating ROS accumulation and callose deposition. The role of Ca\textsuperscript{2+} signaling pathway in non-host resistance to Xoc is previously unknown. Our results provide evidence for the contribution of CNGC-mediated Ca\textsuperscript{2+} signaling pathway to this non-host resistance.

FIGURE 4 | Silencing of SlCNGC1 and SlCNGC14 compromised cytosolic Ca\textsuperscript{2+} accumulation. Production of Ca\textsuperscript{2+} flux elicited by 100 nM flg22 was detected using a luminescence-based assay in leaf disks of SlCNGC1-, SlCNGC14-silenced plants and the eGFP non-silenced control plants of the aequorin transgenic tomato lines. Data are shown as relative luminescence units (RLU) and represent the mean ± SE of three independent experiments.
Roles of CNGCs in plant disease resistance seem to be complex. Results from previous studies using Ca\(^{2+}\) channel blockers indicated that cytosolic Ca\(^{2+}\) elevation in response to PAMPs such as flg22 is required for PAMP induced defense responses including ROS burst (Jeworutzki et al., 2010; Ranf et al., 2011; Segonzac et al., 2011), suggesting a positive role of the possibly involved CNGCs in PTI defenses. However, CNGC genes likely play different roles in various types of resistance. For instance, T-DNA insertion knockout mutants for AtCNGC11 and AtCNGC12 exhibit unaffected flg22 induced PTI responses but show a partial breakdown of resistance against avirulent, but not virulent *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae*, indicating that both AtCNGC11 and AtCNGC12 might be not involved in PTI but act as positive regulators of R gene-mediated resistance responses (Yoshioka et al., 2006; Moeder et al., 2011). Differently, null mutants for AtCNGC2 and AtCNGC4 display impaired HR cell death, while maintaining resistance against avirulent pathogens, exhibiting enhanced broad-spectrum resistance against virulent pathogens, elevated levels of SA, and constitutive expression of PR genes (Yu et al., 1998; Clough et al., 2000; Yu et al., 2000; Balagué et al., 2003; Jurkowski et al., 2004; Genger et al., 2008), and thus these AtCNGCs seem to play positive role in HR while have negative role in R gene-mediated resistance and basal resistance. Additionally, AtCNGC2 was reported to positively contribute to Pep-elicited immunity (Qi et al., 2010; Ma et al., 2012, 2013) and LPS-triggered Ca\(^{2+}\) influx that led to nitric oxide production and consequent HR formation (Ali et al., 2007; Ma et al., 2007), but might be not involved in flg22-triggered immunity (Jeworutzki et al., 2010). In this study, we found the negative role of SlCNGC1 and SlCNGC14 in Xoc-induced HR and non-host resistance, adding further complexity to the function of plant CNGCs. It is likely that different members of CNGC family might have different roles in resistance and at least some CNGCs are multifunctional and differentially regulate various types of resistance against different pathogens. SlCNGC1 and SlCNGC14, which exhibit similar function in Xoc-induced HR and non-host resistance, belong to different groups of CNGCs, with SlCNGC1 to group I as AtCNGC11 and AtCNGC12, while SlCNGC14 to group III, indicating that the function of CNGCs is not group-dependent, as we suggested previously (Saand et al., 2015b). Mechanisms underlying plant CNGC-mediated resistance against various pathogens await further elucidation.

## CONCLUSION

Gene silencing analyses demonstrated that SlCNGC1 and SlCNGC14 negatively regulate non-host resistance related HR cell death and non-host resistance to *X. oryzae* pv. *oryzicola* (*Xoc*) in tomato. These two SlCNGCs repress callose deposition and ROS accumulation to alleviate non-host resistance and likely also PTI. Silencing of SlCNGC1 and SlCNGC14 strongly reduced cytosolic Ca\(^{2+}\) accumulation, suggesting that SlCNGC1 and SlCNGC14 function as Ca\(^{2+}\) channels and negatively regulate non-host resistance and PTI through modulating cytosolic Ca\(^{2+}\) accumulation. SlCNGC14 likely played a stronger regulatory role in the non-host resistance and PTI than SlCNGC1. Our results reveal the contribution of CNGC-mediated Ca\(^{2+}\) signaling pathway to non-host resistance and PTI.

## AUTHOR CONTRIBUTIONS

The project was coordinated by X-ZC. X-RZ conducted the gene silencing analyses. X-RZ and Y-PX carried out the gene expression, designed and analyzed all statistical data. X-ZC conceived of the study, and participated in its design and coordination. X-ZC and X-RZ prepared the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.00285/full#supplementary-material

## REFERENCES

Ali, R., Ma, W., Lemtiri-Chlieh, F., Tsialtas, D., Leng, Q., von Bodman, S., et al. (2007). Death don’t have no mercy and neither does calcium: *Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2* and innate immunity. *Plant Cell* 19, 1081–1095. doi: 10.1105/tpc.106.045096

Balagué, C., Lin, B., Alcon, C., Flottes, C., Malmström, M., Köhler, C., et al. (2003). HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide–gated channel ion channel family. *Plant Cell* 15, 365–379. doi: 10.1105/tpc.006999

Bednarek, P., and Osbourn, A. (2009). Plant-microbe interactions: chemical diversity in plant defense. *Science* 324, 746–748. doi: 10.1126/science.1171661

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., et al. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512. doi: 10.1126/science.1178811

Jeworutzki, V., Rösel, R., Balagué, C., Malmström, M., von Bodman, S., et al. (2010). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512. doi: 10.1126/science.1178811

Qi, Y., Ali, R., Wang, F., Yu, H., Liu, C., Liu, L., et al. (2010). Death don’t have no mercy and neither does calcium: *Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2* and innate immunity. *Plant Cell* 19, 1081–1095. doi: 10.1105/tpc.106.045096

Yu, H., Clough, S., Lin, B., Alcon, C., Flottes, C., Malmström, M., et al. (1998). Death don’t have no mercy and neither does calcium: *Arabidopsis CYCLIC NUCLEOTIDE GATED CHANNEL2* and innate immunity. *Plant Cell* 19, 1081–1095. doi: 10.1105/tpc.106.045096

Balagué, C., Lin, B., Alcon, C., Flottes, C., Malmström, M., Köhler, C., et al. (2003). HLM1, an essential signaling component in the hypersensitive response, is a member of the cyclic nucleotide–gated channel ion channel family. *Plant Cell* 15, 365–379. doi: 10.1105/tpc.006999

Bednarek, P., and Osbourn, A. (2009). Plant-microbe interactions: chemical diversity in plant defense. *Science* 324, 746–748. doi: 10.1126/science.1171661

Boch, J., Scholze, H., Schornack, S., Landgraf, A., Hahn, S., Kay, S., et al. (2009). Breaking the code of DNA binding specificity of TAL-type III effectors. *Science* 326, 1509–1512. doi: 10.1126/science.1178811
Bogdanove, A. J., Schornack, S., and Lahaye, T. (2010). TAL effectors: finding plant genes for disease and defense. *Curr. Opin. Plant Biol.* 13, 394–401. doi: 10.1016/j.pbi.2010.04.010

Boussadegh, M., Willmann, M. R., McMormack, M., Lee, H., Shan, L., He, P., et al. (2010). Differential innate immune signalling via Ca2+-sensor protein kinases. *Nature* 464, 418–422. doi: 10.1038/nature08794

Cai, L., Cao, Y., Xu, Z., Ma, W., Zakria, M., Zou, L., et al. (2017). A transcription activator-like effector Ta37 of *Xanthomonas oryzae* pv. oryzae activates rice gene Os09g29100 to suppress rice immunity. *Sci. Rep.* 7:5089. doi: 10.1038/s41598-017-04800-8

Chin, K., DeFalco, T. A., Moeder, W., and Yoshioka, K. (2013). The Arabidopsis cyclic nucleotide-gated ion channels AtCNGC2 and AtCNGC4 work in the same signaling pathway to regulate pathogen defense and floral transition. *Plant Physiol.* 163, 611–624. doi: 10.1104/pp.113.225680

Chin, K., Moeder, W., and Yoshioka, K. (2009). Biological roles of cyclic-nucleotide-gated ion channels in plants: what we know and don’t know about this 20 member ion channel family. *Botany* 87, 668–677. doi: 10.1139/B08-147

Clough, S. J., Fengler, K. A., Yu, I. C., Lippok, B., Smith, R. K. Jr., and Bent, A. F. (2000). The Arabidopsis dnd1 “defense, no death” gene encodes a mutated cyclic-gated ion channel. *Proc. Natl. Acad. Sci. U.S.A.* 97, 9332–9338. doi: 10.1073/pnas.150005697

DeFalco, T. A., Marshall, C. B., Munro, K., Kang, H. G., Moeder, W., Ikura, M., et al. (2016a). Multiple calmodulin-binding sites positively and negatively regulate Arabidopsis CYCLIC NUCLEOTIDE-GATED CHANNEL12. *Plant Cell* 28, 1738–1751. doi: 10.1105/tpc.16.008776

DeFalco, T. A., Moeder, W., and Yoshioka, K. (2016b). Opening the gates: insights into cyclic nucleotide-gated channel-mediated signaling. *Trends Plant Sci.* 21, 903–906. doi: 10.1016/j.tplants.2016.08.011

Du, L., Ali, G. S., Simons, K. A., Hou, J., Yang, T., Reddy, A. S., et al. (2009). Ca2+-calmodulin regulates salicylic-acid-mediated plant immunity. *Nature* 457, 1154–1158. doi: 10.1038/nature07612

Fan, J., Crooks, C., Creissen, G., Hill, L., Fairhurst, S., Doerner, P., et al. (2011). *Pseudomonas syringae* genes overcome aliphatic isothiocyanate–mediated non-host resistance in Arabidopsis. *Science* 331, 1185–1188. doi: 10.1126/science.1201477

Fischer, C., DeFalco, T. A., Karta, P., Snelden, W. A., Moeder, W., Yoshioka, K., et al. (2017). Calmodulin as a Ca2+-sensing subunit of Arabidopsis cyclic nucleotide-gated channel complexes. *Plant Cell Physiol.* 58, 1208–1221. doi: 10.1093/pcp/pcx052

Fortuna, A., Lee, J., Ung, H., Chin, K., Moeder, W., and Yoshioka, K. (2015). Crossroads of stress responses, development and flowering regulation—the multiple roles of Cyclic Nucleotide Gated Ion Channel 2. *Plant Signal. Behav.* 10:e989758. doi: 10.4161/15592324.2014.989758

Gao, Q. F., Fei, C. F., Dong, J. Y., Gu, L. L., and Wang, Y. F. (2014). Arabidopsis CNGC18 is a Ca2+-permeable channel. *Plant Mol. Biol.* 79, 739–743. doi: 10.1007/s11103-014-00876-x

Genger, R. K., Jurkowski, G. I., McDowell, J. M., Lu, H., Jung, H. W., Greenberg, J. T., et al. (2008). Signaling pathways that regulate the enhanced disease resistance of Arabidopsis ‘defense, no death’ mutants. *Plant Mol. Microbe Interact.* 10, 1285–1296. doi: 10.1094/PMPI-21-10-1285

Jeworutzki, E., Roelfsema, M. R., Anschutz, U., Krol, E., Elzenga, J. T., Felix, G., et al. (2010). Early signaling through the Arabidopsis pattern recognition receptors FLS2 and EFR involves Ca2+-dependent protein kinases, nitric oxide, and reactive oxygen species are downstream from the early Ca2+ signal. *Plant Physiol.* 163, 1459–1471. doi: 10.1104/pp.110.171174

Maimbo, M., Ohnishi, K., Hikichi, Y., Yoshioka, H., and Kiba, A. (2010). S-glycoprotein-like protein regulates defense responses in *Nicotiana* plants against *Ralstonia solanacearum*. *Plant Physiol.* 152, 2023–2035. doi: 10.1104/pp.109.148189

Moeder, W., Ureqhart, W., Ung, H., and Yoshioka, K. (2011). The role of cyclic nucleotide-gated ion channels in plant immunity. *Mol. Plant* 4, 442–452. doi: 10.1093/mp/ssr018

Monaghan, J., Matschi, S., Shorinola, O., Rovenich, H., Matei, A., Segonzac, C., et al. (2014). The calcium-dependent protein kinase CPK28 buffers plant immunity and regulates BIK1 turnover. *Cell Host Microbe* 16, 605–615. doi: 10.1016/j.chom.2014.10.007

Moscou, M. J., and Bogdanove, A. J. (2009). A simple cipher governs DNA recognition by TAL effectors. *Science* 326:1501. doi: 10.1126/science.1178817

Mysore, K. S., and Ryu, C. M. (2004). Nonhost resistance: How much do we know? *Trends Plant Sci.* 9, 97–104. doi: 10.1016/j.tplants.2003.12.005

Niks, R. E., and Marcel, T. C. (2009). Nonhost and basal resistance: How to explain specificity? *New Phytol.* 182, 817–828. doi: 10.1111/j.1469-8137.2009.02849.x

Nino-Liu, D. O., Ronald, P. C., and Bogdanove, A. (2006). *Xanthomonas oryzae* pv. Oryzae reveals novel signaling components. *Cell Host Microbe* 3, 418–422. doi: 10.1016/j.chom.2006.07.004

Pinosa, F., Buhot, N., Kwaaitaal, M., Fahberg, P., Thordal-Christensen, H., Ellerström, M., et al. (2013). Arabidopsis phospholipase D5 is involved in basal defense and nonhost resistance to powdery mildew fungi. *Plant Physiol.* 163, 896–906. doi: 10.1104/pp.113.223503

Qi, Z., Verma, R., Gehring, C., Yamaguchi, Y., Zhao, Y., Ryan, C. A., et al. (2010). Ca2+-signaling by plant *Arabidopsis thaliana* Pep2 peptides depends on AtPepR1, a receptor with guanylyl cyclase activity, and cGMP-activated Ca2+-channels. *Proc. Natl. Acad. Sci. U.S.A.* 107, 21193–21198. doi: 10.1073/pnas.1000191107

Rahman, H., Hu, Y. P., Zhang, X. R., and Cai, X. Z. (2016a). *Brassica napus* genome possesses extraordinary high number of CAMTA genes and CAMTA3 contributes to PAMP-triggered immunity and resistance to *Sclerotinia sclerotiorum*. *Front. Plant Sci.* 7:581. doi: 10.3389/fpls.2016.00581

Rahman, Y., Yang, J., Xu, Y. P., Munyampundu, J. P., and Cai, X. Z. (2016b). Phylogeny of plant CAMTAs and role of AtCAMTAs in nonhost resistance to *Xanthomonas oryzae pv. oryzae*. *Front. Plant Sci.* 7:117. doi: 10.3389/fpls.2016.00177

Ranf, S., Eschen-Lippold, L., Pecher, P., Lee, J., and Scheel, D. (2011). Interplay between calcium signalling and early signalling elements during defence responses to microbe- or damage-associated molecular patterns. *Plant J.* 68, 100–113. doi: 10.1111/j.1365-313X.2011.04671.x

Rojas, C. M., Senthil-Kumar, M., Wang, K., Ryu, C. M., Kaundal, A., and Mysore, K. S. (2012). Glycolate oxidase modulates reactive oxygen species—mediated...
signal transduction during nonhost resistance in *Nicotiana benthamiana* and *Arabidopsis*. Plant Cell 24, 336–352. doi: 10.1105/tpc.111.093245
Saand, M. A., Xu, Y. P., Li, W., Wang, J. P., and Cai, X. Z. (2015a). Cyclic nucleotide gated channel gene family in tomato: genome-wide identification and functional analyses in disease resistance. Front. Plant Sci. 6:303. doi: 10.3389/fpls.2015.00303
Saand, M. A., Xu, Y. P., Munyampundu, J. P., Li, W., Zhang, X. R., and Cai, X. Z. (2015b). Phylogeny and evolution of plant cyclic nucleotide-gated ion channel (CNGC) gene family and functional analyses of tomato CNGCs. DNA Res. 22, 471–483. doi: 10.1093/dnares/dsv029
Schulze-Lefert, P., and Panstruga, R. (2011). A molecular evolutionary concept connecting nonhost resistance, pathogen host range, and pathogen speciation. Trends Plant Sci. 16, 117–125. doi: 10.1016/j.tplants.2011.01.001
Segonzac, C., Feike, D., Gimenez-Ibanez, S., Hann, D. R., Zipfel, C., and Rathjen, J. P. (2011). Hierarchy and roles of pathogen-associated molecular pattern-induced responses in *Nicotiana benthamiana*. Plant Physiol. 156, 687–699. doi: 10.1104/pp.110.1711249
Senthil-Kumar, M., and Mysore, K. S. (2013). Nonhost resistance against bacterial pathogens: retrospectives and prospects. Annu. Rev. Phytopathol. 51, 407–427. doi: 10.1146/annurev-phyto-082712-102319
Sun, T., Zhang, Y., Li, Y., Zhang, Q., Ding, Y., and Zhang, Y. (2013). ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity. Nat. Commun. 4:10159. doi: 10.1038/ncomms10159
Talke, I. N., Blaudez, D., Maathuis, F. J., and Sanders, D. (2003). CNGCs: prime targets of plant cyclic nucleotide signalling? Trends Plant Sci. 8, 286–293. doi: 10.1016/S1360-1385(03)00099-2
Wang, J. P., Xu, Y. P., Munyampundu, J. P., Liu, T. Y., and Cai, X. Z. (2016). Calcium-dependent protein kinase (CDPK) and CDPK-related kinase (CRK) gene families in tomato: genome-wide identification and functional analyses in disease resistance. Mol. Genet. Genomics 291, 661–676. doi: 10.1007/s00438-015-1137-0
Wang, L., Tsuda, K., Sato, M., Cohen, J. D., Katagiri, F., and Glazebrook, J. (2009). Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. PLoS Pathog. 5:e1000301. doi: 10.1371/journal.ppat.1000301
Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L. V., Katagiri, F., et al. (2011). CBP60g and SARD1 play partially redundant, critical roles in salicylic acid signaling. Plant J. 67, 1029–1041. doi: 10.1111/j.1365-313X.2011.04655.x
Wang, Y., Kang, Y., Ma, C., Miao, R., Wu, C., Long, Y., et al. (2017). CNGC2 is a Ca"2+ influx channel that prevents accumulation of apoplastic Ca"2+ in the leaf. Front. Plant Physiol. 17, 1342–1354. doi: 10.1016/j.pmed.2016.01.022
Wang, Y. F., Munemasa, S., Nishimura, N., Ren, H. M., Robert, N., Han, M., et al. (2013). Identification of cyclic GMP-activated nonselective Ca"2+ permeable cation channels and associated CNGCs and CNGCs genes in Arabidopsis guard cells. Plant Physiol. 163, 578–590. doi: 10.1104/pp.113.225045
Yoshioka, K., Moeder, W., Kang, H. G., Kachroo, P., Masmoudi, K., Berkowitz, G., et al. (2006). The chimeric *Arabidopsis CYCLIC NUCLEOTIDE-GATED ION CHANNEL11/12* activates multiple pathogen resistance responses. Plant Cell 18, 747–763. doi: 10.1105/tpc.105.038786
Yu, I. C., Fengler, K. A., Clough, S. J., and Bent, A. F. (2000). Identification of Arabidopsis mutants exhibiting an altered hypersensitive response in gene-for-gene disease resistance. Mol. Plant Microbe Interact. 13, 227–286. doi: 10.1094/MPMI.2000.13.3.277
Yu, I. C., Parker, J., and Bent, A. F. (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis *dnd1* mutant. Proc. Natl. Acad. Sci. U.S.A. 95, 7819–7824. PMID: 9636234; PMCID: PMC22769
Zhang, H., and Wang, S. (2013). Rice versus *Xanthomonas oryzae pv. oryzae*: a unique pathosystem. Curr. Opin. Plant Biol. 16, 188–195. doi: 10.1016/j.pbi.2013.02.008
Zhang, J., Yin, Z., and White, F. (2015). TAL effectors and the executor R genes. Front. Plant Sci. 6:641. doi: 10.3389/fpls.2015.00641
Zhao, Y., Liu, W., Xu, Y. P., Cao, J. Y., Braam, J., and Cai, X. Z. (2013). Genome-wide identification and functional analyses of calmodulin genes in Solanaceous species. BMC Plant Biol. 13:70. doi: 10.1186/1471-2229-13-70

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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