Characterization of tomato protein kinases embedding guanylate cyclase catalytic center motif

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Guanylate cyclases (GCs) are enzymes that catalyze the reaction to produce cyclic GMP (cGMP), a key signaling molecule in eukaryotes. Nevertheless, systemic identification and functional analysis of GCs in crop plant species have not yet been conducted. In this study, we systematically identified GC genes in the economically important crop tomato (Solanum lycopersicum L.) and analyzed function of two putative tomato GC genes in disease resistance. Ninety-nine candidate GCs containing GC catalytic center (GC-CC) motif were identified in tomato genome. Intriguingly, all of them were putative protein kinases embedding a GC-CC motif within the protein kinase domain, which was thus tentatively named as GC-kinases here. Two homologs of Arabidopsis PEPRs, SlGC17 and SlGC18 exhibited in vitro GC activity. Co-silencing of SlGC17 and SlGC18 genes significantly reduced resistance to tobacco rattle virus, fungus Sclerotinia sclerotiorum, and bacterium Pseudomonas syringae pv. tomato (Pst) DC3000. Moreover, co-silencing of these two genes attenuated PAMP and DAMP-triggered immunity as shown by obvious decrease of flg22, chitin and AtPep1-elicited Ca2+ and H2O2 burst in SlGC-silenced plants. Additionally, silencing of these genes altered the expression of a set of Ca2+ signaling genes. Furthermore, co-silencing of these GC-kinase genes exhibited stronger effects on all above regulations in comparison with individual silencing. Collectively, our results suggest that GC-kinases might widely exist in tomato and the two SlPEPR-GC genes redundantly play a positive role in resistance to diverse pathogens and PAMP/DAMP-triggered immunity in tomato. Our results provide insights into composition and functions of GC-kinases in tomato.

Guanosine 3′,5′-cyclic monophosphate (cGMP) is a well known intracellular second messenger that regulates a broad range of physiological responses in animals, fungi and prokaryotes. However, the existence and function of cGMP in higher plants has been controversial for many years1. The level of cGMP in higher plants is much lower than that in animals2. Nevertheless, with the improvement of cGMP determination methods, it has been generally recognized that cGMP is present in plants3–5. Furthermore, searching analyses with a motif deduced from representatives of annotated catalytic domains of GCs from prokaryotes and eukaryotes ([RKS] [YFW] [CTGH] [VIL] [FV] G [DNA] x [VIL] x{4} [KR]) demonstrated that sequences carry the motif of functional amino acid (AA) residues of the GC catalytic center (GC-CC) do exist in plants6–8. In this motif, the AA at position 1 forms the hydrogen bond with the guanine, the residue at position 3 confers substrate specificity for GTP, the residue at position 14 stabilizes the transition state from GTP to

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cGMP, while two or three AAs away from the C-terminal end of the motif is the residue that interacts with the Mg$^{2+}$/Mn$^{2+}$ ions$^{35,36}$. Searches using this motif led to identification of several Arabidopsis thaliana proteins that exhibit GC activity in vitro$^{35,37}$. These include; A. thaliana guanylate cyclase 1 (AtGC1), brassinosteroid receptor (AtBRI1), wall associated kinase-like 10 (AtWAKL10), Pep receptor 1 (AtPEPR1), phospholipase (PSK) receptor (AtPSKR1), nitric oxide (NO) dependent guanylate cyclase 1 (NOGCG), and plant natriuretic peptide receptor (AtPNP-R1)$^{22,38-43}$. The conserved 14-AA GC-CC motif of these functionally confirmed AtGCs is [KS] [YF] [GCS] [VIL] [VILFG] [DVIL] [VILADG] [EPVIL] [DVIL] [TVIL] [WST] [PDRG] [KEG] [KR] [x{2,3}] [DHSE]$^{35}$ as query. All retrieved non-redundant sequences were collected and subjected to GC-CC motif prediction analysis using GCpred tool$^{50}$ and domain analysis using the Pfam (http://pfam.sanger.ac.uk/) and Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/) programs. The predicted GC-CC motif or full length amino acid sequences were aligned using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default settings and were viewed by GeneDoc program.

Methods
Identification of GC proteins in tomato genome. BLASTp search was performed against tomato (Solanum lycopersicum L.) genome in Phytozome (http://www.Phytozome.net) and NCBI (http://www.ncbi.nlm.nih.gov/) using known Arabidopsis GC proteins as queries. Meanwhile, pattern-hit initiated BLAST (phiBLAST) search was conducted in NCBI database using the plant GC-specific GC-CC motif [KS] [YF] [GCS] [VIL] [VILFG] [DVIL] [VILADG] [EPVIL] [DVIL] [TVIL] [WST] [PDRG] [KEG] [KR] x{2,3} [DHSE]$^{35}$ as query. All retrieved non-redundant sequences were collected and subjected to GC-CC motif prediction analysis using GCpred tool$^{50}$ and domain analysis using the Pfam (http://pfam.sanger.ac.uk/) and Conserved Domain Database (CDD) (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi/) programs. The predicted GC-CC motif or full length amino acid sequences were aligned using ClustalW2 program (http://www.ebi.ac.uk/Tools/msa/clustalw2/) with default settings and were viewed by GeneDoc program.

Phylogenetic analysis of tomato and Arabidopsis candidate GCs. Multiple sequence alignments of the full-length candidate GC protein sequences from tomato and Arabidopsis were conducted using clustalW2 program$^{35}$. The phylogenetic tree was constructed using MEGA 5.0$^{52}$ with maximum likelihood (ML) method and a bootstrap test was performed with 1,000 replicates.

Virus-induced gene silencing (VIGS) analyses. To ensure the specificity to target an individual gene member, gene primers for the VIGS target fragments of SIGC17 and SIGC18 were designed from the gene specific regions. The target fragments of SIGC genes were amplified by PCR using gene specific primers (Supplementary Table S1) with BamHI (ggattc) and EcoRI (gaattc) restriction sites for forward and reverse primers, respectively. These fragments were cloned and ligated into the TRV-based VIGS vector pYL156, and were subsequently electroporated into Agrobacterium tumefaciens strain GV3101 for VIGS analyses. Agro-inoculation were conducted as described$^{24,56}$, respectively, while recombinant pYL156 with Agrobacterium tumefaciens strain GV3101 for VIGS analyses. Agro-inoculation were conducted as described$^{53,54}$ except that recombinant pYL156 with Agrobacterium tumefaciens strain GV3101 for VIGS analyses. Agro-inoculation were conducted as described$^{53,54}$ except that recombinant pYL156 with AtPEPR1 ($^{35}$) were added and immediately H$_2$O$_2$ were measured for 35 min as luminescence using a Microplate Luminometer (TITERTEK BERTHOLD, Germany).

Plant inoculation and disease resistance analysis. At three weeks post agro-infiltration, the tomato plants were inoculated with a variety of pathogens including Sclerotinia sclerotiorum, Pseudomonas syringae pv. tomato (Pst) DC3000 and Xanthomonas oryzae pv. oryzae (Xoo). The inoculation and disease resistance evaluation including Sclerotinia sclerotiorum caused lesion size measurement and bacterial number counting in Pst DC3000- and Xoo-inoculated leaf areas were conducted as described$^{24,56}$.

ROS detection. For in situ H$_2$O$_2$ detection, Pst DC3000 and Xoo inoculated leaves of tomato plants were detached and stained with 3,3-diamino benzidine hydrochloride (DAB) (1 mg/mL) as previously described$^{57}$. For quantitative analyses, tomato leaf disks of 3 mm at diameter were dipped in 200 μL of distilled water in a 96-well plate in dark overnight. Water was replaced with 200 μL solution containing 100 μL luminol (Sigma-Aldrich) and 1 μg of horseradish peroxidase. The H$_2$O$_2$ elicitors; bacterial PAMP Hlg22 (100 nM), fungal PAMP chitin (100 μg mL$^{-1}$), DAMP AtPep1 (10 nM) and Pst DC3000 bacterial cells (OD$_{600}$ = 0.1) were added and immediately H$_2$O$_2$ were measured for 35 min as luminescence using a Microplate Luminometer (TITERTEK BERTHOLD, Germany).
Calcium detection. Transient increase of cytosolic Ca\(^{2+}\) concentration was monitored in the Aequorin gene transgenic tomato line\(^{28}\). Leaf discs dispatched on a 96-well plate were incubated overnight in 12.5 mM coenzyme (LUX Innovate). Before measurement, the solution was removed and 100 mL of assay solution containing a PAMP or DAMP or Pst DC3000 bacterial cells as described above was added to the wells. Luminescence was measured using a Microplate Luminometer (TITERTEK BERTHOLD, Germany).

Gene expression analyses by real time PCR. Quantitative real time PCR (qRT-PCR) analyses and consequent statistical analyses were conducted as described\(^{59}\). A tomato rRNA gene was used as internal control\(^{56}\). The gene-specific primers used in qRT-PCR analyses were listed in Supplementary Table S1.

Detection of in vitro catalytic activity of SIGC17\(^{724-1105}\) and SIGC18\(^{788-1104}\) to generate cGMP. The cDNA sequences corresponding to the intracellular domain of SIGC17 and SIGC18 proteins (SIGC17\(^{724-1105}\) and SIGC18\(^{788-1104}\)) were amplified from tomato cv. Moneymaker through RT-PCR using gene specific primers (SIGC17\(^{724-1105}\) F: 5'-GGCAAAATACGGAACCCGA-3'; SIGC17\(^{724-1105}\) R: 5'-CTGGTTGGCTGTGCTATAGCTAAC-3'; SIGC18\(^{788-1104}\) F: 5'-CGCAAAATGTTCTGGGAAAGG-3'; SIGC18\(^{788-1104}\) R: 5'-GTACTTTGCTGATATCTGAATTTG-3'), which were then cloned into prokaryotic expression vector pET32a and were expressed in *Escherichia coli* BL21 (DE3) pLysS (Trans-Gen Biotech, Beijing, China). His-tagged SIGC17\(^{724-1105}\) and SIGC18\(^{788-1104}\) recombinant proteins were affinity purified using His antibody following manufacturer's instructions (TaKaRa, Japan).

The in vitro GC activity of purified SIGC17\(^{724-1105}\) and SIGC18\(^{788-1104}\) was manifested as the produced cGMP content measured using the Amersham Biosciences cGMP enzyme immunoassay Biotrak system (GE Healthcare, USA, code RPN226). Ten μg of each protein was incubated in 100 μl of 50 mM Tris-HCl (pH 8.0) containing 2 mM isobutylmethylxanthine (IBMX), 5 mM MgCl\(_2\) and 1 mm GTP. Background cGMP levels in the reaction mixture were determined using SSR/AF-Pkinase/Pkinase_Tyr proteins, while 26 and 17 solely possessed a Pkinase or Pkinase_Tyr domain, 18 were MB_Lectin-Pkinase_Tyr/Pkinase proteins with additionally one of or both SGP and PANL domains, 5 were MB_Lectin-Pkinase_Tyr proteins, 6 were CB_EGF-Pkinase proteins with one carrying an extra WAK domain in the N-terminus, 23 were MB_Lectin-Pkinase proteins, while 26 and 17 solely possessed a Pkinase or Pkinase_Tyr domain, respectively. The reaction was terminated by addition of 10 mM EDTA. Tubes were then boiled for 3 min, cooled on ice for 2 min and centrifuged at 2300 × g for 3 min. The supernatant was collected for cGMP content measurement based on highly specific anti-cGMP antibody using the system mentioned above following the Protocol 2 as described in the supplier's manual.

In order to verify the enzyme immunoassay result, the produced cGMP in the reactions was further identified by mass spectrometry (MS) assay. MALDI-TOF-MS analyses were performed using a Bruker UltraFlexTreme mass spectrometer equipped with a laser (355 nm, 2000 Hz) (Bruker Daltonics, Bremen, Germany). 3-Hydroxy picolinic acid (3-HPA) was used as matrix. One μl matrix was spotted onto the Ground steel and dried in air. One μl reaction mixture or cGMP standard (Sigma-Aldrich, USA, G7504) was then spotted onto the dried matrix and dried in air, which was subjected to MS analyses. Reflector positive mode was used for detection. Spectra data were collected by FlexControl software and processed using the FlexAnalysis software.

Statistical analysis. For VIGS and resistance evaluation analyses, at least 10 plants per pathogen for each gene were used in each experiment. For *H*₂*O*₂ and Ca\(^{2+}\) detection as well as gene expression analyses, 10 leaves from 10 plants for each treatment were collected in each experiment. All experiments were conducted three times independently. The data from quantitative analyses were statistically analyzed using SPSS software (Version 19.0, IBM, USA) and represent means ± standard error (SE) of three independent experiments. Significant difference is analyzed by Duncan’s multiple range test (DMRT, *p* < 0.05).

Results

Identification of candidate GCs in tomato genome. To identify GC candidates in tomato, two ways of BLAST searches were performed. First, BLASTp search was conducted against tomato genome in Phytozone and NCBI using known Arabidopsis GC proteins as queries. Meanwhile, pattern-hit initiated BLAST (phi-BLAST) was carried out against tomato genome in NCBI using the plant GC-specific GC catalytic center (GC-CC) motif ([KS] [YF] [GCS] [VIL] [VILFG] [DVIL] [VILAGD] [EPVIL] [DVIL] [TVIL] [WST] [PDGR] [KEG] [KR] x(2,3) [DHSE]\(^{50}\)) as query. Resultantly, 99 non-redundant sequences were retrieved. These sequences were then further subjected to prediction for GC-CC motif including the amino acid implicated in co-factor (Mg\(^{2+}\)/Mn\(^{2+}\)) binding using GCPred tool\(^{60}\). All sequences except sequence #41 were predicted to contain one to several potential GC-CC motifs (Supplementary Table S2). When the sequence #41 was aligned with AtGCs, a GC-CC motif was also predicted (Supplementary Table S3). Alignment of the 98 GC-CC motifs predicted using GCPred with the highest confidence together with the one for sequence #41 predicted from alignment analysis revealed that all these motifs contained [SK]1, [GSC]3 and [KR]14, the three AAs associated with catalysis function in the plant GC-specific GC-CC motif, and thus the 99 sequences were recognized as tomato GC candidates (Fig. 1). The 99 tomato GC candidates were listed in Supplementary Table S3.

The GC-CC motif was embedded within the kinase domain of the identified tomato GC candidates. Domain composition analyses showed that all the 99 tomato GC candidates contained a protein kinase domain, either Ser/Thr-type or Tyr-type (Fig. 2), demonstrating that they were likely protein kinases. The putative GC protein sequences represented 13 types of domain combinations of 10 different domains. The domains included LRR-NT, LRR, WAK, Ca\(^{2+}\)-binding EGF (CB_EGF), PAN-like (PANL), D-mannose binding lectin (MB_Lectin), S-locus glycoprotein (SGP), salt stress response/antifungal (SSR/AF), Pkinase and Pkinase-Tyrosine (PKinase_Tyr) (Fig. 2). Out of the 99 putative tomato GCs, 27 were LRR-Pkinase/Pkinase_Tyr proteins, 27 were CB_EGF-Pkinase proteins with one carrying an extra WAK domain in the N-terminus, 18 were MB_Lectin-Pkinase_Tyr/Pkinase proteins with additionally one of or both SGP and PANL domains, 5 were SSR/AF-Pkinase/Pkinase_Tyr proteins, while 26 and 17 solely possessed a Pkinase or Pkinase_Tyr domain.
respectively (Supplementary Table S3). These results indicate that GC-CC tends to combine with other domains especially protein kinase domains to form multifunctional proteins.

Intriguingly, the GC-CC motif was embedded within the kinase domain for all 99 sequences (Fig. 2), indicating that kinase domain is the preferable sequence to hide the GC-CC motif and that the GC and kinase dual functional proteins are widely present in the tomato genome. These GC-CC-motif embedded kinases were therefore abbreviated as GC-kinases hereafter in this paper.

**Figure 1.** Alignment of the GC catalytic center (GC-CC) regions of the 99 candidate GCs identified in tomato. The GC-CC motif conserved in tomato GCs are deduced from the alignment and shown at top. The most conserved amino acid residues required for GC catalysis function are highlighted in different colors and their positions are indicated at bottom of the alignment.
The GC-CC motif region of the 99 putative tomato GC-kinases was aligned. This alignment revealed a tomato GC-CC motif as ([SK] [FYA] [GS] [VN] [VI] [LVFMID] [LAMV] [EDM] [LTAE] [LVEDA] [TSMLIC] [GNSRDEC] [RKPQLM] [RK] x{0,2} [DSEG]), where the amino acids in a certain position were listed in the order of frequency from high to low and existed in at least two SlGCs) (Fig. 1). Comparison of this motif with the one for plant as described above demonstrated that the tomato GC-CC motif was as conserved as the plant GC-CC motif for the functionally associated AAs at positions 1, 3 and 14, while was more relaxed at some other positions. It was notable that A2, N4, D8, T9, [ED]10, M11, [GN]12, [RPQL]13 appeared frequently in predicted tomato GCs but not included in plant GC-CC motif. This result suggests that the plant GC-CC motif needs to be relaxed for these positions when used for plant GC prediction through phi-BLAST search.

Phylogenetic relationship between tomato and Arabidopsis GCs. To examine the phylogenetic relationships and functional associations of candidate tomato GCs, a total of 140 plant GC protein sequences including 99 tomato and 41 Arabidopsis GC candidates were used to construct a phylogenetic tree based on maximum likelihood (ML) method (Fig. 3). The phylogenetic analysis indicated that 99 tomato GCs clustered into seven groups (group I-VII). Six tomato GCs (SIGC1-SIGC6) and six Arabidopsis GCs gathered in group I, which included experimentally confirmed GCs AtNOGC1 and AtBRI139,42. Nineteen tomato GCs (SIGC7-SIGC25) and 9 Arabidopsis GCs formed group II, which included AtPEPR1 and AtPSKR1, two well studied GCs22,41. Five tomato GCs (SIGC26-SIGC30) and 5 Arabidopsis GCs clustered into group III. Sixteen tomato GCs (SIGC31-SIGC46) and 9 Arabidopsis GCs comprised group IV, which included functional GCs AtWAKL10 and AtGC138,39 and seven other AtWAKL kinases. In addition, group V was comprised of 22 tomato GCs (SIGC47-SIGC68) and 2 Arabidopsis GCs (AtBIK1 and AtARSK1), group VI constituted of 9 tomato GCs (SIGC69-SIGC77) and one Arabidopsis GC (kinase-L2) while group VII consisted of 22 tomato GCs (SIGC78-SIGC99) and 9 Arabidopsis GCs among which were 7 AtCRK proteins (Fig. 3).

Interestingly, SIGC6 and AtBRI1, SIGC17/SIGC18 and AtPEPRs, as well as SIGC24/SIGC25 and AtPSKR1 clustered in the same clade of group I, II and III, respectively, in the phylogenetic tree (Fig. 3). Full-length amino acid sequence alignment further showed that they shared with high sequence homology (Supplementary Fig. S1). These results indicate that these putative tomato GCs may be orthologs of the corresponding Arabidopsis GCs.

Silencing of putative tomato PEPR-GC genes SIGC17 and SIGC18 altered resistance to diverse pathogens. The phylogenetic and sequence alignment analyses indicated that SIGC6 and SIGC17/SIGC18 are the homologs of the functionally confirmed GCs AtBRI1 and AtPEPR1, respectively (Fig. 3, Supplementary Fig. S1). Moreover, AtPEPR1 plays an important role in plant disease resistance as a receptor of the Pep elicitors22,23. These findings prompted us to investigate whether these putative SIPEPR-GCs function in calcium regulated plant disease resistance through virus-induced gene silencing (VIGS) analyses.

Gene specific target sequences of SIGC6, SIGC17 and SIGC18 were amplified and inserted into the tobacco rattle virus (TRV)-based VIGS vector pYL156 for silencing analyses, while non-silenced eGFP fragment-inserted recombinant pYL156 vector was used as a negative control35. To examine the functional redundancy of SIGC17 and SIGC18, co-silencing for both genes was analyzed in addition to individual gene silencing. At three weeks after agro-infiltration, the tomato plants for co-silencing of SIGC17 and SIGC18 exhibited clear mosaic symptoms.
in leaves; those for SlGC18 silencing displayed mild mosaic symptoms, while those for SlGC6 and SlGC17 individual silencing grew normally, as observed for control eGFP plants (Fig. 4A). Gene expression analysis revealed that transcripts of TRV1 replicase gene accumulated over 3 and 7 folds higher, while those of TRV2 2b gene were near 3 and 5 folds higher in SlGC18-silenced and SlGC17/SlGC18-co-silenced plants, respectively, in comparison to eGFP control plants (Fig. 4B).

To clarify whether the SlGC genes had been efficiently silenced in the agro-infiltrated tomato plants, transcripts of these genes in the agro-infiltrated plants were quantified with qRT-PCR. Result showed that transcripts of SlGC6, SlGC17 and SlGC18 in the agro-infiltrated plants all dropped to lower than 30% of those in the eGFP control plants (Fig. 4C). This result demonstrated that the SlGC genes had been efficiently silenced in these plants. Taken together, silencing of SlGC18 and co-silencing of SlGC17/SlGC18 resulted in the TRV viral symptoms and higher level of virus accumulation. Thus, SlGC17 and SlGC18 redundantly and positively affect tomato resistance to TRV.

To further probe function of these three tomato GC genes in disease resistance, the silenced tomato plants were inoculated with three different pathogens including bacterial pathogen Pseudomonas syringae pv. tomato (Pst) DC3000, nonhost bacterial pathogen Xanthomonas oryzae pv. oryzae (Xoo) and a fungal pathogen Sclerotiania sclerotiorum. Resistance to all three pathogens in the SlGC6-silenced tomato plants appeared to be similar to that in the eGFP-control plants, as both plants developed similar severity of hypersensitive response (HR) or necrosis symptoms and accumulated similar levels of pathogens (Fig. 5). Nonhost resistance to Xoo in the SlGC17 and SlGC18 individually silenced and co-silenced plants was also similar to that in the eGFP control plants, judged by both HR symptoms and bacterial number counting results (Fig. 5C). These results indicated that the tomato BRI1-GC gene SlGC6 might be not important in tomato resistance to the examined three pathogens and the putative tomato PEPR1-GC genes SlGC17 and SlGC18 might have no significant role in tomato nonhost resistance to Xoo.

Figure 3. Phylogenetic tree of tomato and Arabidopsis candidate GCs. The phylogenetic tree was constructed using MEGA 5.0 with maximum likelihood (ML) method and a bootstrap test was performed with 1,000 replicates. Tomato candidate GCs are marked with a solid red circle while Arabidopsis candidate GCs and functionally confirmed GCs are indicated with a green and a blue square box, respectively.
However, when inoculated with S. sclerotiorum, necrotic symptoms of the leaves of both SIGC17 and SIGC18 individually silenced and co-silenced plants were significantly more severe than that of the eGFP control plants (Fig. 5A). The lesions in the SIGC-silenced plants, 1.2 ~ 1.4 cm at diameter, were significantly larger in size than those in control plants (1.1 cm at diameter) at 24 hpi (Fig. 5A). Interestingly, the lesion size was larger in co-silenced plants (1.35 cm at diameter) than that in individually silenced plants (<1.25 cm at diameter), indicating the redundancy of SIGC17 and SIGC18 in positively affecting tomato resistance to S. sclerotiorum.

Results of inoculation with Pst DC3000 showed that the necrotic disease symptoms of the leaves of SIGC-silenced plants were less severe than those in the eGFP control plants (Fig. 5B). Furthermore, Pst DC3000 bacterial growth in SIGC17 and SIGC18 co-silenced plants was significantly increased by approximately one order of magnitude, while that in SIGC17 and SIGC18 individually silenced plants was increased by about 0.6 order of magnitude, compared with that in the eGFP control plants at 3 d post inoculation (Fig. 5B). These data revealed that the SIGC17 and SIGC18 redundantly and positively regulate resistance to Pst DC3000 in tomato plants.

To examine whether H2O2 production was associated with SIGC17 and SIGC18-mediated resistance to Pst DC3000, leaves of SIGC-silenced and eGFP control plants were stained in situ with 3,3-diamino benzidine hydrochloride (DAB) at 3 d post Pst DC3000 inoculation. The result showed that the leaves of SIGC-silenced plants were stained weaker than those of the eGFP control plants (Fig. 5B), manifesting that the former accumulated less H2O2 than the latter. This result indicated that oxidative burst appears to be associated with SIGC17 and SIGC18-mediated resistance to Pst DC3000.

Collectively, the putative PEPR-GC genes SIGC17 and SIGC18 redundantly and positively regulate resistance to S. sclerotiorum and Pst DC3000 in tomato plants. Furthermore, oxidative burst seems to participate in SIGC17 and SIGC18-mediated resistance.

**Silencing of SIGC17 and SIGC18 in tomato reduced PAMP-triggered Ca2+ and ROS burst.**

To further explore functions of SIGC17 and SIGC18 in pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and the mechanisms underlying SIGC17 and SIGC18-mediated resistance, effect of SIGC17 and SIGC18 gene silencing on the accumulation of Ca2+ and H2O2 elicited by fungal PAMP flg22 and bacterial PAMP DC3000 bacterial cells was monitored. Ca2+ burst was detected by an aequorin-based luminescence approach in aequorin-expressed transgenic (Aeq-OE) tomato plants. Both Ca2+ and H2O2 signals were measured and indicated as relative luminescence (RLU). In response to PAMP chitin, in eGFP control Aeq-OE plants, Ca2+ increased rapidly and peaked to 828 RLU at 12 min post chitin application, whereas the SIGC17 and SIGC18 individually or both silenced Aeq-OE plants, respectively (Fig. 6A). In the SIGC17- and SIGC18-silenced Aeq-OE plants, Ca2+ accumulation but exhibited significantly lower peak value with obvious difference (Fig. 6A). In the SIGC17- and SIGC18-silenced Aeq-OE plants, Ca2+ culminated to 392 and 322 RLU, respectively, while in the co-silenced Aeq-OE plants, the Ca2+ peak value was significantly reduced to 209 RLU, which was only one fourth of that in the control plants. These differences in Ca2+ burst was also observed for these plants in response to PAMP flg22, where Ca2+ peaked to 948 RLU in the eGFP control Aeq-OE plant, but culminated only to 289 RLU in SIGC17/SIGC18 co-silenced Aeq-OE plants, and 553 and 414 RLU in SIGC17- and SIGC18-silenced Aeq-OE plants, respectively (Fig. 6B). In addition to the PAMPs, the response of these plants to Pst DC3000 bacterial cells was also monitored. In this case, Ca2+ peaked to 297 RLU in control Aeq-OE plants, 195 RLU in the SIGC17-silenced plants, 154 RLU in the SIGC18-silenced plants, and 127 RLU in the SIGC17 and SIGC18 co-silenced plants (Fig. 6C). These data...
demonstrated that although the response was weaker, the difference in Ca\(^{2+}\) burst elicited by *Pst* DC3000 bacterial cells was still clear between the SlGC-silenced plants and the eGFP control plants. Collectively, these results demonstrated the positive role of *SlGC17* and *SlGC18* in pathogen *Pst* DC3000-elicited and PAMP-triggered Ca\(^{2+}\) burst and the redundancy of these two genes in this role.

As shown in Fig. 7, the dynamics of H\(_2\)O\(_2\) accumulation were similar to what were observed for Ca\(^{2+}\) measurement. Compared to the eGFP control plants, H\(_2\)O\(_2\) accumulation was reduced most significantly in the *SlGC17* and *SlGC18* co-silenced plants, followed by in the *SlGC18*-silenced plants, and less significantly in the *SlGC17*-silenced plants, no matter in response to PAMPs chitin and flg22 or *Pst* DC3000 bacterial cells. The only difference was that the reduction of H\(_2\)O\(_2\) accumulation caused by *SlGC17* silencing was less obvious in comparison to that of Ca\(^{2+}\) burst especially in response to flg22 and *Pst* DC3000 bacterial cells (Figs. 6 and 7).

Together, these results revealed that *SlGC17* and *SlGC18* redundantly play a positive role in pathogen *Pst* DC3000-elicited and PAMP-triggered Ca\(^{2+}\) and H\(_2\)O\(_2\) burst.

Figure 5. Silencing of *SlGC17* and *SlGC18* in tomato reduced resistance to *Sclerotinia sclerotiorum* (A) and *Pst* DC3000 (B) but not to *Xoo* (C). (A) *S. sclerotiorum* mycelial plug inoculation caused necrosis symptoms in silenced plants and statistical analysis of lesion diameter. Photographs were taken at 24 hpi. (B) *Pst* DC3000 cell suspension infiltration-inoculation caused disease symptoms in silenced plants and bacterial number counting assay of the inoculated areas. Photographs were taken at 3 dpi. (C) *Xoo* cell suspension infiltration-inoculation caused HR in silenced plants and bacterial number counting assay of the inoculated areas. Photographs were taken at 24 hpi. H\(_2\)O\(_2\) in bacteria-inoculated leaves was detected by DAB staining (B,C). All above data represent mean ± SE of three independent experiments. Significant difference between the values for the silenced plants and those for the eGFP-control plants is indicated as small letters (p < 0.05, DMRT).

Silencing of *SlGC17* and *SlGC18* in tomato attenuated DAMP-triggered Ca\(^{2+}\) and ROS accumulation. AtPEPR1 recognizes AtPeps thereby activate plant defense responses including Ca\(^{2+}\) and H\(_2\)O\(_2\) burst\(^{22,23}\). Therefore, function of *SlGC17* and *SlGC18* in DAMP-triggered Ca\(^{2+}\) and H\(_2\)O\(_2\) burst in tomato was evaluated. In the eGFP control Aeq-OE tomato plants, Ca\(^{2+}\) increased rapidly and peaked to 1335 RLU at 10 min post AtPep1 application, while the *SlGC17* and *SlGC18* silenced Aeq-OE plants displayed similar dynamics of Ca\(^{2+}\) accumulation but showed lower peak value. The Ca\(^{2+}\) peak value dropped to 416 RLU in the *SlGC17* and
Silencing of $\text{SlGC17}$ and $\text{SlGC18}$ in tomato altered expression of defense-related \(\text{Ca}^{2+}\) signaling genes. To further elucidate the molecular mechanisms underlying $\text{SIGC}$-mediated disease resistance, we monitored expression of a series of defense-related \(\text{Ca}^{2+}\) signaling genes to clarify whether they are involved in $\text{SIGC}$-mediated resistance. The checked genes under this expression analysis included three CNGC genes $\text{SlCNGC16}$, $\text{SlCNGC17}$ and $\text{SlCNGC18}$, two calmodulin (CaM) genes $\text{SlCaM2}$ and $\text{SlCaM6}$, two calcium-dependent protein kinase (CDPK) genes $\text{SlCDPK2}$ and $\text{SlCDPK11}$, which are tomato homologs of $\text{AtCPK2}$ and $\text{AtCPK11}$, and one CaM-binding transcription activator (CAMTA) gene $\text{SlCAMTA3}$. All these genes play an important role in regulating disease resistance $^{24,39-44}$. The expression result showed that silencing of $\text{SlGC17}$ and $\text{SlGC18}$ individually or together unanimously reduced the expression of $\text{SlCNGC16}$, $\text{SlCNGC17}$, $\text{SlCDPK2}$, and $\text{SlCDPK11}$ but only co-silencing of $\text{SlGC17}/18$ lowered the expression of $\text{SlCaM2}$ and $\text{SlCaM6}$ by 40% and 60%, respectively. In addition, silencing of $\text{SlGC18}$ and co-silencing of $\text{SlGC17}/\text{SlGC18}$ increased the
expression of \( S\text{ICNGC}18 \) by 3.0 and 2.5 folds and that of \( S\text{ICAMTA3} \) by 2.2 and 3.5 folds, respectively (Fig. 9). Both \( S\text{ICNGC}18 \) and \( S\text{ICAMTA3} \) have been found to be negative regulators of plant resistance to pathogens including \( S. \text{Sclerotiorum} \)\textsuperscript{24,60,63,64}. These results indicated that CNGCs, CaMs, CDPKs, and CAMTAs may play a role in the \( S\text{ICGC}17 \) and \( S\text{ICGC}18 \)-mediated resistance.

**Figure 7.** Silencing of \( S\text{ICGC}17 \) and \( S\text{ICGC}18 \) in tomato reduced PAMP-elicited and \( P\text{st} \text{DC3000} \) bacterial cells-induced ROS burst. \( \text{H}_2\text{O}_2 \) induced by 100 nM flg22 (A), 100 \( \mu \text{g mL}^{-1} \) chitin (B) and \( P\text{st} \text{DC3000} \) bacterial cells (\( \text{OD}_{600} = 0.1 \)) (C) in leaf discs of the silenced plants was measured as luminol-based luminescence signal by using a Microplate Luminometer. Data represent mean ± SE of three independent experiments.

**Figure 8.** Silencing of \( S\text{ICGC}17 \) and \( S\text{ICGC}18 \) in tomato reduced DAMP-triggered \( \text{Ca}^{2+} \) and ROS burst. Detection of \( \text{Ca}^{2+} \) (A) and \( \text{H}_2\text{O}_2 \) (B) induced by 10 nM AtPep1 and the data processing followed what were described in Figs. 6 and 7.
Cytoplasmic domain of SlGC17 and SlGC18 proteins exhibited in vitro GC activity. To examine whether the GC-CC-containing SlGC17 and SlGC18 proteins indeed possess GC activity as predicted by bio-informatics analyses, the intracellular domain of SlGC17 and SlGC18 proteins (SlGC17724–1105 and SlGC18788–1104) was subjected to assays for activity to generate cGMP from GTP. The cDNA sequences corresponding to SlGC17724–1105 and SlGC18788–1104 were amplified from tomato cv. Moneymaker, and His-tagged SlGC17724–1105 and SlGC18788–1104 were expressed in *E. coli* and affinity purified using anti-His antibody, showing a single band in SDS-PAGE gel with an expected molecular mass of approximate 62 kDa and 55 kDa, respectively (Fig. 10A). These purified proteins were then examined for their GC activity.

Results of enzyme immunoassay using highly specific anti-cGMP antibody showed that both SlGC17724–1105 and SlGC18788–1104 exhibited activity to produce cGMP from GTP (Fig. 10B). The cGMP was generated rapidly within 5 min after incubation of SIGC protein and GTP, and reached the maximum value at 10 min after incubation. The peak value of cGMP concentration catalyzed by SlGC17724–1105 is approximately 70 fmol/µg protein, which was 1.8 times as high as that by SlGC18788–1104 (Fig. 10B), suggesting that SlGC17 owns higher catalysis activity than SlGC18.

To validate the results of enzyme immunoassay, cGMP produced from the reactions was identified by more sensitive MALDI-TOF-MS assay. Results of the MS assays again demonstrated that cGMP indeed generated in the incubation solution containing SlGC17724–1105 or SlGC18788–1104 and GTP (shown as a MS peak of about 347 m/z as for cGMP standard), but not in that without SlGC protein (CK-) (Fig. 10C), and thus confirmed the results of enzyme immunoassay.

Collectively, these results of in vitro assays revealed that cytoplasmic domain of SlGC17 and SlGC18 proteins exhibits GC activity.

Discussion

cGMP has been recognized as an important second messenger in plants. However, to how wide range GCs exist and how they function in plants remain poorly understood. As a matter of fact, the majority of studies regarding plant GCs were conducted only in the model plant species Arabidopsis. In this study, we identified GC candidates in tomato genome and explored functions of two PEPR-GC genes in tomato resistance, representing the first genome-wide GC identification and functional study of PEPR-GC candidate genes in a crop plant species.

GC family in tomato. Due to the lack of higher plant sequence with high similarity to known GCs in animals and fungi, no significant progress on identification of plant GCs has been made until motif BLAST search approach was employed. The motif is generated based on the conserved AA residues required for GC catalysis function, including those form the hydrogen bond with the guanine, confer substrate specificity for GTP, stabilize the transition state from GTP to cGMP, and interact with the Mg\(^{2+}\)/Mn\(^{2+}\) ions. Through using this motif BLAST search approach, seven *Arabidopsis thaliana* proteins that exhibit GC activity in vitro have been identified to date. In addition, further motif searching study predicted 41 potential GCs in *A. thaliana* genome. However, genome-wide identification of GCs in other higher plant species has not yet been conducted. In this study, employing phi-BLAST of the plant specific GC motif, we identified 99 candidate GCs in genome of the economically important crop plant tomato (Fig. 1, Supplementary Table S3), representing the largest number of GCs predicted in a plant species. Together with the prediction in Arabidopsis, our work revealed that a large GC family is present in a plant species and it is expected that GCs widely exist in higher plants.

Notably, all the 99 tomato GC candidates identified in this study are kinases and the GC-CC is embedded in the kinase domain (Fig. 2). Our finding is consistent with what was obtained in the previous study in Arabidopsis. Collectively, these results demonstrate that GC-CC is frequently encapsulated in a kinase domain. Here, we name...
these GC-CC-containing kinases as GC-kinases. They appear to exist widely in plants. Nevertheless, it needs to be pointed out that only a portion of kinase repertoire in a plant species belong to GC-kinases. For example, tomato genome contains over 640 receptor-like kinases (RLKs)\(^6\). But we found that only about 100 tomato kinases are GC-kinases. Moreover, whether a kinase embeds a GC-CC motif seems not to correlate with the type of kinase, since diverse types of kinases belong to GC-kinases. Tomato GC-kinases include LRR-Pkinase/Pkinase\(_{Tyr}\), CB-EGF-Pkinase, MB_Lectin-Pkinase\(_{Tyr}/Pkinase\), SSR/AF-Pkinase/Pkinase\(_{Tyr}\), and Pkinase or Pkinase\(_{Tyr}\) type kinases possessing no any other known domain (Fig. 2, Supplementary Table S3). Further, not all members of the same type of kinases in a plant species belong to GC-kinases. For instance, only six members of AtWAKL family and seven members of AtCRK family were predicted to be GC-kinases (Fig. 3, and ref. 35).

Additionally, alignment of the GC-CC motif region of the 99 tomato GCs revealed a tomato GC-CC motif as ([SK] [FYA] [GS] [VNY] [VIL] [LVMID] [LAMV] [EDM] [LITAE] [LIVEDA] [TSM] [FG] [EDM] [LITAE] [LIVEDA] [TSMLIC] [GNSRDIC] [RKPQLM] [RK x(0,2) [DSEG]]) (Fig. 1). Comparison of this motif with the one previously reported for plant ([KS] [YF] [GCS] [VIL] [VILFG] [DVIL] [VILADG] [EPVIL] [DVIL] [TVIL] [WST] [PDRG] [KEG] [KR x(2,3) [DHSE]])\(^3\) demonstrated that the tomato GC-CC motif is more relaxed at some positions not directly involved in catalysis function. For example, A2, N4, D8, T9, [ED]10, [GN]12, [RPQL]13 appeared frequently in predicted tomato GCs but not included in the plant GC-CC motif. These amino acids except [G]12 and [R]13 are the amino acids most frequently present at these positions. In addition, many amino acids present in only one SlGC also do not appear in the plant GC-CC motif. These amino acids include [DVNTP]1, [ACL]4, [NQP]5, [CAW]6, [PT]7, [S]8, [FKNYP]9, [CEMN]10, [FPYN]11, [LHTA]12 and [AIH]13. Collectively, our result suggests that the plant GC-CC motif needs to be relaxed for these positions when used for phi-BLAST search for plant GC prediction.

**Role of SIPEPR-GCs in disease resistance.** In the present study, we focused on the role of GC-CC-containing tomato homologs of AtPEPR (SIPEPR-GC) in disease resistance. AtPEPR1 is a LRR-RLK that recognizes Pep elicitors and triggers immunity signaling in Arabidopsis. It contributes to resistance against the bacterial pathogen *Pst* DC3000\(^2\). Here, we obtained similar results for the two SIPEPR-GCs (SIGC17 and SIGC18). Silencing of SIGC17 and SIGC18 reduced tomato resistance to this pathogen (Fig. 5B). Moreover, we demonstrated that SIGC17 and SIGC18 play a positive role in tomato resistance to other types of pathogens including fungal pathogen *S. sclerotiorum* and viral pathogen TRV (Figs. 4 and 5). In addition, SIGC17 and SIGC18 are also LRR-RLK proteins (Supplementary Table S3) and more importantly play a positive role in AtPep1-triggered Ca\(^{2+}\) and H\(_2\)O\(_2\) burst (Fig. 8). Together, our results indicate that SIGC17 and SIGC18 are most probably the functional orthologs of AtPEPR and play important roles in disease resistance against diverse pathogens.
**Figure 11.** A hypothetical model for cGMP signal transduction in plant resistance. Pathogen-related stimuli are recognized by plant receptor proteins. This recognition leads to activation of cytoplasmic or transmembrane GC-kinases and consequently an accumulation of cGMP, which causes cytosolic Ca\(^{2+}\) influx through opening of CNGC channels. The cytosolic Ca\(^{2+}\) modulates CDPK-mediated and RBOH-dependent ROS accumulation as well as CAMTA3-mediated defense signaling, thereby regulates HR and disease resistance. Abbreviations: CaM, calmodulin; CAMTA, calmodulin binding transcriptional activator; CDPK, calcium-dependent protein kinase; CNGC, cyclic nucleotide gated channel; GTP, guanosine triphosphate; cGMP, cyclic guanosine monophosphate; GC, guanylate cyclase; HR, hypersensitive response.

**SIGC17** and **SIGC18** exhibited identical direction but different level in all functions examined in this study including affecting resistance to various pathogens, PAMP and DAMP triggered Ca\(^{2+}\) and H\(_2\)O\(_2\) burst as well as Ca\(^{2+}\) signaling gene expression. **SIGC18** displayed stronger regulatory role in comparison with **SIGC17**, while **SIGC17** and **SIGC18** together showed more substantial effects in all these functions compared with single gene (Figs. 4–9). These results revealed that **SIGC17** and **SIGC18** function redundantly but **SIGC18** acts more strongly than **SIGC17**. The overlapped functions have been observed for AtPEPRs\(^{23}\).

It has been reported that AtPEPR1 has GC activity, generating cGMP from GTP, and that cGMP can activate AtCNGC2-dependent cytosolic Ca\(^{2+}\) elevation and act in pathogen defense signaling cascades in Arabidopsis\(^{22,23}\). Our evidences support **SIGC17** and **SIGC18** to be GCs. First, **SIGC17** and **SIGC18** both contain a GC-CC motif which carries all AA residues required for GC catalysis activity (Fig. 1). Second, silencing of **SIGC17** and **SIGC18** significantly reduced Ca\(^{2+}\) burst evoked by PAMPs (flg22 and chitin), DAMP (AtPep1) and Pst DC3000 living bacterial cells (Figs. 6 and 8), demonstrating the important positive role of **SIGC17** and **SIGC18** in these Ca\(^{2+}\) burst responses. Moreover, cGMP and tomato homologs of AtCNGC2 contribute to resistance in tomato against various pathogens including *S. sclerotiorum* \(^{56,59,63,64,66,67}\). In addition, our previous studies reveal that various Ca\(^{2+}\) signaling related genes including some CaMs, CDPKs and related genes as well as CAMTA3 are involved in various types of resistance in different plant species including tomato\(^{22,23,65}\), and silencing of **SIGC17** and **SIGC18** altered the expression of a set of CNGC, CDPK, CaM and CAMTA3 genes (Fig. 9). Finally and more importantly, enzyme immunoassay and MALDI-TOF-MS assays revealed that the GC-CC-containing cytoplasmic domain of **SIGC17** and **SIGC18** exhibits *in vitro* GC activity (Fig. 10).

The elucidation of functional mechanism of GC-kinases is complicated by the finding that the GC-CC is embedded in the kinase domain. It will be intriguing to clarify the relationship of GC-CC and kinase by analyzing the mutual effects on each other in regulating biological processes such as disease resistance. Do they separately contribute to the functions of GC-kinases, or coordinate in the regulating functions? The currently available examples suggest that this might be GC-kinase-dependent. AtPSKR1 seemed to support separation model while AtBRI1 appeared to favor coordination model\(^{45,68}\). Muleya *et al.*\(^{46}\) reported that calcium acted as the switch in the moonlighting dual function of the ligand-activated receptor kinase AtPSKR1. However, in case of AtBRI1, a functional kinase was required for GC activity to generate cGMP, which rapidly potentiates phosphorylation of the downstream substrate BSK1\(^{45}\). Additionally, considering that kinase exists in a plant species as a superfamily with large number of members, the embedding of a GC-CC in a kinase domain should efficiently broaden the range of processes for GC regulation. This may also at one layer account for the wide range of GC/cGMP-regulated biological processes.

Based on our findings, we propose a working model for cGMP signal transduction in the resistance of tomato (Fig. 11). In this model, stimuli including pathogens such as *S. sclerotiorum*, and *Pst* DC3000 as well as PAMPs such as chitin and flg22 and DAMPs such as AtPep1 activate GCs embedded within kinases of transmembrane RLKs or cytoplasmic RLCKs to generate cGMP, which activates Ca\(^{2+}\) channels such as CNGCs through direct binding to them or promoting their phosphorylation, leading to cytosolic Ca\(^{2+}\) influx. The cytosolic Ca\(^{2+}\) signal is transduced by various Ca\(^{2+}\) sensor proteins including CaM, which regulates CAMTA3. CAMTA3 directly binds to the CCGG cis-elements in the promoter of defense-related target genes and regulates their expression, which alters plant disease resistance. Simultaneously, the increased cytosolic Ca\(^{2+}\) also enhances RBOH activity by direct binding or CDPK-mediated phosphorylation, resulting in ROS accumulation and thereby affecting hypersensitive response (HR) and plant disease resistance (Fig. 11).
Conclusions

Ninety-nine candidate GC-kinases, which embedded a GC catalytic center (GC-CC) motif within the protein kinase domain, were identified in tomato genome. Two homologs of Arabidopsis PEPRs, SlGC17 and SlGC18 exhibited in vitro GC activity. Co-silencing of SlGC17 and SlGC18 genes significantly reduced resistance to a variety of pathogens including Sclerotinia sclerotiorum and Pseudomonas syringae pv. tomato (Pto) DC3000, and attenuated PAMP- and DAMP-elicited Ca\(^{2+}\) and H\(_2\)O\(_2\) burst. Additionally, silencing of these genes altered the expression of a set of Ca\(^{2+}\) signaling genes including SICNGCs, SICaMs, SICDPKS and SICAMTA3. Co-silencing of SlGC17 and SlGC18 caused stronger effects than individual silencing. Collectively, our results reveal that GC-kinases widely exist in tomato and the two SIPEPR-GC genes redundantly play a positive role in resistance to diverse pathogens and PAMP/DAMP-triggered immunity in tomato.

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Author contributions
H.R., X.Y.W. and Y.P.X. conducted the experiments. H.R., X.Y.W. and Y.H.H. performed bioinformatics analyses. H.R., X.Y.W. and Y.P.X. designed and analyzed all statistical data. X.Z.C. conceived of the study, and participated in its design and coordination. X.Z.C., Y.P.X. and H.R. prepared the manuscript.

Competing interests
The authors declare no competing interests.

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