Capsicum annuum transcription factor WRKYa positively regulates defense response upon TMV infection and is a substrate of CaMK1 and CaMK2

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Plants are constantly exposed to pathogens and environmental stresses. To minimize damage caused by these potentially harmful factors, plants respond by massive transcriptional reprogramming of various stress-related genes via major transcription factor families. One of the transcription factor families, WRKY, plays an important role in diverse stress response of plants and is often useful to generate genetically engineered crop plants. In this study, we carried out functional characterization of CaWRKYa encoding group I WRKY member, which is induced during hypersensitive response (HR) in hot pepper (Capsicum annuum) upon Tobacco mosaic virus (TMV) infection. CaWRKYa was involved in L-mediated resistance via transcriptional reprogramming of pathogenesis-related (PR) gene expression and affected HR upon TMV-P0 infection. CaWRKYa acts as a positive regulator of this defense system and could bind to the W-box of diverse PR genes promoters. Furthermore, we found Capsicum annuum mitogen-activated protein kinase 1 (CaMK1) and 2 (CaMK2) interacted with CaWRKYa and phosphorylated the SP clusters but not the MAPK docking (D)-domain of CaWRKYa. Thus, these results demonstrated that CaWRKYa was regulated by CaMK1 and CaMK2 at the posttranslational level in hot pepper.

The plant defense response against pathogen infection is triggered by the recognition of pathogen-associated molecular patterns (PAMPs) or effectors1,2. When a plant recognizes the PAMP, PAMP-triggered immunity (PTI) activates basal defense such as production of reactive oxygen species (ROS), phytoalexins, and pathogenesis-related (PR) gene expression to prevent pathogen penetration and proliferation3. In contrast, when plant resistance (R) proteins recognize effectors, effector-triggered immunity (ETI) activates stronger and faster resistance responses often accompanied by hypersensitive response (HR) cell death4. In both basal defense and R gene-mediated resistance, plant mitogen-activated protein kinase (MAPK) cascades play a central role in signal transduction via three-kinase phosphorelay systems composed of the MAPK, MAPK kinase (MKK) and MAPK kinase kinase (MKKK)4. Approximately 23 MAPKs, 10 MKKs, and 80 MKKKs exist in Arabidopsis and the cascade combinations of the components make complex signal transduction networking5. These cascade events affect various cellular processes such as resistance to biotic or abiotic agents and plant development via phosphorylation or protein-protein interaction with diverse target proteins. However, few target proteins of MAPKs have been identified and regulation of downstream signaling pathway is poorly understood.

WRKY transcription factors have a conserved WRKY domain of about 60 amino acids containing a 'WRKYGQK' heptapeptide and zinc finger-like motif that can bind to W-box (TTGAC[C/T]) in target genes to regulate gene expression upon abiotic/biotic stress conditions6,7. In plants, the WRKY family has been identified in almost all the plant species including Arabidopsis (74 members), soybean (197 members), poplar (104 members), sorghum (68 members), barley (more than 45 members), and rice (more than 100 members). WRKY genes are mainly classified into group I, IIA + IIB, Ic, IId, and III based on sequence homology6-11. The WRKY genes from several plant species are highly upregulated by various stimuli such as pathogen infection, wounding stress, and exogenous defense-related plant hormone treatments. WRKYs also participate in defense responses as a positive or negative regulator through regulation of downstream gene expression7,12. Furthermore, WRKY proteins are activated or inactivated by MAPK-dependent phosphorylation or the conformational change of binding partner protein. Substrates of MAPKs were enriched in WRKY transcription factors and validated phosphorylation of WRKYs by MAPKs13,14. Recently, it was shown that salicylic acid (SA)-induced protein kinase

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(SIPK), wound-induced protein kinase (WIPK), and tobacco mitogen-activated protein kinase 4 (NTF4) directly phosphorylated Nicotiana benthamiana WRKY8 (NbWRKY8), which is involved in plant innate immunity\(^\text{14,15}\). Interestingly, the interaction of NbWRKY8 with MAPKs is dependent on a MAPK-docking domain (D-domain) and phosphorylation efficiency is affected by serine-proline (SP) clusters located in the NbWRKY8 N-terminal region\(^\text{15}\). In Arabidopsis, MPK3 and MPK6 can phosphorylate AtWRKY33 upon Botrytis cinerea infection and phosphorylated AtWRKY33 activates phytoalexin deficient 3 (PAD3) expression\(^\text{16}\). The NbWRKY8 and AtWRKY33 are classified as group I WRKY family members. Group I members contain conserved D-domain and at least five SP clusters at N-terminal region\(^\text{15}\). Thus, WRKY transcription factors of group I could have a possibility of being substrates of MAPKs.

In barley, MLA10 encodes a nucleotide binding (NB) and leucine-rich repeat (LRR) type R protein that recognizes a fungal avirulence A10 (AVR\text{A}_{10}) effector protein and then localizes to the nucleus. The Coiled-coil (CC) domain of MLA10 interacts with the transcriptional repressors HvWRKY1 or HvWRKY2, and then another unknown WRKY protein might activate defense gene expression\(^\text{17}\). The Arabidopsis genome encodes the Resistant to Ralstonia solanacearum\text{1-R} (RRS1-R) protein which has an N-terminal Toll and interleukin-1 receptor, resistance protein (TIR) NB-LRR domain and C-terminal WRKY DNA binding domain\(^\text{18}\). The sensitive to low humidity 1 (shl1) mutant, which possesses an additional amino acid in the WRKY domain of RRS1-R, exhibits impaired DNA binding affinity and causes activation of defense response and hypersensitive cell death\(^\text{19}\). Thus, WRKY domains might be involved in both basal defense and effector-triggered immunity by R protein.

Hot pepper (Capsicum annuum L.) is important crop. The hot pepper plant contains L gene alleles which encode CC-NB-LRR type resistance proteins and the L gene confers resistance to Tobacco mosaic virus (TMV) by restricting virus spread at the primary infection site\(^\text{20}\). Previously, we verified WRKY and MAPK genes were responding to TMV-P\text{0} infection via DNA microarray analysis of a hot pepper expressed sequence tag (EST) database\(^\text{21}\). Specifically, some WRKY genes were involved in HR upon TMV-P\text{0} infection and positively regulated expression of PR genes\(^\text{22-24}\). In this study, we performed functional study of CaWRKY\text{A} via virus-induced gene silencing (VIGS) and demonstrated CaWRKY\text{A} positively regulated PR gene expressions upon TMV-P\text{0} infection. Furthermore, CaMK\text{1} and CaMK\text{2} directly interacted with and phosphorylated CaWRKY\text{A} which contains D-domain and SP clusters at N-terminus. These results indicated that phosphorylation of CaWRKY\text{A} by two TMV-P\text{0}-responsive MAPKs (CaMK\text{1} and CaMK\text{2}) could play a role in TMV defense response in hot pepper plant.

**Results**

**Sequence analysis of CaWRKY\text{A}**. We previously isolated a CaWRKY\text{A} cDNA clone from library screening and deduced the amino acid sequence of CaWRKY\text{A}, which contains two ‘WRKY’ domains and thus CaWRKY\text{A} was assigned to group I compared with other groups (Fig. 1a). Recently some group I member WRKYS were found to contain MAPK-docking sites (D-domain) and Ser or Thr followed by Pro (SP) clusters at the N-terminus\(^\text{15,17}\). By amino acid analysis, we found CaWRKY\text{A} contained a D-domain and five SP clusters at N-terminal region (Fig. 1a). CaWRKY\text{A} exhibited 91% and 90% amino acid sequence homology with Solanum chacoense (Sc) WRKY1 and Lycopersicon peruvianum (Lp) WRKY1, respectively (Fig. 1b). LpWRKY1 was phosphorylated by unknown 44 kD and 67 kD protein kinases which are shown to be transiently activated in response to an elicitor-preparation derived from the wilt inducing fungus Fusarium oxysporum lycopersici (E-FOL)\(^\text{25}\). These results suggest that CaWRKY\text{A} is a member of group I WRKY and has putative phosphorylation sites.

**CaWRKY\text{A} is involved in L-mediated resistance upon TMV-P\text{0} infection**. The roles of CaWRKY\text{A} in the L gene-mediated resistance against TMV-P\text{0} infection in hot pepper plants were studied by using VIGS system based on Tobacco rattle virus (TRV) vector using a partial fragment of CaWRKY\text{A} containing 3’ untranslated region (UTR) (Fig. 2a). The expression level of CaWRKY\text{A} was reduced about 67% in TMV-P\text{0}-infected TRV2-CaWRKY\text{A} plants compared with the TMV-P\text{0}-infected TRV2 control plants (Fig. 2b). To investigate whether silencing of CaWRKY\text{A} affects HR cell death upon TMV-P\text{0} infection trypan blue staining and conductivity measurement were performed. At 3 days post-inoculation (dpi), the HR numbers of CaWRKY\text{A}-silenced plants were reduced by about 40% and ion conductivities were decreased by about 30% compared with TMV-P\text{0}- inoculated TRV2 plants (Fig. 2c and d). To test whether silencing of CaWRKY\text{A} affects L-mediated resistance upon TMV-P\text{0} infection, we performed RT-PCR analysis of TMV-P\text{0}-cost protein (CP) gene expression in the local inoculated leaves and systemic non-inoculated upper leaves. In the local inoculated leaves of TRV2-CaWRKY\text{A} plants, TMV-P\text{0}-CP mRNA was still detected compared with TRV2 control plants (Fig. 2d). In the systemic non-inoculated upper leaves, TMV-P\text{0}-CP was detected only in the CaWRKY\text{A}-silenced plants but not in the TRV2 control plants (Fig. 2e). Thus, L-mediated resistance was affected by silencing of CaWRKY\text{A}. These results imply that suppression of CaWRKY\text{A} expression leads to reduced resistance to TMV-P\text{0} and CaWRKY\text{A} is a component of the L-mediated TMV resistance response in hot pepper.

**CaWRKY\text{A} positively regulated expression of PR genes and some plant hormone related genes upon TMV-P\text{0} infection**. WRKY transcription factors play a crucial role in regulating multiple defense response genes such as PR genes\(^\text{6}\). We have shown the L-mediated resistance was diminished in hot pepper plants upon TMV-P\text{0} infection when the expression of CaWRKY\text{A} was suppressed in CaWRKY\text{A}-silenced plants by VIGS (Fig. 2d and e). Furthermore, we showed that CaWRKY\text{A} bound to the L-box (TTGAC(C/T)) but not to a mutated version in vitro (Supplementary Fig. S1a and b). Following infection with TMV-P\text{0}, we tested whether expression of PR genes was affected by suppression of CaWRKY\text{A} expression. Expressions of CaPR\text{1}, 2, 4, 5 and 10 were strongly reduced in the CaWRKY\text{A}-silenced plants compared to the TRV2 control plants (Fig. 3). To determine whether CaWRKY\text{A} activates these PR genes, we checked activity of the CaPR\text{10promoter}-GUS reporter when co-expressing CaWRKY\text{A}-GFP in tobacco leaves by Agrobacterium-mediated infiltration\(^\text{24}\). The activity of CaPR\text{10pro-GUS} was increased about 37% when it was co-transformed with CaWRKY\text{A}-GFP compared to GFP (Supplementary Fig. S1c). These results suggest that CaPR\text{10} might be one of the target genes of CaWRKY\text{A} and CaWRKY\text{A} might be a positive regulator of other PR genes by binding to the L-box-containing promoter.

Furthermore, gene expression level of CaICS\text{1} was also diminished in CaWRKY\text{A}-silenced plants upon TMV-P\text{0} infection (Fig. 3). In general, PR\text{1}, PR\text{2}, and PR\text{5} are involved in salicylic acid (SA)-dependent signaling pathway and isochorismate mutase 1 (ICS\text{1}) encodes one of the major enzymes of SA biogenesis\(^\text{26}\). Non-race-specific disease resistance 1 (NDR\text{1}), a major regulator of R gene-mediated resistance, was also downregulated in the CaWRKY\text{A}-silenced plant\(^\text{27}\) (Fig. 3). Lipoxigenase (LOX) genes are involved in jasmonic acid (JA) biogenesis pathway and associated with plant defense\(^\text{28,29}\). In CaWRKY\text{A}-silenced plant, CaLOX\text{2} induction was reduced (Fig. 3). These data demonstrate that CaWRKY\text{A} regulates the expression of defense-related genes and signaling pathway genes. These regulations could contribute to the decreased resistance of CaWRKY\text{A}-silenced plant to TMV-P\text{0} infection.

**CaMK\text{1} and CaMK\text{2} are induced during HR to TMV-P\text{0} infection**. Previously, using hot pepper microarray analysis, we characterized CaMK\text{1} and CaMK\text{2} as TMV-P\text{0}-induced genes during HR in hot pepper plants upon TMV-P\text{0} infection. To test whether these CaMK\text{1} and CaMK\text{2} are induced during HR to TMV-P\text{0} infection, we transiently co-transformed CaMK\text{1} and CaMK\text{2} constructs with GFP reporter plasmid and performed transient co-expression analysis of TMV-P\text{0}-infected hot pepper leaves (Fig. 4a). These results suggest that CaMK\text{1} and CaMK\text{2} are induced during HR to TMV-P\text{0} infection.
Figure 1 | Analysis of CaWRKYa amino acid sequence. (a) CaWRKYa amino acid sequence was aligned with other group I WRKY members from various plants by MegAlign software. Members of group I typically contain two WRKY domains (yellow box). The docking domains (D-domain) for MAPKs are conserved at the N-terminal region of WRKYs (blue box). Some WRKYs do not contain D-domain. At the N-terminal region WRKYs contain some SP clusters which are potential phosphorylation sites by MAPKs (red box). Accession numbers; NaWRKY6 (AAS13440), NbWRKY7 (BAI63295), NtWRKY-6 (BAA61053), NtWRKY1 (BAA82107), ScWRKY1 (AAQ72790), LpWRKY1 (AB195141), CaWRKYa (AAR26657), NbWRKY8 (BAI63296), StWRKY8 (BAI63294), AtWRKY33 (NP_181381). (b) Phylogenetic analysis of CaWRKYa and other group WRKY members by Clustal W method. Accession numbers; WRKY51 (AED97953), AtWRKY6 (AEE33948), AtWRKY18 (AEE85961), AtWRKY11 (AEE85927), AtWRKY22 (AEE81999), AtWRKY38 (AED93044).
CaMK1 and CaMK2 were identified as encoding stress-inducible protein kinases which respond to wounding, UV-C, and cold. However, the function of CaMK1 and CaMK2 in plant immunity was not verified. CaMK1 is classified as group A according to the amino acid sequence analysis (Supplementary Fig. S2). The amino acid sequence analysis between CaMK1 and LeMPK3 or WIPK showed 95% and 94% identity, respectively (Supplementary Fig. S2). Thus, we speculated that CaMK1 and CaMK2 were involved in plant immunity.

### Figure 2 | CaWRKYa is involved in L-mediated resistance upon TMV-P0 infection

(a) A schematic illustration of VIGS region of CaWRKYa. Partial 3’ UTR fragment of CaWRKYa cDNA was used for VIGS. (b) qRT-PCR analysis of CaWRKYa-silenced plants. To confirm silencing of CaWRKYa, TRV2-CaWRKYa and TRV2 control plants were inoculated with TMV-P0. After 3 day post inoculation (dpi), total RNA was extracted from three independent samples and qRT-PCR was performed (Student’s t-tests; ***P<0.0001). (c) Trypan blue staining analysis for detecting the hypersensitive response (HR) cell death. Numbers of HR were quantified by counting (Student’s t-tests; ***P<0.0001). (d) Ion conductivity was measured by electrolyte leakage assay upon TMV-P0 inoculation or mock-inoculation in the TRV2 control and TRV2-CaWRKYa plants at 3 dpi. The error bars show the mean of the standard deviation (SD) of the replicate samples. (e) RT-PCR analysis of TMV-P0-coat protein (CP) expression in the local inoculated tissue and systemic non-inoculated upper tissue. Total RNA was extracted from three independent samples and RT-PCR was performed.
CaMK2 might have similar function in L-mediated plant defense response of hot pepper to TMV infection.

To elucidate CaMK1 and CaMK2 function in L-mediated resistance, we checked whether CaMK1 and CaMK2 could be induced specifically during HR upon TMV-P0 infection using qRT-PCR. We first confirmed that CaWRKYα expression level was induced by an avirulent strain, TMV-P0, but not by a virulent strain, PMMoV-P1,2,3 (Fig. 4a). The mRNA levels of CaMK1 and CaMK2 were increased within 24 h after TMV-P0 inoculation (Fig. 4b and c). Thus, CaMK1 and CaMK2 are responsive genes to TMV-P0 infection. In particular, CaMK1 gene expression level was highly increased by TMV-P0 inoculation at 24 h and slightly induced upon PMMoV-P1,2,3 infection (Fig. 4b). Furthermore, mRNA levels of CaWRKYα and CaMK1 were increased at early time points by exogenous application of salicylic acid (SA), methyl jasmonic acid (MeJA), and ethephon (ET) treatments but CaMK2 mRNA level was not increased (Supplementary Fig. S3a, c, and d).

Figure 3 | CaWRKYα silencing affects some PR and defense-related genes expression upon TMV-P0 infection. In TMV-P0-inoculated CaWRKYα-silenced and TRV2 control plants, qRT-PCR was performed with some PR genes and defense-related genes primers. Asterisks indicate significant differences in TMV-P0-inoculated TRV2-CaWRKYα compared with TRV2 control plants. (Student’s t-tests; *P < 0.05, **P < 0.001, ***P < 0.0001).
cing of hot pepper plants exhibited reduced HR lesions upon TMV-
P₀ infection (Supplementary Fig. S4). We thus postulated that func-
tion of CaWRKYα might be linked to the CaMK1 and CaMK2 and
regulated by CaMK1/CaMK2 via post-translational modification.

CaMK1 and CaMK2 can interact with CaWRKYα in vivo. CaWRKYα can be classified as a WRKY group I member, having two WRKY domains, and D-domain and SP clusters at the N-
terminal region (Fig. 1a). Many substrates of MAPK were identified
as transcription factors, such as WRKYs, in the nucleus to regulate gene
expression. To determine whether CaWRKYα is localized in the
nucleus, as other WRKY proteins are, a construct expressing GFP-
CaWRKYα fusion was transformed into hot pepper protoplasts
along with Tsi1-RFP construct as a nucleus-target control. Signal
of GFP-CaWRKYα predominantly was merged with Tsi1-RFP signal
in the nucleus although the CaWRKYα did not contain any obvious
nuclear localization signal (NLS) (Fig. 5a, panel i). Thus, CaWRKYα
localizes to the nucleus in hot pepper protoplasts. Most of interacting
proteins generally co-localize to the same or interactive subcellular
space. Consequently, we tested the subcellular localization of CaMK1
and CaMK2. The construct of GFP fused to N-terminus of CaMK1 or
CaMK2 was transformed into hot pepper protoplasts by polyethylene
glycol (PEG)-mediated transformation with Tsi1-RFP construct as
nucleus-localization marker. GFP signals of GFP-CaMK1 and GFP-
CaMK2 protein were merged with red signals of Tsi1-RFP but some
GFP signals were also detected in the cytosolic region (Fig. 5a, panels ii

![Figure 4](https://www.nature.com/scientificreports)

**Figure 4** Gene expression analysis of CaWRKYa, CaMK1, and CaMK2 upon TMV-P₀ infection. (a–c) The expanded leaves of six-week-old hot pepper plants were rubbed with TMV-P₀ or PMoV-P₁,₁,₂,₃. Inoculated samples were harvested at the indicated time points and total RNA was extracted. qRT-
PCR was performed with gene-specific primers. These experiments were performed with three independent samples. CaActin and Ca18S were used as the internal controls. (Student’s t-tests; *P < 0.05, ***P < 0.0001).
Figure 5 | CaWRKYa associates with CaMK1 and CaMK2 in vivo. (a) Localization analysis of CaWRKYa, CaMK1, and CaMK2 using GFP in the hot pepper protoplasts. (Panel, i–iii) The combination of GFP-CaWRKYa/Tsi1-RFP, GFP-CaMK1/Tsi1-RFP, and GFP-CaMK2/Tsi1-RFP constructs were cotransformed into the hot pepper protoplasts by PEG-mediated transformation. Tsi1-RFP was used as a nucleus localization control. GFP and RFP signals were detected using Axioplan 2 imaging fluorescence microscope. Bars indicate 20 μm. (b) CaWRKYa associates with CaMK1 and CaMK2 in the split YFP analysis. Panel i, Combination of YFPN-CaMK1 and CaWRKYa-YFPC constructs was introduced into hot pepper protoplasts by PEG-mediated transformation. Bars indicate 20 μm. Panel ii, Combination of CaMK2-YFPN and CaWRKYa-YFPC constructs was introduced into hot pepper protoplasts by PEG-mediated transformation. Reconstructed YFP fragments exhibited YFP signals in the nucleus. Red signals indicate chloroplast auto-signals. Panel iii, Combination of bZIP-YFPN and bZIP-YFPC constructs was introduced into hot pepper protoplasts by PEG-mediated transformation. bZIP transcription factor forming homodimer complex in the nucleus was used as a positive control for BiFC. (c) CaWRKYa can associate with CaMK1 and CaMK2 in the co-IP. Co-IP was performed using anti-HA beads and then detected by anti-Myc and -HA antibodies. Combinations of Myc-CaMK1 and CaWRKYa-HA or CaMK2-Myc and CaWRKYa-HA were introduced into the hot pepper protoplasts by PEG-mediated transformation. Combinations of CaWRKYa-HA and Myc empty vector or Myc-CaMK1/CaMK2-Myc and HA empty vector were used as a negative control. Specific signal bands are indicated by asterisks. N.S indicates non-specific bands.
CA MK1 and CA MK2 phosphorylated the SP clusters of CA WRKY a.

To test whether CA MK1 and CA MK2 are catalytically active in vitro, the MBP-CA MK1 and MBP-CA MK2 fusion proteins were purified from E. coli (Supplementary Fig. S5a). Next the proteins were subjected to an in vitro auto-phosphorylation activity assay. Both MBP fusion recombinant CA MK1 and CA MK2 proteins exhibited auto-phosphorylation activity (Fig. 6a). To test if CA MK1 and CA MK2 are able to trans-phosphorylate the target protein the myelin basic protein (MyBP), which is a commonly used model protein substrate of MAPKs, was used as a substrate. MyBP was phosphorylated by CA MK1 and CA MK2 in the presence of [γ-32P]ATP (Fig. 6b). Thus, recombinant CA MK1 and CA MK2 could be verified as catalytically active protein kinases that can phosphorylate MyBP as a substrate. Former results clearly demonstrated that CA MK1 and CA MK2 interacted with CA WRKY a in vivo (Fig. 5b and c). To assess the role of interaction between hot pepper MAPKs (CA MK1 and CA MK2) and CA WRKY a, we focused on the possibility of CA WRKY a as a substrate of CA MK1 and CA MK2. We expressed CA WRKY a fused with MBP protein to test the possibility that CA WRKY a acts as a substrate of CA MK1 and CA MK2 protein kinases (Fig. 6c). When affinity-purified MBP-CA WRKY a protein was incubated with each of CA MK1 and CA MK2 in the presence of [γ-32P]ATP, both protein kinases could phosphorylate CA WRKY a in vitro (Fig. 6d), indicating that CA WRKY a is indeed a substrate of CA MK1 and CA MK2.

Some group I WRKY proteins contain the conserved D domain and SP clusters at the N-terminal region and CA WRKY a also has these domains (Fig. 1a). The D-domain is known to be important for the interaction of a substrate with MAPKs and phosphorylation of SP clusters. To investigate the phosphorylation site of CA WRKY a, we made mutant protein of MBP-CA WRKY a N-terminal region, which was mutated either at the D-domain (mD) or SP clusters (mSP) (Fig. 6c). Interaction with MAP kinase could be achieved through the putative docking domain of CA WRKY a located at the N-terminus. Thus, if we mutate the corresponding region, we could expect significant decrease of enzymatic activity of both MAP kinases. However, WT CA WRKY a or CA WRKY a mD protein were phosphorylated by recombinant CA MK1 and CA MK2 (Fig. 6d).

These findings suggested that CA MK1 and CA MK2 might use non-identified docking motif in CA WRKY a. To further investigate the role of SP clusters for phosphorylation, a set of SP cluster mutants were constructed and tested for phosphorylation. MBP-fused recombinant CA WRKY a mSP protein, which has four mutations in the SP cluster region (Fig. 6c), did not show phosphorylation signal by CA MK1 and CA MK2 (Fig. 6d). Recombinant MBP fused D-domain mutant CA WRKY a mD was not affected in phosphorylation by CA MK1 and CA MK2 (Fig. 6d). These data indicated that the D-domain of CA WRKY a is not required for phosphorylation of CA MK1 and CA MK2 although interaction site is not clear in our experiments. However, multiple SP clusters of CA WRKY a have an important role in phosphorylation of CA WRKY a by CA MK1 and CA MK2.

A previous report suggested that phosphorylation of WRKY proteins affected DNA binding affinity of WRKY proteins to the W-box.

To test whether DNA binding affinity of CA WRKY a is enhanced by CA MK1 and CA MK2, we performed EMSA using purified GST fused with full length recombinant CA WRKY a protein with or without MBP-CA MK1 and -CA MK2 (Supplementary Fig. S5a). CA WRKY a incubated with CA MK1 or CA MK2 show slightly enhanced binding affinity to the W-box in vitro (Supplementary Fig. S5b). On the contrary, CA WRKY a with higher amount of CA MK1 was inhibited in DNA binding activity but CA MK2 still positively affects DNA binding affinity (Supplementary Fig. S5b). This result indicated that CA MK1 and CA MK2 function might be related to the regulation of stability and/or DNA binding affinity of CA WRKY a.

Discussion

In the tobacco and TMV interaction model system, MEK1, NTF6, WRKY, and MYB are involved in N-mediated resistance to TMV.

This suggests that TMV resistance response is regulated by diverse regulators and especially that transcription factor-MAPK cascades might be a major regulation pathway of R-mediated resistance via posttranslational step. We have studied the hot pepper-TMV-P0 interaction model system and two WRKY proteins that were previously reported as being involved in L-mediated resistance to TMV via regulating PR genes expression. In this study, we characterized CA WRKY a as a positive regulator in L-mediated resistance to TMV-P0 by regulating PR genes and SA/JA biogenesis genes. Furthermore, CA WRKY a was identified as a substrate of CA MK1 and CA MK2 in hot pepper. However, it is still largely unknown how other WRKY proteins are regulated by MAPKs or other regulators in hot pepper.

WRKY transcription factors are localized to the nucleus and then bind to W-boxes of target gene promoters to regulate gene expression upon stress conditions. However, WRKYs do not work alone in recognizing the signals from the stimulus. R-mediated signal transduction or MAPK signal cascade could be necessary for relaying the defense signal transduction to WRKYs or other transcription factors. However, some WRKYs could directly regulate R-mediated signal transduction. The slh1 mutant was isolated as a single amino acid insertion mutant in the WRKY domain of RRS1-R, and slh1 showed enhanced disease resistance to pathogen infection. Thus, this WRKY protein has evolved the capacity to play a dual role in defense signal recognition and regulation for plant immunity.

LeMPK3 can be activated in Cf-4/Avr4 interaction in tomato and is involved in HR upon pathogen infection. WIPK is activated in N gene-mediated cell death in tobacco upon TMV infection. Ca MK2 also belongs to group A and shows high identity to LeMPK2 (95%), NTF4 (94%), and NISIPK (88%) (Supplementary Fig. S2). LeMPK2 is required for prosystemin-mediated resistance to Manduca sexta and NTF4 is known to be involved in pathogen-induced HR cell death in tobacco. Furthermore, WIPK and SIPK also are activated by TMV and positively regulate N gene-mediated TMV resistance in tobacco. Interestingly, NbWRKY8 was identified as a substrate of...
Figure 6 | Analysis of CaWRKYa as a substrate of CaMK1 and CaMK2. (a) MBP-CaMK1 and -CaMK2 had an autophosphorylation activity. In vitro kinase assay was performed with MBP-CaMK1 (1 μg) and -CaMK2 (1 μg) incubated with no substrate, and MBP protein (1 μg) was used as a negative control. Proteins were incubated in the reaction buffer containing [γ-32P]-ATP for 1 h at RT, respectively. After heating for 5 min, the samples were run in SDS-PAGE gel. The gel was dried in gel dryer for 1 h and phosphorylation signals were detected by BAS reader. Arrows indicate phosphorylated CaMK1 and CaMK2. (b) MyBP protein (1 μg) was phosphorylated by MBP-CaMK1 and -CaMK2 in vitro kinase assay. Arrows indicate phosphorylated CaMK1, CaMK2 and MyBP. (c) A schematic illustration of CaWRKYa mutant constructs and confirmation of the purified proteins in SDS-PAGE gel. MBP-CaWRKYaN, MBP-CaWRKYaN-mSP (S85A, S97A, S104A, S116A), and MBP-CaWRKYaN-mD (K66A, K68A, L75A, M77A) proteins were made by mutagenesis. Purified proteins were checked via 15% SDS-PAGE gel. Asterisk indicates specific band. (d) MBP-CaMK1 (1 μg) or -CaMK2 (1 μg) were incubated with CaWRKYaN (1 μg), CaWRKYaN-mSP (1 μg), and CaWRKYaN-mD (1 μg) proteins and then in vitro kinase assay was performed. MBP was used as a negative control. N.S indicates non-specific bands.
the indicated time points. Control plants were also treated with virus-inoculation buffer containing the carborundum only. For SA, MeJA, and ET treatments, 3-week-old plants were sprayed with solutions of 1 mM SA, 100 μM MeJA, and 1 mM ET, respectively. Control plants were sprayed with water containing 0.01% ethanol. Three independent samples were harvested and total RNA was prepared using Qiagen RNA kit. Quantitative RT-PCR was performed with SYBR Green (Kapa Biosystems) in the LightCycler 480 Real-Time PCR System (Roche Applied Science) according to the manufacturer’s instructions. The primers used for real-time PCR reactions are listed in Table S1.

Trypan blue staining. HR cell death was detected with trypan blue staining. TMV-P0-inoculated leaves of TRV2 and TRV2-CaWRKYa plant were stained with lactophenol-trypsin blue solution (10 ml lactic acid, 10 ml glycerol, 10 ml acidophenol, 0.02 g trypan blue, and 10 ml water) and chloral hydrate solution (Fluka chemical) was used as a destaining solution.

Subcellular localization determination of CaWRKYa, CaMK1, and CaMK2. The CaWRKYa, CaMK1, and CaMK2 coding regions were fused to the green fluorescence protein (GFP)-coding region at the N-terminal region in 326 GFP vector. The DNA of GFP-CaWRKYa, GFP-CaMK1, and GFP-CaMK2 constructs was introduced into the hot pepper protoplasts by modified polyethylene glycol (PEG)-mediated transformation. GFP signal was excited at 488 nm laser and was collected using 495-510 nm bandwidths using an Axiosplan 2 imaging fluorescence microscope (Carl Zeiss).

Bimolecular fluorescence complementation (BiFC) and co-immunoprecipitation (co-IP) assay. BiFC assays were performed as previously described. Briefly, CaMK1 clone was inserted into the pUC18-backboned vector which contains partial YFP-N fragment (1-155 amino acid) tagged with c-Myc at the N-terminus using the primers; 5'-GGGATCCATGGTGATGCAAATATGGGTGGCGGGCT-3’ and 5'-GGGATCCCTTATGGCGTGAGATTTTATATATG-3’. The CaWRKYa clone was inserted into the pUC18-backboned vector which contains partial YFP-N fragment (1-155 amino acid) tagged with c-Myc at the C-terminus using the primers; 5'-GGGATCCATGGTGATGCAAATATGGGTGGCGGGCT-3’ and 5'-GGGATCCCTTATGGCGTGAGATTTTATATATG-3’. The CaWRKYa construct was inserted into the pUC18-backboned vector which contains partial YFP-C fragment (155-239 amino acid) tagged with HA at the C-terminus using the primers; 5'-GGGATCCATGGTGATGCAAATATGGGTGGCGGGCT-3’ and 5'-GGGATCCCTTATGGCGTGAGATTTTATATATG-3’. Combination of CaWRKYa-YFPN and YFP-Coding CaMK1 or CaMK2-YFP constructs were transformed into the hot pepper protoplasts by PEG-mediated transformation. Reconstructed yellow fluorescent protein (YFP) signals was excited at 514 nm laser and was collected using 560–640 nm bandwidths using an Axiosplan 2 imaging fluorescence microscope (Carl Zeiss).

To confirm these BiFC interactions, co-IP was performed with the hot pepper protoplasts co-expressing CaWRKYa-YFPN and YFP-Coding CaMK1 or CaMK2-YFP constructs. Briefly, after PEG-mediated transformations into protoplasts from the hot pepper plant, the cells were harvested by quick spin. The cells were suspended in extraction buffer (150 mM NaCl, 50 mM Tris-Cl pH 7.5, 5 mM EDTA, 1% Triton X-100, 1 mM DTT, and protease inhibitor cocktail) and then total proteins were extracted by sonication. After centrifugation, the supernatants were mixed with HA-agarose beads and incubated at 4°C for 2 h. The samples were run in SDS-PAGE gel and then western blot was performed with ECL kit (Amersham).

In vitro phosphorylation assay. The N-terminal CaWRKYa fragment (CaWRKYa<sup>3</sup>) protein and its mutant constructs were fused with maltose-binding protein (MBP) using pMAL vector.
incubated with 1 proteins were purified. MBP-CaMK1 (1AAACATATTGAGATGGTTGACGGTA-3'. To make WRKYa D-
CATGGCTTCTTCAGGTGGAAATACG-3' and 5'-GGATCCTTG-
TCGCGTCTTTTCCACCTTCTTCTGCGCCTGCGATCTCTTCT-
T3'- and CaWRKYa-5'-TGAAGAAGATGCAGCAGGCAGA-GAGAAGATGGTGGAAGACGCGACCTACAT-
CaPR10 promoter reporter was co-transformed into tobacco leaves by
TCA-3' and CaWRKYaN-mD-5'-TGAAGAAGAGATCGCAGGCGC-
TCGCGTCTTTTCCACCTTCTTCTGCGCCTGCGATCTCTTCT-
CaWKya D-

For in vitro phosphorylation assay, MBP fused CaWRKYaN, CaMK1, and CaMK2 were expressed in E. coli and recombinant proteins were purified. MBP-CaMK1 (1 μg) or CaMK2 (1 μg) were incubated with 1 μg of its substrates myelin basic protein (MyBP) or MBP-CaWRKYaN, -CaWRKYaN-mD, and -CaWRKYa-mSP proteins in reaction buffer (50 mM Tris-HCl pH7.5, 10 mM MgCl2, 2 mM MnCl2, 1 mM DTT, 50 μM γ-[32P]-ATP) for 1 h at RT. The reaction was stopped by adding the sample buffer. After heating for 5 min, the samples were run in SDS-PAGE gel and then stained and destained shortly. The gels were dried in gel dryer for 1 h and the signal was detected by BAS reader.

Electrophoretic mobility shift assay (EMSA). GST-fused full length CaWRKYaN recombinant protein was purified according to the GST gene fusion system protocol provided by Amersham. EMSA was performed as described21. Briefly, double-stranded synthetic W-box and mutant W-box oligonucleotides were labeled with [γ-32P]-ATP using T4 polynucleotide kinase (TaKaRa). The labeled probes were incubated with purified GST-CaWRKYa protein (5 μg) or GST protein (5 μg) at 4 °C for 30 min. DNA and protein complexes were resolved on a 5% polyacrylamide gel in 0.5 X TBE buffer. The gels were dried and the signals were detected by BAS reader.

GUS promoter activity assay. CaWRKYa-GFP construct is used as an effector protein and GFP construct is used as a negative control. CaPR10 promoter-GUS is used as a reporter31. Each effector and reporter was co-transformed into tobacco leaves by Agrobacterium-mediated infiltration. The leaves were harvested after 3 days at three intervals after infiltration and extracted with GUS extraction buffer (50 mM NaHPO4 pH7.0, 10 mM 2-mercaptoethanol, 10 mM Na2EDTA, 0.1% sodium laurel sarcosine, 0.1% Triton X-100) for GUS activity analysis. The 4-methyl-umbelliferyl-glucuronide (4-MU) as a substrate was used and protein concentrations of the extracts were determined by BSA assay. GUS activity was measured using a Mithras LB940-luminometer (Berthold Technologies)32. The excitation wavelength was 365 nm and the emission wavelength 455 nm.

Electrolyte leakage assay. The electrolyte leakage assay was performed as described previously32. Briefly, three leaf discs were taken from one leaf of each TMV-P0 inoculated plant. The conductivity is measured by portable conductivity meter (Thermo Orion). The error bars show the mean value of the standard deviation (SD) of the replicate samples (n = 3).

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Author contributions

S.U.H., G.J.L., Y.J.K., J.H.J., Y.S.K. and K.H.P. wrote the manuscript. S.U.H. and K.H.P. reviewed the manuscript. J.H.J. performed the experiments. S.U.H., G.J.L., Y.S.K. and K.H.P. analysed the data. S.U.H., G.J.L., J.H.J., Y.S.K. and K.H.P. designed the experiments. S.U.H., G.J.L., Y.S.K. and K.H.P. wrote the manuscript. CaWRKY2, a chili pepper transcription factor WRKYa positively regulates defense response upon TMV infection and is a substrate of CaMK1 and CaMK2. Sci. Rep. 5, 7981; DOI:10.1038/srep07981 (2015).

Additional information

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