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Calcium channel CNGC19 mediates basal defense signaling to regulate colonization by Piriformospora indica in Arabidopsis roots

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Abstract

The activation of calcium signaling is a crucial event for perceiving environmental stress. Colonization by Piriformospora indica, a growth-promoting root endosymbiont, activates cytosolic Ca\(^{2+}\) in Arabidopsis roots. In this study, we examined the role and functional relevance of calcium channels responsible for Ca\(^{2+}\) fluxes. Expression profiling revealed that CYCLIC NUCLEOTIDE GATED CHANNEL 19 (CNGC19) is an early-activated gene, induced by unidentified components in P. indica cell-wall extract. Functional analysis showed that loss-of-function of CNGC19 resulted in growth inhibition by P. indica, due to increased colonization and loss of controlled fungal growth. The cngc19 mutant showed reduced elevation of cytosolic Ca\(^{2+}\) in response to P. indica cell-wall extract in comparison to the wild-type. Microbe-associated molecular pattern-triggered immunity was compromised in the cngc19 lines, as evidenced by unaltered callose deposition, reduced cis-(+)-12-oxo-phytodienoic acid, jasmonate, and jasmonoyl isoleucine levels, and down-regulation of jasmonate and other defense-related genes, which contributed to a shift towards a pathogenic response. Loss-of-function of CNGC19 resulted in an inability to modulate indole glucosinolate content during P. indica colonization. CNGC19-mediated basal immunity was dependent on the AtPep receptor, PEPR. CNGC19 was also crucial for P. indica-mediated suppression of AtPep-induced immunity. Our results thus demonstrate that Arabidopsis CNGC19 is an important Ca\(^{2+}\) channel that maintains a robust innate immunity and is crucial for growth-promotion signaling upon colonization by P. indica.

Keywords: Arabidopsis thaliana, callose, cell-wall extract, cellotriose, CNGC19, indole glucosinolates, phytohormones, Piriformospora indica, Serendipita indica

Introduction

Piriformospora indica (syn. Serendipita indica) is a cultivable, root-colonizing endophytic fungus belonging to Sebacinales (Basidiomycota) (Verma et al., 1998; Weiβ et al., 2016). It colonizes many plant species including Arabidopsis and promotes their growth (Varma et al., 1999; Peškan-Berghöfer et al., 2004; Vadassery et al., 2009), enhances nutrient uptake (Yadav et al., 2010; Rani et al., 2016; Bakshi et al., 2017; Prasad et al., 2018), and imparts tolerance to abiotic and biotic stresses to a wide range of its hosts (Waller et al., 2005; Baltruschat et al., 2008; Jogawat et al., 2013, 2016; Sun et al., 2014). It colonizes the root epidermal and cortex cells without penetrating the central cylinder, and displays a biphasic colonization strategy (Deshmukh et al., 2006; Zuccaro et al., 2011). However, the establishment of a beneficial plant–microbe interaction is
not always harmonious, and rejection of the invading symbiont or control of its colonization can occur due to active plant defense (Vadassy and Oelmüller, 2009). Basal plant defense relies on the recognition of conserved microbial structures called microbe-associated molecular patterns (MAMPs), and is termed MAMP-triggered immunity (MTI) (Millet et al., 2011). Recognition of MAMPs triggers downstream early plant-defense responses such as elevation of cytosolic calcium (Ca\(^{2+}\)) and a burst of reactive oxygen species (ROS), which further activates mitogen-activated protein (MAP) kinase and various phytohormone pathways that stimulate defense-related pathways (Harper and Harmon, 2005; Ranf et al., 2013; Steinhorst and Kundla, 2013). Similar to plant pathogens, mutualists such as \textit{P. indica} are also confronted with an effective innate immune system in roots, and the colonization success depends on the evolution of strategies for immunosuppression (Van Wées et al., 2008; Jacobs et al., 2011). During colonization by \textit{Serendipita indica} on Arabidopsis, eATP, which acts as a damage-associated molecular pattern (DAMP) accumulates in the apoplast. \textit{Serendipita indica} secretes SIE5\(^{NT}\), an enzymatically active nucleotidase capable of hydrolysing eATP, in the apoplast and thus suppresses immunity (Nizam et al., 2019). During the early stages of mycorrhiza formation and \textit{P. indica} colonization, H\(_2\)O\(_2\) is produced and its production declines when a mutualistic interaction is established (Fester and Hause, 2005; Matsuo et al., 2015). \textit{Piriformospora indica} also actively represses ROS accumulation by activating ROS-scavenging genes (Matsuo et al., 2015). To achieve a harmonious interaction with plants, \textit{P. indica} also regulates biosynthesis and signaling of several phytohormones such as jasmonic acid (JA), gibberellins (GA), and ethylene (Camehl et al., 2010; Sun et al., 2014; Vahabi et al., 2015; Pan et al., 2017; Xu et al., 2018). \textit{Piriformospora indica} association also alters callose deposition and defense-related metabolites, such as phytoalexins and glucosinolates (GS) (Jacobs et al., 2011; Lahrmann et al., 2015). Indole glucosinolates (iGS) are an important part of MTI in plants (Clay et al., 2009; Böhm et al., 2014) and they are found to be essential in balancing the beneficial interaction between \textit{P. indica} and Arabidopsis (Nongbri et al., 2012; Lahrmann et al., 2015). In addition, \textit{P. indica} also suppresses innate immunity upon encountering the flagellin 22 elicitor from bacteria (Jacobs et al., 2011). An active plant immunity and its suppression is thus critical for controlled \textit{P. indica} colonization. The early-activated plant defense genes that are responsible for regulating the entry of the symbiont and its subsequent colonization are unknown.

Ca\(^{2+}\) is a universal second messenger, activated very early in signaling cascades upon recognition of both pathogens and symbionts. Rhizobacteria-mediated nodulation and mycorrhiza formation are associated with oscillations in nuclear Ca\(^{2+}\) in host plants. These oscillations upon perception of rhizobia and mycorrhiza activate induction of the common genes that are important for the establishment of the symbioses (Oldroyd, 2013). Colonization by \textit{P. indica} in Arabidopsis is independent of these common arbuscular mycorrhiza symbiotic genes (Banhara et al., 2015). However, elevation of Ca\(^{2+}\) is common with other symbiotic interactions, as \textit{P. indica} cell-wall extract (PiCWE) elevates root Ca\(^{2+}\) and is crucial for growth promotion in Arabidopsis (Vadassy et al., 2009). Using the elevation of Ca\(^{2+}\) as a marker, Johnson et al. (2018) identified cellotriose (CT) as the major elicitor in crude PiCWE. It was further confirmed that CT targets a poly(A)-specific ribonuclease in order to modulate plant responses such as elevation of Ca\(^{2+}\) generation of ROS, expression of defense-related genes, phytohormonal signaling, and growth promotion. Elevation of Ca\(^{2+}\) requires entry of Ca\(^{2+}\) either across the plasma membrane or from intracellular compartments. In Arabidopsis, ligand-gated channels such as cyclic nucleotide gated channels (CNGCs), glutamate receptor-like channels (GLRs), stretch-activated Ca\(^{2+}\) channels (OSCAs), and the M1D1-complementing activity (MCA) families are the four main plasma membrane Ca\(^{2+}\)-permeable channels, whilst the slow vacuolar two-pore channel 1 (TPC1) is the key vacuolar channel (Dodd et al., 2010). The Arabidopsis genome encodes 20 members of the CNGC family, with roles in plant development and a functions related to biotic and biotic stresses (Meena and Vadassy, 2015; DeFalco et al., 2016). CNGC2, CNGC4, CNGC11, and CNGC12 have been reported to play crucial roles in defense against bacterial and fungal pathogens (Yoshioka et al., 2001; Ahn, 2007), and we have recently identified a role of the CNGC19 Ca\(^{2+}\) channel in herbivory-induced Ca\(^{2+}\) flux and plant defense against \textit{Spodoptera litura} (Meena et al., 2019).

CNGC15 has been identified as critical nuclear channel that generates oscillatory Ca\(^{2+}\) signals during arbuscular mycorrhizal symbiosis with \textit{Medicago truncatula} roots (Charpentier et al., 2016). In \textit{Lotus japonicus}, a mutation in the \textit{AtCNGC19} homolog \textit{BRUSH} is reported to result in impaired infection by nitrogen-fixing rhizobia due to a leaky channel (Chiasson et al., 2017). The identity of the channel involved in the elevation of Ca\(^{2+}\) that is induced by \textit{P. indica} is not yet known. The \textit{P. indica} elicitor CT induces expression of GLR Ca\(^{2+}\) channels in Arabidopsis roots; however, (Johnson et al., 2018) found no functional roles for GLR3.3, GLR2.4, GLR2.5, and TPC1 in this response. Expression levels of CNGCs are altered upon PiCWE treatment in plant roots and these are the only other type of Ca\(^{2+}\) channel known to be involved in the interaction (Vadassy et al., 2009). We therefore hypothesized that CNGCs might be involved in the generation of elevated Ca\(^{2+}\) in Arabidopsis roots and in the downstream signaling in response to \textit{P. indica} mutualism. Our results point to a role of Arabidopsis CNGC19 as an important gatekeeper to regulate \textit{P. indica} colonization.

### Materials and methods

#### Plant and fungal material and conditions

\textit{Piriformospora indica} (Verma et al., 1998) was grown and maintained on Kafer's medium at 28±2 °C at 110 rpm (Varma et al., 1999; Hill and Kafer, 2001). For \textit{P. indica} co-cultivation we used \textit{Arabidopsis thaliana} wild-type Columbia (Col-0), the T-DNA mutant lines of \textit{AtCNGC19} (Ar3g17690) SALK\(-129200C\) (eng19-2) and SALK\(-027306\) (eng19-1), which were provided by TAIR. (Alonso et al., 2003), and the \textit{pepr1 pepr2} double-mutant line provided by Prof. Gerald Berkowitz (University of
Connecticut, USA). Adult plants were grown at 22 °C with a 10/14 h light/dark photoperiod and a light intensity of 150 μmol m⁻² s⁻¹ in a growth room (Perceval Scientific). For Ca²⁺ measurements, we used transgenic Col-0 expressing cytosolic aequorin, (referred to as WT: ::aq; Knight et al., 1997), and the oeg19 and pep1 pep2 mutants transformed with the pMAQ2 vector (referred to as oeg19: ::aq and pep1 pep2: ::aq, respectively). The T2 generation was used.

Plant and fungal interactions in soil and co-cultivation media
For soil experiments, seeds were sown in pots containing soilrite, Irish peat moss, and exfoliated vermiculite (1:1:1, w:w:w) and kept for 2 d at 4 °C in the dark for stratification. The soil was pre-mixed with 1% P. indica mycelia (w/w), and plants were grown for 6 weeks after stratification. Control plants had no P. indica mycelia in the soil. The plants were grown at 22 °C with a 10/14 h light/dark photoperiod and a light intensity of 150 μmol m⁻² s⁻¹ in the growth room. Samples were harvested at 42 d post-inoculation (dpi).

For plate experiments, seeds were surface-sterilized, stratified under the conditions described above, and placed on half-strength MS plates supplemented with 1% sucrose and 0.8% agar, and germinated for 7 d. The seedlings were grown at 22 °C with a 10/14 h light/dark photoperiod and a light intensity of 150 μmol m⁻² s⁻¹ in the growth room. They were then transferred to 1× PNM medium for co-cultivation with P. indica discs for 14 d (Johnson et al., 2011) under similar conditions. Samples were harvested at 2, 7, and 14 dpi.

Preparation of P. indica cell-wall extract and application on roots
Piriformospora indica cell-wall extract (PiCWE) was prepared as described by Vadassery et al., 2009. In brief, the mycelia from 14-d-old liquid cultures were homogenized, filtered using nylon membranes, and washed three times with water, twice with chloroform/methanol (1:1), and finally twice with acetone. The mycelial cell wall material obtained was dried at room temperature, suspended in water, and autoclaved for 30 min at 121 °C. It was then filter-sterilized using a 0.22-μM filter, and 50 μl of the resulting extract was used per seedling root for experiments. The active elicitor of PiCWE was recently identified as celtotriose (CT; Johnson et al., 2018). CT (Sigma, C1167) and cellbioso (Sigma, C7252) were used for some experiments.

Plant treatments and gene expression analysis
Seedlings at 10 d old were treated with 100 μl PiCWE or 10 μM CT by adding to the MS media and were harvested at 0, 15, 30, 45, and 60 min. For the co-cultivation experiment, the seedlings were harvested at 2, 7, and 14 dpi. Each sample consisted of six seedlings and was ground to a fine powder in liquid N₂, and total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer’s protocol. Four replicate samples were used. An additional DNAs (Turbo DNAse, Ambion) treatment was included to eliminate any contaminating DNA. cDNA synthesis was performed using a High Capacity cDNA kit (Applied Biosystems). Gene-specific primers were designed using the NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and are listed in Supplementary Table S1 at JXBN online. qRT-PCR was performed in optical 96-well plates on a CFX96 Real-Time PCR Detection System (Bio-Rad) using iQ™ universal SYBR green Mix (Bio-Rad). Actin2 (At3g18780) was used as the endogenous control for normalization of transcripts. The fold-induction values of the target genes were calculated using the ΔΔC(T) method (Livak and Schmittgen, 2001) and were expressed relative to the mRNA level of the genes in the control seedlings, the values of which were set as 1.

Detection and measurement of P. indica colonization
For tracking of colonization, a green fluorescent protein (GFP)-tagged P. indica strain was utilized (Hilbert et al., 2012). Roots colonized with tagged P. indica were harvested at 2, 7, and 14 dpi, and were cleaned, mounted, and observed using fluorescence microscopy (Nikon 80i).

For confocal microscopy, the roots were treated with propidium iodide and observed under a confocal microscope (Leica TCS M5) at an emission wavelength of 505–530 nm with excitation at 470 nm and digital sectioning of 4–5 μm of root thickness. The relative amount of fungal DNA was determined using real-time qPCR, utilizing Arabidopsis Actin2 (At3g18780) and P. indica TeF1 (Bütehorn et al., 2000). Relative changes in fungal DNA content were calculated using the C(T) values of P. indica, which were normalized by the C(T) values of Actin2 using the ΔΔC(T) equation and setting the P. indica DNA content of the control roots as 1 (Vadassery et al., 2008).

Tissue localization by GUS assays
Transgenics with a fusion of the CNGC19 promoter and β-glucoronidase (GUS) were constructed as previously described by Meena et al., 2019. Arabidopsis seedlings of ProCNGC19::GUS-expressing transgenic plants (T3 generation) were co-cultivated with P. indica, and were carefully harvested at 2 dpi and 7 dpi. They were vacuum-infiltrated with GUS staining solution and incubated in the dark at 37 °C. Tissues were decolorized by treating with 70% ethanol at 65 °C and then observed under a light microscope (Nikon 80i).

Glucosinolate analysis
For analysis of glucosinolates (GS), samples of plants were harvested at 2, 14, and 42 dpi. At 2 dpi and 14 dpi, 40 whole seedlings per replicate were harvested, whilst at 42 dpi whole rosettes were harvested. Four replicate samples were used at all time points. The samples were frozen in liquid N₂, lyophilized, and ground to a fine powder in TissueLyser II (Qiagen). Total GS were extracted with 80% methanol solution containing 0.05 mM 4-hydroxybenzylglucosinolate as an internal standard. Extracts were loaded onto DEAE Sephadex A 25 columns and treated with arylsulfatase for desulfuration (Sigma-Aldrich). The eluted desulfohglucosinolates were separated using HPLC (Shimadzu CLASS-VP V6.14) on a reversed-phase C-18 column (50×0.46 mm internal diameter) with a water–acetonitrile gradient as follows: 0–1.5% acetonitrile from 0–1 min, 1.5–5% acetonitrile from 1–6 min, 5–7% acetonitrile from 6–8 min, 7–21% acetonitrile from 8–18 min, 21–29% acetonitrile from 18–23 min, 29–100% acetonitrile from 23–24 min, 100–1.5% acetonitrile from 24–28 min. This was followed by a washing cycle with a flow of 1 ml min⁻¹ (Vadassery et al., 2012). Detection was performed using a photo-diode array detector and peaks were integrated at 229 nm. The following response factors were used for quantification of individual glucosinolates: aliphatic glucosinolates, 2.0; indole glucosinolates, 0.5; and 2-phenylethyl glucosinolate, 2.0 (Burrow et al., 2006).

Estimation of phytohormones
Phytohormones were quantified as described previously (Vadassery et al., 2012; Meena et al., 2019). Seedlings were sampled at 2 dpi and 14 dpi. The samples were harvested, frozen immediately in liquid N₂, lyophilized, and ground to a fine powder. Weighed, powdered samples (25 mg) were extracted using 1.5 ml of methanol containing internal standards of
60 ng d$_4$-jasmonic acid (HPC Standards GmbH, Cunkenrode, Germany), 60 ng salicylic acid-d$_4$ (Santa Cruz Biotechnology), 60 ng abscisic acid-d$_8$ (Toronto Research Chemicals), and 12 ng d$_4$-jasmonic acid-isoleucine conjugate (HPC Standards GmbH). A triple-quadrupole LC-MS/MS system was used for phytohormone quantification (SCIEX 6500).

Callose staining, microscopy, and quantification
Seedlings at 2, 7, and 14 dpi were treated with Aniline Blue (0.001%) according to the protocol described by Schenk and Schikora (2015). Colonized and non-colonized seedlings, and control and AtPep1-treated (100 nM) 7 d old seedlings were incubated at room temperature in acetic acid and ethanol (1:3) for decolorization, washed in 150 mM K$_2$HPO$_4$, and stained with Aniline Blue (0.001%) solution. Slides for observing callose deposition were prepared using 50% glycerol under a Nikon 80i microscope at 358 nm excitation and 460 nm emission. Relative callose intensities were calculated by dividing callose pixels and total pixels using the digital photograph analysis software GIMP (Scalschi et al., 2015).

Phylogenetic analysis
A total of 123 complete CNGC sequences from seven different plants were selected for construction of the phylogenetic tree, namely Arabidopsis thaliana, Glycine max, Medicago truncatula, Solanum lycopersicum, Zea mays, Oryza sativa, and Lotus japonicus. All the amino acid sequences encoding CNGCs were retrieved from previously published reports (Moeder et al., 2011; Nawaz et al., 2014; Charpentier et al., 2016; Chiasson et al., 2017; Moeder and Yoshioka, 2017) and are listed in Supplementary Table S2. A phylogenetic tree was constructed using the MEGA 10 software (https://www.megasoftware.net/; Kumar et al., 2018), in which the sequences were aligned by MUSCLE with default parameters. These aligned sequences were used to build the phylogenetic tree using the maximum likelihood (ML) method and the evolutionary distances were computed using a Jones–Taylor–Thornton matrix-based method with 1000 bootstrap replications.

Statistical analysis
Statistical differences between treatments were analysed using two-tailed Student’s t-tests or one-way ANOVA followed by Tukey’s test in SigmaPlot 13.0. Figures were generated using Origin 6.0 (www.originlab.com).

Results
CNGC19 expression is activated by P. indica cell-wall extract
To identify the role of CNGCs in the perception of P. indica by Arabidopsis, we applied either crude cell-wall extract (PiCWE) or its identified active elicitor cellobiose (CT) to Arabidopsis seedlings for 30 min (Fig. 1A). Upon treatment with PiCWE, five CNGCs were found to be induced in Arabidopsis roots, namely CNGC19 (14.3-fold increase), CNGC3 (6.22-fold), CNGC13 (5.9-fold), CNGC10 (5-fold), and CNGC6 (3.7-fold). In response to CT, nine CNGCs were induced, with the highest expression being found for CNGC3 (17.5-fold); however, CNGC19 was not induced by CT. Since it was the highest expressed upon PiCWE treatment and the only transcript specifically induced by PiCWE but not by CT, we hypothesized that CNGC19 was induced by unidentified elicitors. We examined the patterns of CNGC19 expression in wild-type (WT) roots and found that it increased from 10–45 min in response to PiCWE but there was no response to CT (Fig. 1B). We then examined roots of plants co-cultivated with P. indica, and found that expression of CNGC19 was increased by 7-fold at 2 d post-colonization, before returning to the basal level at later time-points (Fig. 1C), thus indicating a potential role in the colonization process. To identify the tissue-specific expression pattern of CNGC19 in Arabidopsis after colonization by P. indica, we used ProCNGC19::GUS. CNGC19 promoter activity was observed in the root primordia and primary vasculature at 2 dpi and 7 dpi (Fig. 1D, E). Interestingly, we also observed a systemic expression of CNGC19 in the leaf vasculature at the same time. The results therefore indicated that CNGC19 is an early-activated gene that is expressed in the vasculature, and it is induced in the roots by unidentified components in PiCWE and systemically in leaves.

The growth of cngc19 mutants is inhibited by P. indica
To identify the functional role of CNGC19 in the promotion of growth induced by P. indica, we utilized the cngc19-2 and cngc19-1 mutant T-DNA lines (Meena et al., 2019). In contrast to the WT plants, we observed no growth promotion in the cngc19 mutants in response to P. indica for parameters such as fresh weight (Fig. 2A, B) and root length (Supplementary Fig. S1) when plants were grown in culture medium. Indeed, the mutants showed growth inhibition at 7 dpi and 14 dpi compared to the WT (Fig. 2B). We found similar effects when we repeated the experiment with plants grown in soil, with the growth of the mutants being strongly inhibited at 42 dpi whereas growth promotion was observed in the WT in response to P. indica (Supplementary Fig. S2A–C).

Colonization by P. indica is enhanced in cngc19 roots
We tested the hypothesis that the reduced growth in cngc19 mutants upon P. indica inoculation was due to enhanced colonization. The roots of the WT and cngc19-2 were co-cultivated with GFP–tagged P. indica and were used for microscopic analyses. We observed no differences between P. indica colonization in the WT and cngc19-2 at 2 dpi and 7 dpi (Supplementary Fig. S3); however, at 14 dpi cngc19 roots had increased colonization compared to the WT and clumps of fungal mycelia and spores could be observed (Fig. 2C). This was supported by quantification of the relative fungal DNA content, which increased at 14 dpi (grown in medium) and 42 dpi (grown in soil) in cngc19-2 roots relative to the WT (Fig. 2D). Thus, the loss-of-function of CNGC19 resulted in increased colonization and loss of controlled P. indica growth in the plant–fungal interaction at the post-establishment phase.

The CNGC19 channel is involved in PiCWE-mediated elevation of cytosolic Ca$^{2+}$
CNGC19 is a plasma membrane–localized Ca$^{2+}$–permeable channel (Meena et al., 2019) and we therefore hypothesized that it could be involved in generating the elevation in cytosolic Ca$^{2+}$ in response to PiCWE. We used the WT::aeq and cngc19::aeq lines to examine intracellular Ca$^{2+}$, upon application of PiCWE and CT to the roots. The substrate of CT, cellobiose (CB), was also used as an unrelated elicitor control. Both CT (Fig. 3A) and cellobiose (Supplementary Fig.
S4) induced elevation of Ca\textsuperscript{2+}\textsubscript{cyt} in the WT::seq and \textit{ange}19 roots at similar levels. When PiCWE was added, the elevation in Ca\textsuperscript{2+}\textsubscript{cyt} was reduced in the \textit{ange}19::seq line relative to the WT::seq (Fig. 3B), both in the initial peak and for several minutes thereafter. These results suggest that CNGC19 is a crucial channel that is involved in sensing as yet unidentified elicitors in PiCWE and in activating the elevation of Ca\textsuperscript{2+}\textsubscript{cyt}.

**Callose deposition in response to \textit{P. indica} colonization is delayed in \textit{cngc19}**

Increased callose deposition has been reported in Arabidopsis roots colonized by \textit{P. indica} and indicates the activation of MTI (Jacobs \textit{et al.}, 2011). In the WT plants, callose deposition was increased at 2 dpi and 7 dpi and then remained unchanged at 14 dpi during the established colonization phase (Fig. 4A, C). In contrast, in the \textit{ange}19-2 mutant the callose deposition was unaltered compared to the control at both 2 dpi and 7 dpi, indicating a reduced defense in these plants at these early stages. Callose deposition was not induced in \textit{ange}19-2 until 14 dpi. Thus, plant defense was lowered in the \textit{ange}19 mutant during the initial stages of the plant–fungal interaction, leading to increased colonization.

**CNGC19-mediated basal immunity upon perception of \textit{P. indica} is dependent on \textit{AtPep-PEPR}**

Plants roots encounter damage-associated molecular patterns (DAMPs) upon microbial invasion (Boller and Felix, 2009; Albert, 2010).
Role of the Ca\(^{2+}\) channel CNGC19 in \textit{P. indica}–Arabidopsis mutualism

Fig. 2. Effects of \textit{P. indica} colonization on Arabidopsis cngc19 mutants. (A) Representative images of the wild-type (WT), and the cngc19-2 and cngc19-1 lines after co-cultivation with \textit{P. indica} (Pi-treated) for 14 d compared with non-inoculated controls. (B) Fresh weights of the WT, cngc19-2, and cngc19-1 at 2–14 d post-inoculation (dpi) with \textit{P. indica} compared with non-inoculated controls. Data are means (±SE), n=20 seedlings. Different letters indicate significant differences among the means as determined using one-way ANOVA and a post hoc Tukey test (\(P\leq0.05\)). (C) Colonization patterns of \textit{P. indica} on the WT and cngc19-2 as determined by confocal microscopy. GFP-tagged \textit{P. indica} was visualized at 14 dpi and arrows indicate chlamydospores and hyphae. DIC, differential interference contrast images. (D) Quantification of \textit{P. indica} colonization in the WT and cngc19-2 mutant grown on plates (14 dpi) and in soil (42 dpi). The relative fungal colonization was calculated by subtracting the C\(_T\) values of \textit{P. indica} Tef1 from the C\(_T\) values of Arabidopsis \textit{Actin2}. Data are means (±SE) of four replicates, each of which consisted of the combined roots of six seedlings. Significant differences were determined using two-tailed Student’s \(t\)-tests (*\(P\leq0.05\)).

Fig. 3. Concentrations of cytosolic calcium (Ca\(^{2+}\)\(_\text{cyt}\)) in Arabidopsis in response to treatment with \textit{P. indica}-related elicitors. Roots of transformed 10-d-old seedlings of the wild-type (WT) and cngc19 expressing cytosolic apoaequorin were treated with (A) cellotriose (10 \(\mu\)M) or (B) \textit{P. indica} cell-wall extract (50 \(\mu\)l). Data are means (±SE), n=5. The experiment was repeated three times with similar results and the data from one experiment are shown. Water was used as the control and gave background readings in the WT and cngc19. The arrows indicate the time of treatment with the elicitors. Different letters indicate significant differences between the WT and cngc19 during the selected periods enclosed in the boxes, as determined using one-way ANOVA and a post hoc Tukey test (\(P\leq0.001\)).
In Arabidopsis, a family of endogenous elicitor peptides referred to as AtPeps acts as DAMPs (Huffaker and Ryan, 2007; Bartels et al., 2013) and the plasma membrane Pep-receptors PEPR1 and PEPR2 perceive them (Yamaguchi et al., 2006, 2010; Krol et al., 2010). We tested the possibility that P. indica may suppress AtPep-induced defense, and the role of CNGC19 in such a process. We found that callose deposition induced by application of AtPep1 was suppressed in WT seedlings inoculated with P. indica at 2 dpi (Fig. 4B, D). For the cngc19 mutant, the AtPep1-induced deposition of callose was constitutively lower than that of the WT for all the treatments, and it was not suppressed by colonization by P. indica (Fig. 4D). Thus, the results indicated that CNGC19 was also crucial for P. indica-mediated suppression of DAMP-triggered immunity. The Pep-receptors PEPR1 and PEPR2 have a putative guanyl cyclase domain that generates cyclic nucleotides and they are upstream of CNGC2 (Ma et al., 2012). In order to determine the role of PEPR1 and PEPR2 in the Arabidopsis–P. indica association, we examined their expression. In WT seedlings, expression of PEPR1 and PEPR2 was found to be induced by PiCWE treatment (up to 3.5-fold; Fig. 5A) and by P. indica colonization (up to 6-fold; Supplementary Fig. S5A). To examine the dependency of CNGC19 activation on PEPR, we measured the expression of CNGC19 in the pepr1 pepr2 background in response to treatment with PiCWE, and
found that it was reduced at 45 min and 60 min relative to the WT (Fig. 5B). We then examined the role of PEPRs in *P. indica*–induced growth promotion. Colonization by *P. indica* was relatively high in pepr1 pepr2, and instead of growth promotion, inhibition was observed both on plates (Fig. 5C, D) and in soil (Supplementary Fig. S6A, B). However, elevation of Ca$_2^+$ cyt was unaltered when compared to the WT in pepr1 pepr2::aeq upon treatment with PiCWE (Fig. 5E) and CT (Supplementary Fig. S5B). PEPR signaling contributes to the JA signaling pathway upon herbivory (Klauser et al., 2015; Meena et al., 2019). The level of the JA marker VSP2 was found to be reduced in pepr1 pepr2 upon both treatment with PiCWE and colonization by *P. indica* (Fig. 5E, Supplementary S5C), indicating its function downstream of CNGC19 via jasmonate signaling.

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**Fig. 5.** The roles of PEPR1 and PEPR2 in the Arabidopsis–*P. indica* interaction. Expression of (A) PEPR1 and PEPR2 in wild-type (WT) and (B) expression of CNGC19 in the pepr1 pepr2 double-mutant in response to treatment with *P. indica* cell-wall extract (PiCWE) in 10-d-old seedlings. Transcripts levels were normalized to AtActin2 mRNA and the fold-change in expression is relative to the value at time zero, which was set as 1. Data are means (±SE) of four replicates, each of which consisted of six seedlings. Significant differences were determined using two-tailed Student’s t-test (*P<0.05). (C) Effects of *P. indica* colonization on the fresh weight of the WT and the pepr1 pepr2 double-mutant in non-inoculated controls in response to colonization by *P. indica* at 14 d post-inoculation (dpi). Data are means (±SE), n=30. Different letters indicate significant differences among the different treatments, as determined using one-way ANOVA and a post hoc Tukey Test (P≤0.001). (D) Effect of *P. indica* colonization on fungal colonization in the WT and the pepr1 pepr2 double-mutant. Plants were co-cultivated with or without fungal discs on 1× PNM agar plates and the roots were harvested at 14 dpi. The relative fungal colonization was calculated by subtracting the C$_T$ values of *P. indica* Tef1 from the C$_T$ values of Arabidopsis Actin2. Data are means (±SE) of four replicates, with six seedlings per replicate. The significant difference was determined using a two-tailed Student’s t-test (**P≤0.005). (E) Response of cytosolic calcium (Ca$_2^+$ cyt) to treatment with *P. indica* cell-wall extract (PiCWE, 50 µl) in roots of transformed 10-d-old seedlings of the WT and pepr1 pepr2 expressing cytosolic apoaequorin. Data are means (±SE), n=5. The experiment was repeated three times with similar results and the data from one experiment are shown. Water was used as the control and gave background readings in the WT and pepr1 pepr2. The arrow indicates the time of treatment with the elicitor. (F) Expression of the defense-related gene VSP2 in response to treatment with PiCWE in roots of transformed 10-d-old seedlings of the WT and pepr1 pepr2 expressing cytosolic apoaequorin. Transcripts levels were normalized to AtActin2 mRNA and the fold-change in expression is relative to the value at time zero, which was set as 1. Data are means (±SE), n=4.) Significant differences were determined using two-tailed Student’s t-test (*P≤0.05; **P≤0.005).
Loss-of-function of CNGC19 down-regulates jasmonate biosynthesis upon Piriformospora indica colonization

*P. indica* activates the JA signaling pathway in Arabidopsis, and this is crucial for colonization and for balancing the beneficial interaction between the two organisms (Stein et al., 2008; Vahabi et al., 2015). To identify the role of various phytohormones in *P. indica* colonization in the *cngc19* mutant, we measured their levels at 2 dpi and 14 dpi. The levels of JA and jasmonoyl isoleucine (JA-Ile) were increased in WT plants in response to *P. indica* at 2 dpi and 14 dpi, and cis-(+)-12-oxo-phytodienoic acid (cis-OPDA) was also strongly increased at 2 dpi (Fig. 6A-C). In contrast, no significant effects of colonization were observed for *cngc19*. The lack of an effect on the levels of cis-OPDA, JA, and JA-Ile at 2 dpi may have contributed to uncontrolled colonization in *cngc19* roots. JA-Ile-OH also showed similar trends (Supplementary Fig. S7A), but no changes in ABA and salicylic acid (SA) were observed in response to *P. indica* colonization in either genotype at either time-point (Supplementary Fig. S7B, C).

Phytohormone- and defense-related genes are down-regulated in *cngc19* during colonization

Since colonization by *P. indica* was enhanced in the *cngc19* lines, we examined the expression of marker genes of different defense pathways such as those of JA, ROS, and phytoalexin. The JA markers *VSP2*, *PDF1.2*, and *LOX1* were found to be induced by *P. indica* in WT plants at both 2 dpi and 14 dpi compared to non-inoculated controls, whereas these genes were found to be down-regulated in *cngc19* except *LOX1* at 2 dpi (Fig. 7A). The ROS markers *RBOHD*, *RRTF1*, and *OXI1* were selected based on their known functional roles in the Arabidopsis–*P. indica* interaction (Camehl et al., 2011; Matsuo et al., 2015; Johnson et al., 2018). *RRTF1* was found to be up-regulated in the WT but not in *cngc19* (Fig. 7B), *RBOHD* was up-regulated in both the WT and *cngc19* and did not differ between the two, and *OXI1* was strongly up-regulated at 2 dpi in the WT but showed no change in *cngc19* compared to non-inoculated controls. In contrast, at 14 dpi *OXI1* was observed to be strongly up-regulated in *cngc19*, which may have been due to increased colonization. In addition, the ROS-related genes *SOD1*, *GSTF8*, and *APX1* were found to be up-regulated in the WT and down-regulated in *cngc19* at 2 dpi (Supplementary Fig. S8), and at 14 dpi *GR1* and *CAT2* were found to be up-regulated in *cngc19* but unaltered in the WT. *WRKY33* and *PAD3*, which are related to phytoalexin biosynthesis, were also found to be up-regulated in the WT but not in *cngc19* at 2 dpi (Fig. 7C). At 14 dpi, *WRKY33* was up-regulated in both the WT and *cngc19* and did not differ between the two, whilst *PAD3* was also up-regulated in both

**Fig. 6.** Effects of *P. indica* colonization on levels of phytohormones in seedlings of the Arabidopsis wild-type (WT) and the *cngc19* mutant. (A) Jasmonates (JA), (B) the JA bioactive form (+)-jasmonoyl isoleucine (JA-ILE), and (C) the JA precursor cis-(+)-12-oxo-phytodienoic acid (cis-OPDA) at 2 d post-inoculation (dpi) and 14 dpi. Data are means (±SE) of three replicates, each of which consisted of 40 seedlings. Different letters indicate significant differences between the WT and *cngc19*-2 plants at both time-points as determined using one-way ANOVA and a post hoc Tukey Test (*P*≤0.001).
Role of the Ca\(^{2+}\) channel CNGC19 in \(P.\) indica–Arabidopsis mutualism

but had a greater increase in the WT. Thus, the loss-of-function of CNGC19 affected JA-responsive genes, and genes involved in ROS signaling and defense, and was associated with over-colonization by \(P.\) indica in cngc19 plants.

CNGC19-mediated defense signaling in roots acts via indole glucosinolates

CNGC19 loss-of-function results in constitutively reduced levels of aliphatic GSs in Arabidopsis rosettes, which are crucial for defense against herbivory (Meena et al., 2019). Upon plant–microbe interactions, accumulation of antimicrobial indole glucosinolates (iGSs) and camalexin trigger immunity (Bednarek et al., 2009; Clay et al., 2009; Böhm et al., 2014).

The iGS pathway is critical for mutualistic \(P.\) indica colonization and for uncompromised plant immunity (Nongbri et al., 2012; Lahrmann et al., 2015). We therefore decided to test the effects of mutation in CNGC19 on iGS levels. We found that the constitutive levels of both iGSs and aliphatic GSs in non-inoculated (control) seedlings were the same in the WT and cngc19 at 14 dpi (Fig. 8A, B). At the rosette stage in non-inoculated plants grown in soil (42 dpi), the level of iGSs did not differ between the WT and cngc19 whilst aliphatic GSs were significantly lower in cngc19, as also reported by Meena et al., 2019. We then looked at the effects of \(P.\) indica colonization and found that iGSs were increased in the WT at 14 dpi but were not affected in cngc19 (Fig. 8A). The levels of aliphatic GSs also showed a similar trend (Fig. 8B). After a prolonged period of colonization by \(P.\) indica (42 dpi, grown in soil), iGS levels were reduced significantly in WT plants but were unaltered in cngc19 (Fig. 8A). Aliphatic GS levels did not change at 42 dpi in either of the genotypes (Fig. 8B). We examined the relative expression of key genes related to the iGS biosynthesis pathway and found that they were generally up-regulated in the WT but not in cngc19 at 2 dpi and 14 dpi (Fig. 8C). The results therefore indicate that CNGC19 plays a crucial role in modulating the content of indole GSs during colonization by \(P.\) indica.

Phylogenetic analysis of CNGC19 indicates it has a distinct role in microbial interactions

Unlike rhizobial nodulation and mycorrhizal symbiosis, \(P.\) indica has a broad host range and is a primitive symbiont (Franken et al., 2000). We constructed a phylogenetic tree in order to understand the relationship between AtCNGC19 and its orthologs in other host plants, many of which form symbiotic interactions. Genome-wide analyses in seven different species have identified distinct CNGCs (Mäser et al., 2001; Nawaz et al., 2014; Saand et al., 2015; Charpentier et al., 2016). In our unrooted phylogenetic
tree, 123 CNGCs clustered into four different groups (Groups I–IV; Fig. 9). Among these, Group IV was further subdivided into IVA and IVB. AtCNGC19 and AtCNGC20 were clustered into Group IVA with orthologs from different legume plants that form symbiotic interactions, such as G. max, M. truncatula, and L. japonicas. AtCNGC19 was clustered with BRUSH from L. japonicas, which plays a crucial role in rhizobial symbiosis by regulating Ca²⁺ fluxes (Chiasson et al., 2017). AtCNGC19 also grouped with OsCNGC13 (its ortholog in O. sativa; Moeder and Yoshioka, 2017), which has been shown to be up-regulated by a bacterial pathogen (Nawaz et al., 2014). Nuclear-localized MtCNGC15 has been shown to be critical in generating oscillations in nuclear Ca²⁺ during mycorrhizal symbiosis (Charpentier et al., 2016); however, it was found to be clustered into Group III, which suggests it is evolutionarily divergent from AtCNGC19.

**Discussion**

Plant roots interact with both pathogenic and symbiotic microbes and recognize them as a potential threat by activation of basal MAMP-triggered immunity (MTI). Activation of MTI prevents the establishment of pathogens and regulates colonization by symbionts, and thus the process acts as a gatekeeper (Yu et al., 2019a). Root MTI efficiently restricts penetration and colonization of the mutualist P. indica and prevents over-colonization, with the result that a symbiotic interaction is established (Jacobs et al., 2011). Recognition of P. indica cell-wall extract (PiCWE) by Arabidopsis roots induces elevation of cytosolic Ca²⁺ (Ca²⁺cyt) and is crucial in activating the symbiotic interaction and in promoting growth (Vadassery et al., 2009; Johnson et al., 2018). The identity of the ion channels responsible for the influx of Ca²⁺ and the activation of signaling is currently unknown. In our present study, we identified CNGC19 as an early-activated gene (Fig. 1A) and found that it was induced by unidentified components in PiCWE and not by treatment with cellotriose (CT) (Fig. 1A, B). Cyclic nucleotide gated channels (CNGCs) are altered by treatment with PiCWE and glutamate receptor-like channels (GLRs) are altered by treatment with CT in plant roots (Vadassery et al., 2009; Johnson et al., 2018). PiCWE (which contains many elicitors including CT) and CT also differ in their activation of other pathways. Importantly, CT induces a defense
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The pathway comprising ROS accumulation and the expression of its marker gene RBOHD (Johnson et al., 2018), whereas PiCWE does not activate this pathway (Vadassery et al., 2009). We found that CNGC19 was crucial for P. indica-induced promotion of growth, as its loss-of-function resulted in increased colonization and the complete loss of growth-promotion phenotype (Fig. 2A, B). Importantly, CNGC19 was found to be critical for the generation of the PiCWE-induced elevation of Ca\(^{2+}\) cyt. No other Ca\(^{2+}\) channels have yet been implicated in the growth promotion and elevation of Ca\(^{2+}\) cyt that is induced by P. indica. Since PiCWE-induced Ca\(^{2+}\) signals were not completely abolished in the cngc19 mutant (Fig. 3B), they might be controlled by additional genetic interactions between CNGCs and other unknown channels. A leucine-rich repeat protein mutant, Piriformospora indica-insensitive12 (pii12), has previously been reported to show no promotion of growth when it is associated with P. indica (Shahollari et al., 2007). Overall, our results indicate that the PiCWE-activated CNGC19 is a critical Ca\(^{2+}\) channel for growth-promotion signaling.

Expression of CNGC19 in Arabidopsis is associated with salinity stress (Kugler et al., 2009; Oh et al., 2010). We have previously reported that CNGC19 expressed in the leaf vasculature is crucial for Arabidopsis defense against herbivory by Spodoptera moths by regulating the spread of Ca\(^{2+}\) signals and the levels of jasmonate and aliphatic glucosinolates (Meena et al., 2019). The AtCNGC19 homolog SICNGC15 in tomato is induced by both salinity stress and P. indica colonization (Ghorbani et al., 2019). SICNGC15 is also associated with disease resistance against the necrotrophic fungus Sclerotinia sclerotiorum (Saand et al., 2015). cngc19 and cngc20 mutants are also more susceptible to infection by Botrytis cinerea (Moeder et al., 2011). The receptor kinase BAK1/SERK4 phosphorylates the Ca\(^{2+}\)-channel complex CNGC20/CNGC19 and has crucial role in pathogen-induced cell death (Yu et al., 2019b). The roles of other CNGCs in plant defense have been demonstrated in many studies. AtCNGC2 and AtCNGC4 are known to regulate Ca\(^{2+}\)-induced PAMP-triggered immunity (Chin et al., 2013; Tian et al., 2019). They act as a heterotetrameric Ca\(^{2+}\) channel and are phosphorylated and activated by the kinase BIK1 of the pattern-recognition receptor complex, triggering an increase in the concentration of Ca\(^{2+}\) cyt (Tian et al., 2019). CNGC2 and CNGC4, which are also known as dnd1 (defense no death1) and dnd2, respectively, are involved in the hypersensitivity response and DAMP perception during bacterial infection in Arabidopsis (Ahn, 2007). cngc11 and cngc12 are also hypersusceptible to fungal infection (Yoshioka et al., 2001). In apple, overexpression of MdCNGC1 results in increased susceptibility to fungal infection and in reduced callose deposition when plants are treated with flg22 and chitosan (Zhang et al., 2018). Interestingly, in our study CNGC2, CNGC4, CNGC11, and CNGC12 were not significantly induced by P. indica (Fig. 1A), indicating that a different set of channels is involved in the interaction. The brush mutant has been isolated in a screen of an ethyl-methanesulphonate-mutated population...
for plants defective in symbiotic cell development (Maekawa-Yoshikawa et al., 2009). At 26 °C, brush roots are stunted and infection threads in root hairs do not progress into the cortex, resulting in the formation of non-infected nodules. This has been mapped as a gain-of-function CNC1.IVA mutation and it is orthologous to AtCNGC19 and AtCNGC20, resulting in a leaky tetrameric channel (Chiasson et al., 2017). Similarly, nuclear-localized CNGC15 in Medicago forms a complex with the potassium-permeable channel DMI1, and is responsible for nuclear Ca\textsuperscript{2+} release upon mycorrhizal symbiosis (Charpentier et al., 2016). Our phylogenetic analysis also indicated that CNGCs of diverse plants that clustered in Groups IV and III are involved in both symbiotic and pathogenic interactions (Fig. 9). Thus, activation of Ca\textsuperscript{2+} channels belonging to the CNGC family seems to be a conserved element in symbiotic interactions.

Increased *P. indica* colonization in the *cngc19* mutants (Fig. 2) indicated that the normal functioning of CNC19 is crucial for maintaining controlled colonization, and suggests that it has a role in MTI. Plants deposit callose (β1,3-glucan) into cell walls upon microbial invasion as a part of MTI (Thordal-Christensen, 2003; Nürnberger and Lipka, 2005). Colonization by *P. indica* is known to induce callose deposition and additional exposure to the elicitor flg22 does not increase callose because of *P. indica*-mediated suppression of late MTI (Jacobs et al., 2011). Thus, the suppression of callose deposition is required for progression of *P. indica* colonization. We found that callose deposition was initially unaltered in *cngc19* (Fig. 4A, C), indicating that the plant defense was lowered and hence led to increased colonization and pathogen-like growth. Plants activate robust MAMP perception and subsequent MTI by the action of phytohormones such as salicylic acid, ethylene, and jasmonate to regulate *P. indica* colonization. It is known that colonization increases JA/JA-Ile levels in co-cultivated plants with the result that plant defense responses are altered and tolerances to pathogenic microbes and root herbivory are improved (Vahabi et al., 2013, 2015; Lahrmann et al., 2015; Cosme et al., 2016). It has also been reported that JA signaling is required to suppress late MTI in order to facilitate the progression of *P. indica* colonization at the late biotrophic stage (Jacobs et al., 2011). It is known that the jasmonate insensitive 1-1 (*jins1-1*) and jasmonate resistant 1-1 (*jar1-1*) mutants show no promotion of growth upon *P. indica* colonization (Jacobs et al., 2011). Loss-of-function of CNC19 down-regulated jasmonate biosynthesis and JA-responsive genes upon *P. indica* colonization (Fig. 6 and 7A). This suggests that CNC19-mediated signaling leads to the activation of JA/JA- like signaling (Fig. 10), as has also been observed for defense against *Spodoptera* herbivory in Arabidopsis leaves (Meena et al., 2019). In our study, the *cngc19* mutants also displayed reduced expression of early and late MTI-related genes involved in phytohormone, ROS, and secondary metabolite pathways (Fig. 7A–C), which contributed to unbalancing the mutualistic relationship between the fungus and host plant (Fig. 10). We also found that H\textsubscript{2}O\textsubscript{2}-induced Oxidative Signal Inducible 1 (OXI1) kinase was reduced in *cngc19* plants at an early stage of colonization (Fig. 7B). The *oxi1* mutant together with the *age2-2* (OXI1 kinase homolog) and *pdk1.1* *pdk1.2* (3-PHOSPHOINOSITIDE-DEPENDENT PROTEIN KINASE1) mutants also have reduced growth upon *P. indica* colonization (Camreh et al., 2011). In addition, we also found that genes related to the antioxidant system were altered at early and late stages of *P. indica* colonization, namely SOD1, GSTF8, GR1, CAT2, and APX1 (Supplementary Fig. S8). Thus, CNC19 is critical for MTI responses in Arabidopsis roots (Fig. 10).

Damage-associated molecular patterns (DAMPs) are recognized by leucine-rich repeat (LRR)-like receptors, which activate downstream signaling. AtPep1 is perceived as a stronger danger signal by Arabidopsis roots than the MAMP-like bacterial *flg22* or chitin, and hence AtPep–PEPR signaling is a major component of surveillance in the roots (Poncini et al., 2017). We have previously identified that CNC19 is involved in AtPep1-induced elevation of Ca\textsuperscript{2+}cyc (Meena et al., 2019). Pep-induced PEPR signaling further intensifies the plant defense response together with MTI (Ross et al., 2014). Upon addition of AtPep1, *P. indica*-mediated suppression of callose occurred in the WT but not in the *cngc19* mutant (Fig. 4). *pepr1* *pepr2* double-mutants also showed a growth inhibition phenotype (Fig. 5C), but these genes are not involved in the PiCWE-induced elevation of Ca\textsuperscript{2+}cyc and instead might be acting via the JA pathway (Fig. 5E, F). PEPR signaling maintains basal immunity by regulating JA and SA signaling, locally and systemically (Ross et al., 2014; Yamada et al., 2016), which agrees with the down-regulation of the JA-responsive gene *VSP2* that we observed upon *P. indica* colonization in the *pepr1* *pepr2* mutant (Fig. 5). Thus, PEPR signaling works downstream of CNC19, contributes to the JA pathway, and may interact with unknown receptors and kinases for modulating downstream targets.

It is known that *cngc19* mutants are constitutively deficient in aliphatic glucosinolate accumulation and that they hyperaccumulate its precursor, methionine (Meena et al., 2019). CNC19 modulates aliphatic glucosinolate biosynthesis in tandem with BRANCHED-CHAIN AMINO ACID TRANSAMINASE4 (BCAT4), which is involved in the chain elongation pathway of metionine-derived glucosinolates (Meena et al., 2019). However, this phenotype appeared to be age-dependent as it was absent in the seedlings that we studied, and upon *P. indica* colonization we found that it was the activation of iGS that was important (Fig. 8A). Thus, regulation of glucosinolates by CNC19 is age- and stimuli-dependent. The indolic glucosinolate pathway plays a major role in the growth restriction of *P. indica* (Lahrmann et al., 2015). The loss-of-function mutants *cyp79b2*/*3 and *cyp81f2*, which are genes involved in iGS biosynthesis, exhibit growth inhibition upon *P. indica* colonization similar to what we observed in *cngc19* (Nongbri et al., 2012; Lahrmann et al., 2015). Cytochrome P450 enzymes (*CYP79B2, CYP79B3, CYP81F2*) and transcription regulators of iGS (*MYB51, MYB122, WRKY33*) and other iGS-related genes (*IGMT1, IGMT2, PAD3, PEN2*) have previously been observed to be stimulated during the interaction with *P. indica* (Jacobs et al., 2011; Nongbri et al., 2012; Lahrmann et al., 2015; Peskan-Berghöfer et al., 2015). It has also been shown that genes related to the iGS biosynthesis pathway are essential for callose deposition (Clay et al., 2009). In our study, such genes were found to be down-regulated upon *P. indica* colonization in *cngc19* (Fig. 8), and the levels of
iGS were increased at 14 dpi and reduced at 42 dpi in colonized WT plants, but were unaltered in \textit{cngc19}. The activation of iGS biosynthesis might occur via activation of CNGC19 in the colonized plants. Some other iGS-related mutants (\textit{myb34/51/122, pen2-1}) have also been shown to have higher levels of \textit{P. indica} colonization (Jacobs \textit{et al.}, 2011; Nongbri \textit{et al.}, 2012; Lahrmann \textit{et al.}, 2015). In addition, the mutant of a \(\beta\)-glucosidase (\textit{ΔPYK10}), which is involved in hydrolysing iGS, has also been observed to have no growth promotion upon \textit{P. indica} colonization (Sherameti \textit{et al.}, 2008; Nakano \textit{et al.}, 2017). All these findings place CNGC19 as an upstream element in iGS activation upon \textit{P. indica} colonization.

In conclusion, our results indicate that CNGC19 is activated by as yet unidentified elicitors in the cell-wall extract of \textit{P. indica}. The CNGC19-mediated pathway affects the basal immunity and the levels of phytohormones and glucosinolates in Arabidopsis upon colonization by \textit{P. indica}, and subsequently affects the growth of the plants. These events do not occur in the \textit{cngc19} mutants, and this leads to over-colonization and detrimental effects on plant health and growth (Fig. 10). Thus, CNGC19 plays a central role as a gatekeeper during colonization by \textit{P. indica}, maintaining a robust innate immunity that ensures that the interaction is mutualistic.

**Supplementary data**

Supplementary data are available at \textit{JXB} online.

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Authors contributions
JV designed the experiments; AJ performed the experiments; MKM performed the luminescence experiments and generated the transgenic lines; AK performed the HPLC and LC-MS experiments; MV performed the phloem-genetic tree analysis; AJ and JV analysed the results and wrote the manuscript.

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