Potyviral Gene-Silencing Suppressor HCPro Interacts with Salicylic Acid (SA)-Binding Protein 3 to Weaken SA-Mediated Defense Responses

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The viral infection process is a battle between host defense response and pathogen antagonizing action. Several studies have established a tight link between the viral RNA silencing suppressor (RSS) and the repression of salicylic acid (SA)-mediated defense responses, nonetheless host factors directly linking an RSS and the SA pathway remains unidentified. From yeast two-hybrid analysis, we identified an interaction between the potyviral RSS helper-component proteinase (HCPro) and SA-binding protein SABB3. Co-localization and bimolecular fluorescence complementation analyses validated the direct *in vivo* interaction between *Turnip mosaic virus* (TuMV) HCPro and the *Arabidopsis* homologue of SABB3, AtCA1. Additionally, transient expression of TuMV HCPro demonstrated its ability to act as a negative regulator of AtCA1. When the plants of the AtCA1 knockout mutant line were inoculated with TuMV, our results indicated that AtCA1 is essential to restrict viral spreading and accumulation, induce SA accumulation, and trigger the SA pathway. Unexpectedly, the AtCA1 overexpression line also displayed a similar phenotype, suggesting that the constitutive expression of AtCA1 antagonizes the SA pathway. Taken together, our results depict AtCA1 as an essential regulator of SA defense responses. Moreover, the interaction of potyviral HCPro with this regulator compromises the SA pathway to weaken host defense responses and facilitate viral infection.

Many plant viruses of the genus *Potyvirus* (family *Potyviridae*) cause severe damage in a wide range of economically important crops (Ivanov et al. 2014; Revers and García 2015). They have a conserved genomic organization consisting of a large open reading frame (ORF) and another shorter ORF (pretty interesting potyvirus ORF, PIPO) within the P3 coding sequence in a reading frame different from the polyprotein (Chung et al. 2008; White 2015). These two ORFs generate a large polyprotein and a transframed protein, which are processed by three virus-encoded proteases into 11 mature viral proteins, designated P1, HCPro (helper-component proteinase), P3, P3N-PIPO, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, and CP (coat protein)

(Carrington et al. 1989; García et al. 2014; Riechmann et al. 1992). Most of these proteins are multifunctional and play different roles during virus infection. This infection process has been described as an unending battle between host and pathogen, forcing plant viruses in a perpetual evolution to counteract plant defense responses, such as RNA silencing (Ruiz et al. 1998; Simón-Mateo et al. 2011) or the salicylic acid (SA)-dependent defense pathway (Malamy et al. 1990; Vlot et al. 2009; White 1979).

A perfect illustration of viral strategy to avoid plant defense responses is the well-characterized potyviral protein HCPro, which has RNA silencing suppressor (RSS) activity (Anandalakshmi et al. 1998). Most potyviruses have a second suppressor of RNA silencing, viral genome-linked protein (VPg), whose function is yet poorly characterized; nonetheless, recent study demonstrated that *Turnip mosaic virus* (TuMV) VPg can reduce a key component of the RNA silencing pathway (Cheng and Wang 2017). RNA silencing is a key player in the post-transcriptional regulation of physiological processes of the host, providing virus-specific defense in response to the recognition of a general elicitor, double-stranded (ds)RNA (Bouché et al. 2006; Ruiz et al. 1998; Voinnet 2001). During antiviral-based silencing, viral dsRNAs are recognized and are cleaved into viral short interfering (vsi)RNAs by Dicer-like proteins (DCLs) (Nakahara et al. 2012). These primary vsiRNAs induce host RNA-dependent RNA polymerases to generate secondary vsiRNA to amplify and maintain the antiviral silencing response. Generated vsiRNAs are loaded into specific Argonaute proteins, the effector components of the RNA-induced silencing complex, to target and cleave viral RNA sharing sequence homology with vsiRNAs (Kim et al. 2014; Morel et al. 2002; Wang et al. 2011). HCPro interferes with both micro RNA and short-interfering RNA pathways (Kasschau et al. 2003; Kung et al. 2014; Wu et al. 2010) through its ability to inhibit DCL2- and DCL4-mediated post-transcriptional gene silencing (PTGS) (Kung et al. 2014; Lin et al. 2007). Moreover, HCPro silencing suppressor activity correlates with its ability to bind small RNA (del Toro et al. 2017). Most of HCPro functions are based on the central region of the protein. This central region harbors a highly conserved FRNK motif and involves genome amplification, RNA binding activity, and suppression of both PTGS and virus-induced gene silencing (Kasschau and Carrington 2001; Kung et al. 2014; Plisson et al. 2003; Wu et al. 2010). Other functions of HCPro, such as aphid transmission (N-terminal region of HCPro), virus replication (central region of

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HCPPro), and the protease activity (C-terminal region of HCPPro) have also been described (Plisson et al. 2003). The central and C-terminal regions of HCPPro are also responsible for cell-to-cell and systemic movement of the virus (Plisson et al. 2003). Interestingly, a study demonstrates that one mutation in TuMV HCPPro essential motif FRNK leads to downregulation of viral titer and correlates with the induction of innate immune responses, including SA-mediated defense responses (Kung et al. 2014). In addition, a recent study shows that HCPPro of *Tobacco vein banding mosaic virus* is able to activate the SA pathway by interfering with DNA methylation (Yang et al. 2016). These results suggest a possible crosstalk between RSS and the SA-dependent defense pathway.

The endogenous phytohormone SA plays a central role in plant defense signaling. It is required for the recognition of pathogen infection and subsequent establishment of local resistance in the infected region as well as systemic resistance at the whole-plant level (Carr et al. 2010; Malamy et al. 1990; Shah 2003; Vernooij et al. 1994). SA accumulation is induced upon infection of several pathogens (Delaney et al. 1994; Lu 2009; Métraux et al. 1990). SA is synthesized from chorismate via isochorismate. The enzymes isochorismate synthase (ICS) and isochorismate pyruvate lyase catalyze the two steps from chorismate to SA (Serino et al. 1995). Biosynthesis of SA is mostly controlled by the SA-induction deficient 2 (*SID2*) gene, which encodes a putative chloroplast-localized ICS (Wildermuth et al. 2001). Several plant factors were identified as regulators involved in the SA-mediated defense pathway. The activation of the SA pathway requires a coordinated interplay between enhanced disease susceptibility 1 (*EDS1*) and phytoalexin deficient 4 (*PAD4*), this interaction directly contributing to a positive loop of SA accumulation (Rietz et al. 2011; Wiermer et al. 2005). Beyond *EDS1* and *PAD4*, numerous SA-positive regulators have been identified, including *ACD6* (Lu et al. 2003), *EDS5* (Nawrath et al. 2002), *ALD1* (Song et al. 2004), *WIN3* (HOP 1-1-interacting 3) (Lee et al. 2007), and *MOS* (Palma et al. 2005). Another key regulator of SA signaling is the regulatory protein *NPR1*. Optimal levels of SA allow changes in the cellular redox homeostasis, initiating the reduction of the *NPR1* oligomer (Mou et al. 2003), then, SA would directly interact with *NPR1* to cause the final disassembly of the oligomer (Wu et al. 2012). *NPR1* monomer enters the nucleus to interact with TGA and WRKY transcription factors (Dong et al. 2003; Loake and Grant 2007; Pieterse and Van Loon 2004), leading to transcriptional upregulation of the SA-dependent pathogenesis-related (PR) proteins (Durrant and Dong 2004). The accumulation of SA is required for inducing defense response upon pathogen infection in inoculated leaves and generate one or more multiple mobile signals triggering systemic acquired resistance (SAR), a broad spectrum and long-lasting immune response (Shah and Zeier 2013; Vlot et al. 2008, 2009).

To clarify the role of SA in defense signaling, several SA-binding proteins (SABPs) in tobacco have been identified. The first characterized SGBP possesses a catalase activity, allowing the degradation of H_2O_2 . Its activity is specifically inhibited by SA, leading to an accumulation of reactive oxygen species, which is related to SA-induced cell death (Chen et al. 1993). The second identified SGBP has methyl salicylate (MeSA) esterase activity, catalyzing the hydrolysis of MeSA to SA. This enzyme is required for the activation of SAR (Forouhar et al. 2005; Kumar et al. 2006; Park et al. 2007). It has been suggested that during the activation of SAR, MeSA accumulates and, then, moves from infected leaves to distal leaves. In the distal leaves, SGBP2 hydrolyzed MeSA to produce SA, activating SA-mediated defense responses in the pathogen-free organs (Dempsey and Klessig 2012). The third SGBP, SGBP3,

is tobacco chloroplastic carbonic anhydrase (CA), which is required for the induction of the *Pto:avrPto*-mediated hypersensitive response formation in *Nicotiana benthamiana* (Slaymaker et al. 2002). Furthermore, in *A. thaliana*, the chloroplastic CA1 (*AtCA1* or *AtSABP3*) binding activity to SA as well as its CA activity are essential for the induction of host resistance during pathogen infection (Wang et al. 2009). Altogether, these SABPs appear to be essential for the regulation of the SA-dependent defense pathway.

Our preliminary results, obtained by screening a *Chenopodium quinoa* (Willd.) library by yeast two-hybrid (Y2H) assay, using *Zucchini yellow mosaic virus* (ZYMV) HCPPro as a bait, leads to the identification of a strong interaction between the potyviral RSS HCPPro and a putative *C. quinoa* CA (*CqCA1*). This newly identified protein shows high identity with the SGBP *AtCA1*, indicating a possible implication of HCPPro in the modulation of SA-mediated defense response. Unlike *C. quinoa*, *Arabidopsis thaliana* (L.) Heynh. is commonly used as an ideal model plant for the acquisition of knowledge on basic plant biology and on adaptation to biotic or abiotic stress. Moreover, *A. thaliana* is susceptible to various viral pathogens, making it an ideal host for the characterization of plant-virus interactions. Among potyviruses, TuMV has the ability to infect wild Brassicaceae species as their natural habitats (Pallett et al. 2002; Thurston et al. 2001), this makes the virus a perfect partner to investigate innate defense response.

In this study, we used TuMV as a model virus to investigate the implication of HCPPro and *AtCA1* on the SA-dependent defense pathway in *A. thaliana*. We verified the relevance of the TuMV HCPPro interaction with *AtCA1*. Our results demonstrated that HCPPro acts as a negative regulator of *AtCA1*. Using a knockout mutant line, our results indicated *AtCA1* is essential to restrict virus spread and accumulation, induce SA accumulation, and trigger the SA pathway. Surprisingly, similar results were observed when *AtCA1* was overexpressed, suggesting a possible negative regulation of the SA-dependent defense pathway. Furthermore, we have verified the ability of HCPPro to negatively impact the SA pathway, suggesting its critical role in the modulation of innate defense responses.

RESULTS

TuMV HCPPro interacts with *CqCA1* and its *A. thaliana* homologue *AtCA1*.

In the scope of identifying new host factors interacting with HCPPro, a screening of the *C. quinoa*-expressing library using ZYMV HCPPro (ZHC) as bait was conducted by Y2H analysis. The *C. quinoa* library was constructed in fusion with the GAL4 activation domain (pGBK vector), while ZHC was fused with the GAL4 binding domain (pGAD vector). Given that HCPPro has a self-interacting capability (Lin et al. 2009; Urcuqui-Inchima et al. 1999; Zheng et al. 2011), the combination of pGBK-ZHC and pGAD-ZHC was used as a positive control (Fig. 1A). Among several positive interactions, one sample homologue of *Nicotiana tabacum* SGBP, *NtSABP3*, mentioned as the *C. quinoa* SGBP gene (*CqSABP3*) (Jarvis et al. 2017), revealed a strong interaction with ZHC (Fig. 1A) on selective media lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine. This protein showed high homology with both *NtSABP3* and *AtCA1* (Fig. 1B), including the presence of a conserved cysteine at position 280, corresponding to the Cys280 essential for *AtCA1* enzyme activity (Wang et al. 2009). Altogether, this allowed us to identify one HCPPro-interacting protein as a CA1, this newly identified host-factor was referred as a putative *CqCA1* hereafter.

To confirm that both ZHC and TuMV HCPPro (TuHC) can directly interact with *AtCA1*, ZHC, TuHC, and full-length

cDNA of β -glucuronidase (GUS) were cloned into pMAL-DC-myc vector to generate recombinant proteins ZHC-myc, TuHC-myc, and GUS-myc, respectively, while AtCA1 was cloned into pMAL-DC-HA (hemagglutinin) vector to generate the recombinant protein AtCA1-HA. Monoclonal antibody (MAb) against myc-tag was used to pull down the coimmunoprecipitation (Co-IP) of AtCA1-HA with either ZHC-myc, TuHC-myc, or GUS-myc, while polyclonal antibodies (PAb) against HA-tag were used for revelation. The negative control of the Co-IP of AtCA1-HA and GUS-myc showed a barely detectable signal, while the Co-IP of AtCA1-HA with both ZHC-myc and TuHC-myc revealed strong signals (Supplementary Fig. S1). The observed signal in the negative control could be explained by some unspecific interaction; on the other hand, the strong intensity of the signal detected in AtCA1-HA and ZHC-myc or TuHC-myc Co-IP displays a clear specific interaction. This result indicated that both ZYMV and TuMV HCPros interact with AtCA1 protein under in vitro conditions.

Since the HCPro-CA1 interaction was established using the ZYMV-*C. quinoa* pathosystem, we first evaluated cellular localization of potyviral HCPro and AtCA1. ZHC and TuHC were fused with yellow fluorescent protein (ZHC-YFP and TuHC-YFP, respectively); meanwhile AtCA1 was fused with cyan fluorescent protein (AtCA1-CFP), and protein localization was monitored by fluorescent microscopy. Transient expression in *N. benthamiana* plants with AtCA1-CFP coexpressing with either ZHC-YFP or TuHC-YFP showed that AtCA1 as well as both HCPros were localized in the cytoplasm (Fig. 2, left and

central panels). Likewise, individual expressions of AtCA1-CFP, ZHC-YFP, and TuHC-YFP showed the same localization (data not shown). AtCA1 cytoplasmic localization might be explained by the fact that chloroplastic AtCA1 is produced in the nucleus and is then targeted to the chloroplast, through a chloroplastic transit peptide (Fett and Coleman 1994). The merged image (Fig. 2, right panel) revealed a clear colocalization between AtCA1-CFP and each of ZHC-YFP and TuHC-YFP, displaying that, despite the chloroplastic function of AtCA1, both ZYMV and TuMV HCPros colocalize with the *A. thaliana* homologue of CqCA1, i.e., AtCA1.

Therefore, to validate this link in the *A. thaliana*-TuMV pathosystem, we further conducted a bimolecular fluorescence complementation (BiFC) assay to test the direct interaction between TuHC and AtCA1. The N-terminal fragment of YFP was fused to the N termini of TuHC and AtCA1 to generate YFP^N-TuHC and YFP^N-AtCA1, while the C-terminal fragment of YFP was fused to the N termini of TuHC and TuMV CP (TuCP) to generate YFP^C-TuHC and YFP^C-TuCP. Plants were infiltrated with each construct with pairwise combinations. No fluorescence was detected in the single infiltration (data not shown). Since HCPro was reported to self-interact, coinfiltration of YFP^N-TuHC and YFP^C-TuHC was used as a positive control. We were able to detect YFP fluorescence for the positive control as well as the YFP^N-AtCA1 and YFP^C-TuHC combination (Fig. 3). As another negative control, no YFP signal was observed when YFP^N-AtCA1 was coinfiltrated with the CP of TuMV (YFP^C-TuCP) (Fig. 3). Our BiFC results demonstrated

Fig. 1. Identification of *Chenopodium quinoa* putative carbonic anhydrase by yeast two-hybrid analysis. **A**, Analysis of CqSABP3 direct interaction with *Zucchini yellow mosaic virus* HCPro by yeast two-hybrid. Each cotransformed yeast cell was grown on synthetic defined (SD) medium lacking tryptophan (Trp), leucine (Leu), histidine (His), and adenine (Ade). pGAD vector contained the GAL4 activation domain and pGBK vector contained the GAL4 binding domain. **B**, Amino acid alignment of *Arabidopsis thaliana* carbonic anhydrase (AtCA1), *Nicotiana tabacum* salicylic acid-binding protein 3 (NtSABP3), and partial amino acid sequence of the putative *C. quinoa* SABP3 gene (CqSABP3).

the specificity of interaction between AtCA1 and TuMV HCPro.

HCPro acts as a negative regulator of AtCA1.

We generated two *A. thaliana* mutant lines, an *AtCA1* knockout line (namely, *cal*) obtained by screening homozygous progeny of the Salk_106570, T-DNA insertion line, (Arabidopsis Biological Resource Center [ABRC], Ohio State University, Columbus, OH, U.S.A.) and an *AtCA1* overexpression line (namely, CA1-OE) obtained in our laboratory by floral-dip transformation (Clough and Bent 1998; Zhang et al. 2006) of the *A. thaliana* wild-type Col-0. AtCA1 protein accumulation was assessed in untreated 4-week-old plants, with anti-CA1 polyclonal antibodies, prepared in our laboratory by rabbit immunization with the purified bacteria-expressed AtCA1 protein. We failed to detect AtCA1 protein in the knockout *cal* line, while its accumulation was increased up to 61% in the CA1-OE line as compared with the wild-type Col-0 (Fig. 4A). These results validated these mutant lines.

To determine whether HCPro protein would affect AtCA1 expression, an *Agrobacterium* infiltration system was employed to transiently express TuMV HCPro protein. In this system, *Agrobacterium* strain carrying either HCPro fused with full-length YFP (HC-YFP) at the C terminus or YFP^C-TuCP were infiltrated in leaves of Col-0, *cal*, and CA1-OE plants. The construct expressing YFP^C-TuCP was used as a control. Two days postinfiltration, the expression of *AtCA1* transcript was measured by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), *AtCA1* expression in the control condition (YFP^C-TuCP) was completely impaired in *cal* and was significantly upregulated in CA1-OE as compared with Col-0 (Fig. 4B), consistent with the protein accumulation analysis of mutant lines. When HCPro was transiently expressed, *AtCA1* transcript was reduced by 72 and 45% in Col-0 and CA1-OE, respectively, compared with the control expressing TuMV CP (Fig. 4B). Furthermore, Western blot analysis showed that transient expression of HCPro in the CA1-OE background leads to a 52% reduction of AtCA1 protein accumulation (Fig. 4C). Similarly, in Col-0, we observed a 26% decrease of AtCA1 when HCPro was transiently expressed, as compared with CP (Fig. 4C). These

results revealed that TuMV HCPro protein has the ability to specifically reduce AtCA1 transcript and protein accumulation, suggesting that HCPro acts as a negative regulator of AtCA1.

TuMV accumulation is enhanced in *AtCA1* knockout and overexpression lines.

Since TuMV HCPro negatively regulates AtCA1, we decided to look into the function of AtCA1 during virus infection. For this purpose, TuMV accumulation in inoculated leaves was assessed by challenging Col-0, *cal*, and CA1-OE lines with a TuMV infectious clone expressing the YFP (TuMV-YFP). At 2 days postinoculation (dpi), initial infection spots caused by the virus were monitored by fluorescent microscopy.

Fluorescence microscopy of *cal* inoculated leaves revealed wider initial infection spots as compared with those in Col-0 plants (Fig. 5A). Average size of these loci was estimated using ImageJ software (Slaymaker et al. 2002); analysis of four independent experiments confirmed that initial virus spread on infected leaves was four times higher in *cal* compared with that on Col-0 (Fig. 5B). This means an absence of AtCA1 leads to acceleration of the spreading of initial infection spots, suggesting its important role in TuMV restriction at the early stage of the viral infection. In addition, AtCA1 has been reported as SABP3 (Slaymaker et al. 2002; Wang et al. 2009), implying possible involvement of AtCA1 during SA-mediated defense responses.

Surprisingly, fluorescence microscopy of CA1-OE inoculated leaves also showed increments of virus spread and accumulation (Fig. 5A). Size estimation of infection foci confirmed this observation by showing a fivefold increase of initial infection spots in CA1-OE as compared with Col-0 (Fig. 5B). This result suggested that, similar to the knockout line, AtCA1 overexpression also negatively affects plant defense responses.

AtCA1 is essential to the SA induction during TuMV infection.

To confirm that the phenotypes observed in *cal* and CA1-OE lines are linked to SA-mediated resistance, total SA accumulation was estimated directly, using high-performance liquid chromatography-mass spectrometry (HPLC-MS) at 2 dpi. For

Fig. 2. Spatial colocalization of Zucchini yellow mosaic virus (ZYMV) or Turnip mosaic virus (TuMV) HCPro with *Arabidopsis thaliana* carbonic anhydrase (AtCA1). Transient coexpression of cyan fluorescent protein-tagged AtCA1 protein (AtCA1-CFP) with yellow fluorescent protein-tagged HCPro protein of ZYMV (ZHC-YFP) (upper panel) or YFP-tagged HCPro protein of TuMV (TuHC-YFP) (lower panel) in *Nicotiana benthamiana* leaves. Fluorescent microscopy shows ZHC-YFP and TuHC-YFP in green, AtCA1-CFP in red and the merge combination indicates the cytoplasmic colocalization in yellow. The results are representatives of three independent experiments. Scale bar = 20 μ m.

each line, total SA was extracted from a pool of treated and untreated rosette leaves of two plants either challenged with TuMV or treated with inoculation buffer (Mock).

As observed in Figure 6, SA levels in Col-0 remained low in the absence of TuMV infection while *cal* and CA1-OE lines showed a slight increase of SA accumulation, compared with Col-0. Following TuMV inoculation, SA levels increased drastically in Col-0. On the contrary, in *cal* and CA1-OE lines, TuMV failed to induce SA while CA1-OE displayed a decrease in its SA level (Fig. 6).

This failure of SA induction coupled with TuMV susceptibility enhancement in both AtCA1 knockout and overexpression lines demonstrated that the absence of AtCA1 impairs SA accumulation, resulting in a failure of SA-mediated resistance activation and facilitating the spread of TuMV. Moreover, in the TuMV-infected CA1-OE line, SA accumulation was impaired and dropped to levels lower than in the mock condition, suggesting the activation of a possible negative feedback loop, leading to an outcome similar to that of the AtCA1 knockout line, an inactivation of the SA-mediated defense pathway.

AtCA1 is a key regulator of the SA pathway during TuMV infection.

We decided to look at AtCA1 influence on the SA pathway during TuMV infection. The relative transcript accumulations of two positive regulators of SA as well as two genes induced

by SA were compared in the wild type, *cal*, and CA1-OE lines. We first validated *AtCA1* accumulation and found that, in CA1-OE, accumulation was higher than in the wild type and was constant in both inoculated and mock conditions. As expected, we failed to detect *AtCA1* in *cal*. Regarding Col-0, we noticed that *AtCA1* accumulation was modulated by TuMV and its transcript increased during the first 4 days of infection, followed by a sharp downregulation thereafter (Fig. 7A). When virus accumulation was measured by transcript accumulation of TuMV CP and HCPro, viral RNA followed the same accumulation pattern (data not shown). Our time course experiment showed higher TuMV accumulation in both the knockout *cal* line and the overexpression CA1-OE line as compared with the wild type and, once again, CA1-OE appeared to be more susceptible than *cal* (Fig. 7B). These results are consistent with our virus-spread experiment, indicating the importance of AtCA1 in TuMV restriction.

As positive regulators of SA formation, we chose to evaluate the *SID2* gene involved in SA biosynthesis (Lu 2009; Wildermuth et al. 2001) and the *WIN3* gene involved in SA accumulation (Lee et al. 2007; Lu 2009). In control Col-0, both positive regulators were induced at the beginning of TuMV infection. On the contrary, in the *cal* line, none of these positive regulators were expressed and, in CA1-OE, *SID2* was not expressed while *WIN3* has a lower level of expression than in Col-0 (Fig. 7C and D).

Fig. 3. Bimolecular fluorescence assay of the interaction between *Arabidopsis thaliana* carbonic anhydrase (AtCA1) and *Turnip mosaic virus* (TuMV) HCPro (TuHC) or TuMV coat protein (TuCP). Proteins were transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration. Positive control YFP^N-TuHC/YFP^C-TuHC (upper panel), negative control YFP^N-AtCA1/YFP^C-TuCP (lower panel), and tested interaction YFP^N-AtCA1/YFP^C-TuHC (middle panel) are shown. Bright field displays chlorophyll autofluorescence, signal in the YFP column indicates the successful reconstitution of the chromophore and the merge displays the combination of both bright field and YFP (yellow fluorescent protein). The results are representatives of four independent experiments. Scale bar = 100 μm.

In plants, SA-mediated resistance correlates with the presence of PR proteins (Yalpani et al. 1991). Therefore, we used PR1 and PR5 to evaluate the SA pathway response to TuMV infection. In Col-0, their expression increased over time, and, while CA1-OE had very high levels of PR1 and PR5 at 2 dpi, expression decreased rapidly thereafter. In the case of *ca1*, we failed to detect any expression of both PR1 and PR5 (Fig. 7E and F).

Altogether, our results showed that, in wild type, *AtCA1* expression increased at the beginning of the infection at the

same time positive regulators of SA were activated, and then, in the late stage of infection, *AtCA1* expression decreased while *PR* gene expression increased. In the absence of *AtCA1*, neither positive regulators nor *PR* genes were activated, demonstrating a failure in SA-pathway induction, leading to an enhancement of TuMV susceptibility. In the presence of an abnormal proportion of *AtCA1*, the two positive regulators, *SID2* and *WIN3*, of SA were drastically reduced compared with those of the wild type. *PR* genes appeared to be strongly activated at the beginning

Fig. 4. Protein accumulation and relative expression levels of *Arabidopsis thaliana* carbonic anhydrase (*AtCA1*) in wild-type, *AtCA1* knockout, or overexpression lines. **A**, Upper panel shows the Western blot analysis of seedling extracts of wild type (Col-0), *AtCA1* knockout (*ca1*), or overexpression (CA1-OE) lines of *A. thaliana*, using polyclonal antibodies targeting *AtCA1* (anti-CA1). The results are representative of three independent experiments. **B**, *Turnip mosaic virus* (TuMV) HCPro and TuMV coat protein (CP) were transiently expressed in wild-type (Col-0), *AtCA1* knockout (*ca1*-ko), and overexpression (CA1-OE) seedlings. Two days after infiltration, total RNA was extracted and quantitative reverse transcription-polymerase chain reaction was performed. Relative expression of *AtCA1* was evaluated, using *A. thaliana* Actin-8 for normalization. All values are the mean ($\pm$ standard deviation) of 12 plants taken from four separate experiments. Different letters above each sample represent statistical significance at $P < 0.001$, assessed by student's *t* test. **C**, TuMV HCPro and TuMV CP were transiently expressed in seedlings of the wild-type (Col-0) or overexpression (CA1-OE) lines and total protein extracts were obtained 2 days after infiltration. Upper panel shows the Western blot analysis of CA1-OE infiltrated with agroinfiltration buffer (Mock) or TuMV HCPro (TuHC-YFP) and Col-0 infiltrated with TuMV CP (TuCP) or TuMV HCPro (TuHC-YFP). Polyclonal antibodies targeting *A. thaliana* CA1 (anti-CA1) were used. Histograms show the intensity of Western blot immunostaining of TuHC-YFP-infiltrated CA1-OE and Col-0 leaves relative to the band intensity of their respective controls. The results are representatives of three independent experiments. The lower panels in A and C show similar loading of each lane stained with Coomassie brilliant blue R250, the protein band corresponding to the RuBisCO large subunit (CB) was used as a loading control.

of infection and then decreased after 4 dpi, displaying a completely inverted pattern compared with the wild type.

TuMV HCPro acts as a negative regulator of the SA pathway.

Our previous experiments showed that HCPro acts as a negative regulator of AtCA1, which appeared to be essential to induce SA-mediated defense responses. To prove that HCPro could impact the SA pathway, we induced this pathway by treating *A. thaliana* plants with a 2-mM solution of SA. Two days later, the same plants were agroinfiltrated to transiently express either HCPro or TuMV CP, in which the latter was used as a control condition. Activation of SA-mediated defense responses was monitored the next day by measuring *PR5* gene expression. As a negative control, we used a *NahG* mutant line that constitutively expresses the *Pseudomonas putida NahG* gene that encodes salicylate hydroxylase, an enzyme that converts SA to catechol (Yamamoto et al. 1965), thus resulting in reduction of SA levels and inactivation of the SA pathway in this plant (Delaney et al. 1994). As expected, neither HCPro nor CP was able to induce *PR5* expression in *NahG* plants (Fig. 8). The wild-type and overexpression CA1-OE lines showed a high level of *PR5* under the control condition, resulting from SA-mediated

defense response activated by SA treatment (Fig. 8). Transient expression of HCPro leads to a reduction by 63 and 47% of *PR5* gene expression in Col-0 and CA1-OE, respectively (Fig. 8), demonstrating the ability of TuMV HCPro to negatively impact the SA-mediated defense response. Moreover, we noticed that SA treatment slightly activated *PR5* in the knockout mutant line and ectopic expression of HCPro failed to reduce *PR5* gene expression. This means that, in the absence of AtCA1, HCPro failed to downregulate SA-mediated defense responses, indicating the essential role of AtCA1 for HCPro to modulate the SA pathway.

DISCUSSION

To counteract host defense responses, plant viruses develop strategies of RNA silencing suppression and inhibition of SA-mediated defense responses. A recent study demonstrated that one mutation in TuMV HCPro leads to a variation of SA-mediated defense responses (Kung et al. 2014). Furthermore, a study of diverse plant viruses showed that *Cucumber mosaic virus* (CMV, genus *Cucumovirus*) viral protein Cmv2b, which has silencing suppressor activity by inhibiting DCL2- and DCL4-mediated PTGS (Ziebell et al. 2007), also has an antagonistic

Fig. 5. Visualization and average size of initial infectious spots induced by *Turnip mosaic virus* (TuMV) on wild-type, *Arabidopsis thaliana* carbonic anhydrase (AtCA1) knockout, and overexpression lines. Wild-type (Col-0), AtCA1 knockout (*ca1*), and overexpression (CA1-OE) seedlings of *Arabidopsis thaliana* were challenged with yellow fluorescent protein (YFP)-tagged TuMV (TuMV-YFP). **A**, Two days postinoculation, initial infectious spots localized on the adaxial surface were monitored by fluorescent microscopy and **B**, their surfaces were estimated. Results displayed in **A** are representative of four independent experiments (scale bar = 100 µm), while, in **B**, all values are the mean ($\pm$ standard deviation) of 20 plants taken from four separate experiments. Asterisks (****) indicate $P < 0.0001$.

effect on the SA-mediated virus resistance mechanism (Ji and Ding 2001). And the RSS P6 of *Cauliflower mosaic virus* (CaMV, genus *Caulimovirus*) strongly inhibited SA-dependent responses, including the expression of representative *PR* genes, hypersensitive cell death, and basal defense against biotrophic bacterial pathogens (Love et al. 2007, 2012). Altogether, these studies clearly indicated the existence of a tight link between RSS and the repression of SA-mediated defense responses. To date, possible host factors directly linking HCPro and SA pathway have not identified yet. Here, we report for the first time the interaction of the potyviral RSS HCPro with a chloroplastic CA (AtCA1), which has been described as SABP3 (Slaymaker et al. 2002; Wang et al. 2009). Furthermore, our results revealed that this interaction leads to a weakened SA pathway during viral infection, suggesting a novel way for HCPro to modulate innate immunity.

Several data pointed out a key function of SABP3 and its homologs in disease resistance. A potato homolog of this gene was found to be quickly suppressed during the development of late blight disease, and silencing of SABP3 in *N. benthamiana* resulted in increased pathogen growth (Restrepo et al. 2005). Also, both *N. benthamiana* and *A. thaliana* SABP3 has been shown to be essential to induce a full defense response against bacterial strain (Slaymaker et al. 2002; Wang et al. 2009). It is worth noting that several studies have found chloroplastic proteins interacting with viral proteins (Cheng et al. 2013b; Feki et al. 2005; Huh et al. 2011; Jiménez et al. 2006). In addition, chloroplastic regulators guanosine tetraphosphate and pentaphosphate [(p)ppGpp] were recently found to directly regulate SA accumulation and increase TuMV susceptibility (Abdelkefi et al. 2017). Finally, multiple diverse viruses have been observed to alter chloroplast structure, size, or number (Zhao et al. 2016). In accordance with the chloroplastic localization of AtCA1, our results demonstrated that ectopic expression of TuMV HCPro in *A. thaliana* leads to the downregulation of both its transcript and protein accumulation, depicting HCPro as a negative regulator of SABP3.

To evaluate the effect of AtCA1 on viral spreading and accumulation, an *A. thaliana* mutant line lacking *AtCA1* was challenged with TuMV. We observed that virus spread was enhanced at 2 dpi in inoculated leaves. Furthermore, we noticed that viral accumulation was significantly increased in systemic tissue as compared with the wild type at 6 dpi. Our results indicated that AtCA1 is indispensable to restrict viral spreading, confirming its essential role in the induction of host defense responses. Nevertheless, underlying mechanisms responsible for viral spread enhancement in our study need to be further investigated. Infection efficiency could be controlled by two major factors, replication and movement; thus, to fully understand the observed phenotype, it is essential to study TuMV replication in *cal* and CA1-OE protoplasts in order to distinguish between the two possibilities.

The principal role of CA is to catalyze the reversible hydration of CO₂ into bicarbonate (HCO₃⁻). In C₃ plants, CA is distributed primarily in the stroma of chloroplasts, speculated to facilitate the supply of CO₂ to ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) by maintaining the equilibrium between HCO₃⁻ and CO₂ within the chloroplast (Williams et al. 1996). The CO₂ transformation into HCO₃⁻ mediated by CA is essential to retain inorganic carbon for the primary carboxylation reactions. For example, in multiple plant species, plastidial CA function is required for lipid biosynthesis, possibly by its interaction with acetyl-CoA carboxylase and the enzymes of the fatty acid synthase complex, to process carbon into fatty acid (FA) (Hoang and Chapman 2002). Since, AtCA1 should have the same role and FA-derived signaling has been recognized as one of the most important regulators of defense

pathways (Chandra-Shekara et al. 2007; Weber 2002; Yaeno et al. 2004), we propose that AtCA1 downregulation mediated by HCPro might directly impact plant defense responses and, thus, increase host susceptibility.

FA biosynthesis affects SA pathway regulation (Falk et al. 1999; Jirage et al. 1999; Kachroo et al. 2003, 2005); this, coupled with the SA-binding property of AtCA1, led us to evaluate its possible implication on the induction of the SA pathway during TuMV infection. We demonstrated that the pathogen-dependent induction of SA was compromised in the *cal* line. In this mutant line, we noticed that gene expression of *SID2* and *WIN3*, two positive regulators of SA accumulation, was completely aborted and, also, that *PR1* and *PR5*, the hallmark of the SA pathway induction, failed to be activated during TuMV infection. Our result revealed that, in the absence of AtCA1, SA pathway activation was completely impaired, depicting its implication in the induction of the SA pathway during viral infection. Hence, a likely hypothesis is that the decrease of AtCA1 during viral infection compromises the SA pathway, possibly through its modulation of FA biosynthesis, and weakens SA-mediated defense responses in order to facilitate viral infection.

Surprisingly, our results showed that, similarly to *cal*, AtCA1 overexpression also enhances TuMV spreading and accumulation. In the CA1-OE line, we observed that, 2 days after TuMV infection, the expression of *PR1* and *PR2* gene was abnormally high and their levels decreased rapidly and was almost abolished at 4 dpi. Additionally, the quantification of total SA at 3 dpi revealed that SA accumulation was not only impaired but reduced as compared with the mock condition. Altogether, these results suggest that, after viral infection, AtCA1 overexpression has an adverse effect on SA production.

Since SA plays an important role in plant growth and development (Rivas-San Vicente and Plasencia 2011), its regulation

Fig. 6. Total salicylic acid (SA) accumulation in wild-type, *Arabidopsis thaliana* carbonic anhydrase (AtCA1) knockout, and overexpression lines challenged with *Turnip mosaic virus* (TuMV). Seedlings of wild-type (Col-0), AtCA1 knockout (*cal*), and overexpression (CA1-OE) lines of *Arabidopsis thaliana* were inoculated with TuMV, and a mock treatment with inoculation buffer was used as control. Two days postinoculation, total (free and sugar-conjugate) SA was extracted and subjected to high-performance liquid chromatography-mass spectrometry analysis to estimate the amount of SA measured in nanograms per gram of the fresh weight (FW). All values are the means ($\pm$ standard deviation) of three pools of two plants taken from three separate experiments. Different letters above each sample represent statistical significance at $P < 0.001$, as assessed by student's *t* test.

Fig. 7. Time course of the relative expression levels of hallmark genes of the salicylic acid (SA) pathway in wild-type, *Arabidopsis thaliana* carbonic anhydrase (AtCA1) knockout, and overexpression lines. Wild-type (Col-0), AtCA1 knockout (*ca1*), and overexpression (CA1-OE) seedlings of *Arabidopsis thaliana* were inoculated with *Turnip mosaic virus* (TuMV), and a mock treatment with inoculation buffer was performed as control. After 2, 4, 6, and 10 days, total RNA was extracted and quantitative reverse transcription-polymerase chain reaction was performed. Relative expression levels of each gene were evaluated using *A. thaliana* Actin-8 for normalization. **A**, Transcript accumulation of AtCA1. **B**, RNA accumulation of TuMV coat protein (CP) showing a control of infection. **C** and **D**, Transcript accumulation of the two positive regulators of the SA pathway, SID2 and WIN3. **E** and **F**, Transcript accumulation of two genes, *PR1* and *PR5*, induced during the SA pathway. All values are the means ($\pm$ standard deviation) of 16 plants taken from four separate experiments.

is essential for normal plant physiology. Numerous positive and negative feedback loops allowing stringent regulation of SA accumulation have been described (Delaney et al. 1994; Lu 2009; Shah 2003; Shah et al. 1997; Vlot et al. 2009). Therefore, the increase of susceptibility observed in the CA1-OE line may be explained by two well-accepted antagonistic effects readjusting SA accumulation. First, inside the SA pathway, optimal levels of SA activate NPR1, which controls the onset of SA-mediated defense responses. Interestingly, NPR1 also participates to a negative feedback loop by directly downregulating SA accumulation (Clarke et al. 2000; Delaney et al. 1995; Shah et al. 1999, 2001). Thus, constitutive expression of AtCA1, illustrated by the CA1-OE mutant line, might overexpress the SA pathway, leading to the activation of a NPR1 negative-feedback loop and, thus, shutting off SA production, directly resulting in the enhancement of TuMV susceptibility. Secondly, AtCA1 involvement in lipid biosynthesis could also directly impact the modulation of other plant hormones. Several studies revealed extensive crosstalk between hormonal signaling pathways; therefore, AtCA1 overexpression might trigger the activation of jasmonic acid or abscisic acid, which has the ability to antagonize the SA pathway (Bostock 2005; Mauch-Mani and Mauch 2005; Loake and Grant 2007; Vlot et al. 2009). Besides the essential regulation of SA accumulation, AtCA1 was identified as a target for S-nitrosylation during the expression of disease resistance (Wang et al. 2009). This post-translational modification is crucial to restrain AtCA1 activity and thus contributes to dampened defense responses. Hence, in the CA1-OE line, we cannot exclude the possibility of a direct inactivation of AtCA1 activity by S-nitrosylation, leading to weakened host defense reaction. Nonetheless, our current results prevent us from ruling out any of the above possibilities and the mechanism behind this antagonistic effect still remains to be further investigated. Validation of one or more of these hypotheses should give us a better insight into the function of AtCA1 during plant defense regulation and leads us to fully understand the interplay between HCPro, SABP3, and SA-mediated defense responses.

Overall, our results showed that potyviral HCPro is able to interact and downregulate an essential regulator of the host factor SABP3 (homologue AtCA1), for reducing SA accumulation and weakening SA-mediated defense responses to facilitate virus spread and accumulation. To validate the HCPro ability to antagonize the SA pathway out of a viral infection context, we further transiently expressed HCPro alone in SA-pretreated *A. thaliana*. Our results further validated the involvement of HCPro in the modulation of SA-mediated defense responses and pointed at AtCA1 as an essential partner for this mechanism. To further investigate the role played by AtCA1 during potyviral infection, it is essential to define the critical domain responsible for HCPro-AtCA1 interaction. The generation of plants with a functional AtCA1 variant or mutated form losing its ability to bind HCPro would definitively confirm the essential role of the interaction but, above all, may display enhanced resistance and potentially provide a new resistance mechanism against potyviruses.

MATERIALS AND METHODS

Plant material.

Plants of *C. quinoa*, *A. thaliana*, and *Nicotiana benthamiana* Domin were grown in a greenhouse maintained at $25 \pm 3^\circ\text{C}$ with a 12-h photoperiod. *A. thaliana* wild-type Col-0 and the *NahG* mutant line were provided by S.-S. Lin at National Taiwan University, Taipei, Taiwan. Seeds for the AtCA1 (At3g01500) knockout line (*ca1*) was obtained from the ABRC as a T-DNA insertion line (Salk_106570). Genotyping of the PCR reaction

was performed using primer pairs RP-SALK_106570/LP-SALK_106570 and RP-SALK_106570/LBb1.3 (Table 1), and subsequent sequence analysis of the products was carried out to identify the plants carrying an insertion at At3g01500. Homozygous lines containing a single T-DNA insert in At3g01500 were subjected to two rounds of self-fertilization and progeny analyses.

For the AtCA1 overexpression line (CA1-OE), full-length cDNA of AtCA1 was cloned into the pENTR/D vector (Invitrogen, Carlsbad, CA, U.S.A.) and transferred to pBCo-DC-HA plant expression vector by Gateway cloning (Invitrogen). The obtained vector was transferred into *Agrobacterium tumefaciens* C58C1 and, then, was used for floral-dip transformation (Clough and Bent 1998; Zhang et al. 2006) of the *A. thaliana* wild-type accession Col-0. Transgenic plants were selected on a selection medium containing the herbicide Basta. The ectopic expression of AtCA1 was confirmed by Western blot, using the monoclonal antibody against HA tag (Santa Cruz Biotechnology, Inc., Dallas).

Viral source and inoculation.

TuMV infectious clone p35STuMVcYFPHis, which was modified, by replacement of the green fluorescent protein (GFP) ORF with a YFP ORF, from the previously constructed TuMV-GFP vector p35STuMVcGFPHis (Kung et al., 2014), was mechanically applied onto Carborundum-dusted leaves of *C. quinoa*, using a 20- μl aliquot containing 1 μg of DNA in sterilized water. After 6 or 7 dpi, individual local lesions were isolated and mechanically transferred to *N. benthamiana* plants, and infected leaves were collected and stored at -80°C for further study. TuMV-YFP inoculum was then obtained by crushing inoculated *N. benthamiana* leaves in 10 mM phosphate buffer (1:10, wt/vol) supplemented with Carborundum. AtCA1 knockout or overexpressed *A. thaliana* plants were mechanically inoculated by rubbing the inoculum on four leaves of each plant,

Fig. 8. Relative expression levels of *PR5* in salicylic acid (SA) pretreated wild-type, *Arabidopsis thaliana* carbonic anhydrase (AtCA1) knockout, and overexpression lines. Turnip mosaic virus (TuMV) HCPro and coat protein (TuCP) were transiently expressed in wild-type (Col-0), AtCA1 knockout (*ca1*), or overexpression (CA1-OE) seedlings of *Arabidopsis thaliana* pretreated with a 2-mM solution of SA. Two days after agro-infiltration, total RNA was extracted and quantitative reverse transcription-polymerase chain reaction was performed. Relative expression levels of *PR5* were evaluated, using *A. thaliana* Actin-8 for normalization. All values are the means ($\pm$ standard deviation) of 12 plants taken from four separate experiments. Different letters above each sample represent statistical significance at $P < 0.001$, as assessed by student's *t* test.

using a cotton swab, and inoculum residues were rinsed out by water spray.

Virus visualization.

To determine the cell-to-cell movement and spreading of TuMV-YFP virus in inoculated leaves, YFP fluorescence was observed using fluorescence microscopy (Zeiss AXIO imager A1) at 2 dpi. Size of initial infection spots caused by the tested viruses was assessed with ImageJ software (Schneider et al. 2012).

Vector construction for Y2H analysis, Co-IP, colocalization, and BiFC assay.

(ZHC, TuHC, and TuCP genes were amplified by RT-PCR from ZYMV- and TuMV-infected *N. benthamiana* plants, using primers sets P-ZHC/N-ZHC, P-TuHC/N-TuHC, and P-TuCP/N-TuCP (Table 1), respectively. The ZYMV HC-Pro ORF was amplified by PCR with the forward primer P-ZHCPro *NcoI* and reverse primer N-ZHCPro *BamHI* (Table 1) from the ZYMV infectious clone (Lin et al., 2007). The amplified fragment was cloned in pGBKT7 vector (Clontech, Palo Alto, CA, U.S.A.) to generate pGBKT7/ZHC-Pro as a bait. The *C. quinoa* cDNA library was constructed by the MATCHMAKER library construction and screening kit (Clontech). Described briefly below, the *C. quinoa* mRNA was purified from noninoculated *C. quinoa* plants, and then, the first-strand cDNA library was synthesized with CDSIII oligo-dT and coupled dC tail by RT, then amplified by PCR with SMART III oligonucleotide and CDSIII oligo-dT primers.

The full-length cDNA clone of the *A. thaliana* CA1 gene (AtCA1) was constructed from 2-week-old Col-0 seedlings. Total RNA was extracted using the Plant total RNA purification kit according to manufacturer's instructions (GMBiolab Co. Ltd, Taichung, Taiwan) and RT was performed, using the

SuperScript III first-strand synthesis system (Invitrogen), with polydT primers (Table 1). Subsequent PCR was performed using Ex *Taq* polymerase (Clontech) with the P-CA1/N-CA1 primer pair (Table 1). Full-length GUS cDNA was amplified with specific primers. Individually amplified fragments were cloned in pENTR/D vector (Invitrogen) to generate pENTR/ZHC, pENTR/TuHC, pENTR/TuCP, pENTR/AtCA1, and pENTR/GUS. Then, with LR Clonase enzyme (Invitrogen), ZHC, TuHC, GUS, and AtCA1 fragments were transferred into bacterial expression vectors (pMAL-DC-HA or pMAL-DC-myc) for the generation of pMAL-ZHC-myc, pMAL-TuHC-myc, pMAL-GUS-myc, and pMAL-AtCA1-HA, respectively. The same strategy was employed to transfer ZHC, TuHC, and AtCA1 fragments into plant expression vectors to express either the yellow or cyan fluorescence proteins (pBCo-DC-YFP or pBCo-DC-CFP, respectively) (provided by S.-S. Lin) for the generation of pBCo-ZHC-YFP, pBCo-TuHC-YFP, and pBCo-AtCA1-CFP. And, finally, the same strategy was employed to clone TuHC, AtCA1, and TuCP fragments into BiFC vectors expressing either the N-terminal part (pBA-NE-DC-myc) or C-terminal part (pBA-CE-DC-HA) (provided by S.-S. Lin) of the YFP, to generate pBA-NE-TuHC-myc, pBA-CE-TuHC-HA, pBA-NE-AtCA1-myc, and pBA-CE-TuCP-HA.

Y2H analysis. The pGBKT7/ZHC-Pro, pGADT7-Rec vector, and PCR products were cotransformed into *Saccharomyces cerevisiae* AH109 cells (Clontech). Transformants were grown at 30°C for 2 days on the synthetic defined (SD) medium lacking Leu and Trp, then, individual colonies were picked from plate to liquid SD medium lacking Leu and Trp and were cultured at 30°C overnight. Then, liquid yeast culture was transferred to the SD medium lacking His, Leu, and Trp agar plate. The plasmid of individual colonies was extracted and sequenced. The *C. quinoa* SABP3 gene (*CqSABP3*) amino acid sequence is shown in Figure 1B.

In vitro Co-IP of AtCA1 with both ZYMV and TuHC. *Escherichia coli* BL21 cells were transformed with the individual plasmids expressing ZHC-myc, TuHC-myc, GUS-myc, and AtCA1-HA (pMAL-ZHC-myc, pMAL-TuHC-myc, pMAL-GUS-myc, and pMAL-AtCA1-HA, respectively). Following induced expression of recombinant proteins, *E. coli* cells were pelleted and resuspended in 1 ml of extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 5% glycerol, 1 mM EDTA, and 0.02% NaN₃) containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, U.S.A.), were lysed with a sonicator 250-450 Sonifier Analog Cell Disruptor (Branson, CT, U.S.A.), and were then centrifuged at 12,000 × *g* for 5 min. Crude extract was loaded on amylose column (New England Biolabs, Ipswich, MA, U.S.A.) and was washed 12 times with column buffer (CB) (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, pH 7.4), and then, the recombinant proteins were eluted with 1 ml of CB containing 10 mM maltose, three times. Aliquots of the purified sample containing AtCA1-HA was mixed, in equal volume, with each purified sample containing ZHC-myc, TuHC-myc, or GUS-myc (i.e., AtCA1-HA + ZHC-myc; AtCA1-HA + TuHC-myc; AtCA1-HA + GUS-myc) and was incubated at 4°C for 1 h. MAb against myc-tag (Santa Cruz Biotechnology, Inc.) was added to each reaction mixture, before incubation at 4°C for 1 h. Then, 25 µl Mag Protein A sepharose (GE Healthcare Life Sciences, Uppsala, Sweden) was added and the mixture was incubated further for 1 h. The tubes were kept on a magnetic platform (MagRack6) to capture the Mag Protein A sepharose beads. After washing with 600 µl of extraction buffer four times, the beads were resuspended in 100 µl of sample buffer and the immunoprecipitated proteins were analyzed by Western blotting, using PAb against HA-tag (Santa Cruz Biotechnology, Inc.) to detect the nontagged protein pulled down by myc MAb through Co-IP with the myc-tagged proteins.

Table 1. Primers sequences for cloning, mutant screening, and reverse transcription-quantitative polymerase chain reaction (qPCR)

Name	Sequence (5' to 3')
Mutant line screen	
RP-SALK_106570	ATCGGGTAAGGCTTCAAGAG
LP-SALK_106570	CTTCGTGGTCCGTAACATAGC
LBb1.3	ATTTTGCCGATTTCGGAAC
Protein-protein interaction	
P-ZHCPro <i>NcoI</i>	ATCCATGGTCATCCATGGAAGTTCAGTT
N-ZHCPro <i>BamHI</i>	CCGGATCCGCCAACTCTGTAATGCTTC
P-ZHC	CACCATGTCATCCATGGAAGTTCAGTTCCTC
N-ZHC	GCCAACTCTGTAATGCTTCATC
P-TuHC	CACCATGAGTGCAGCAGGAGCCAAAC
N-TuHC	TCCGACACGGTAGTGTTC
P-TuCP	CACCGCAGGTGAGACGC
N-TuCP	TAACCCCTTAACGCCAAGTAAG
P-CA1	CACCATGTCGACCGCTCCTCTCTC
N-CA1	CAGCTTCCAATGTAGTATGG
poly-dT	TTTTTTTTTTTTTTTTTTTT
Gene expression	
qPCR_CA1-All_F	CCTTCGTGGTCCGTAACATAG
qPCR_CA1-All_R	TAAGGTGTAAGACCGCGTATTC
qPCR_TuHC_F	AGGAATTCTCACAGGCCACG
qPCR_TuHC_R	GGTGGTGTGTTGGTTGGCATC
qPCR_SID2_F	ATGAGATTTCAGCCTCGCTGT
qPCR_SID2_R	TGATGGATCTCCAATCGTCA
qPCR_WIN3_F	ACACGAGCCCTGATGAAGTC
qPCR_WIN3_R	CCCAAGTCTGTGACCCAGTT
qPCR_PR1_F	TTCTTCCCTCGAAAGTCAA
qPCR_PR1_R	AAGGCCACACAGAGTGTATG
qPCR_PR5_F	CGTACAGGTGCAACTTTGA
qPCR_PR5_R	GCGTTGAGGTGACAGACACA
Actin8_F	CCCAAAAGCCAAACAGAGAGA
Actin8_R	CATCACCAGAGTCCAACACAAT

Colocalization and BiFC experiment analyzed by transient expression.

Agrobacterium tumefaciens C58C1 was separately transformed with pBCo-ZHC-YFP, pBCo-TuHC-YFP, or pBCo-AtCA1-CFP vector to observe the subcellular localization and colocalization of ZHC, TuHC, and their target candidate AtCA1. While *Agrobacterium* strain carrying pBA-NE-TuHC-myc, pBA-CE-TuHC-HA, pBA-NE-AtCA1-myc, or pBA-CE-TuCP-HA were used to validate the direct in vivo interaction between TuMV HcPro and AtCA1 by BiFC, intact leaves of 6- to 8-week-old *N. benthamiana* plants were infiltrated with *agrobacterium* for transient expression. The agroinfiltration method was modified from our previous studies (Cheng et al. 2013a; Huang et al. 2015; Kung et al. 2015). In both experiments, the overnight *agrobacterium* cell cultures were resuspended in agroinfiltration buffer (10 mM MgCl₂, 150 μ M acetosyringone, 10 mM MES). Obtained *agrobacterium* suspensions were adjusted to a final optical density at 600 nm (OD₆₀₀) of 1.0, were kept for 3 h at room temperature, and then, were directly injected into the intercellular spaces of leaves, using a needleless plastic syringe. For coinfiltrations, the *agrobacterium* cultures were mixed together in equal amounts. After infiltration, the plants were maintained for 2 to 3 days under controlled conditions in greenhouse facilities (25 $\pm$ 3°C with a 12-h photoperiod).

For colocalization, an individually infiltrated portion was taken at 2 days post-agroinfiltration (dpa) for observation by confocal microscopy. The Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo) was used to examine the subcellular localization of YFP, CFP, and chlorophyll fluorescence. For the imaging of chlorophyll autofluorescence, excitation with a 488-nm argon laser was used and fluorescence was detected at 630 to 690 nm. The excitation wavelength for CFP was 405 nm and its emission was recorded at 440 to 485 nm. For visualization of YFP, excitation at 514 nm was used along with an emission window set at 520 to 550 nm. For the CFP and YFP colocalization experiments, simultaneous imaging was conducted, using the line-sequential multitrack scanning mode of the microscope to exclude the possibility of crosstalk between the fluorophores. For BiFC analysis, infiltrated tissues were examined by fluorescent microscopy (Zeiss AXIO imager A1, Oberkochen, Germany) with YFP filter (Chroma, Bellows Falls, VT, U.S.A.).

Arabidopsis thaliana agroinfiltration.

A. thaliana wild type (Col-0), knockout (*ca1*), and overexpression (CA1-OE) lines were infiltrated with *Agrobacterium tumefaciens* C58C1 harboring either pBCo-TuHC-YFP or pBA-CE-TuCP-HA. According to Lee and Yang (2006), the overnight *agrobacterium* cell cultures were pelleted and resuspended in induction medium (per liter, 10.5 g of K₂HPO₄, 4.5 g of KH₂PO₄, 1 g of (NH₄)₂SO₄, 0.5 g of NaCitrate, 1 g of glucose, 1 g of fructose, and 4 g of glycerol and 1 mM MgSO₄ and 10 mM MES, adjusted to pH 5.6) supplemented with 100 μ M acetosyringone and cultured for 6 h. Cells were then pelleted and resuspended to an OD₆₀₀ of 0.4 in infiltration medium (10 mM MgSO₄, 10 mM MES; adjusted to pH 5.6). Attached leaves of 4-week-old *A. thaliana* plants were infiltrated using a needleless plastic syringe. Two days postinfiltration, qRT-PCR and Western blotting analysis were conducted.

CA1 antiserum preparation.

The full-length cDNA of AtCA1 was cloned into bacterial protein expression vector pET32a (Novagen, Madison, WI, U.S.A.), and was then introduced into *E. coli* BL21 (NEB, Beverly, MA, U.S.A.) to express AtCA1 protein. Bacteria-expressed AtCA1 with six histidine residues at its N-terminus was purified by Ni²⁺-NTA affinity column. The polyclonal

antibody of AtCA1 (anti-CA1) was prepared from a rabbit immunized with four times weekly injections with the purified AtCA1 protein (1 mg/injection/week). At two weeks after the last injection, the antiserum was collected and used for this study.

Purified AtCA1 protein was resolved in a 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel, was transferred to polyscreen polyvinylidene difluoride (PVDF) membrane (PerkinElmer, Waltham, MA, U.S.A.), and was revealed using Coomassie brilliant blue R250, and the AtCA1 concentration was compared with bovine serum albumin standard protein. (Supplementary Fig. S2). Anti-CA1 polyclonal antiserum was tested by Western blot analysis as described below. After transfer, polyscreen PVDF membranes (PerkinElmer) were saturated with 7% milk in phosphate buffered saline (PBS)-Tween and were incubated 1 h with anti-CA1 antibodies diluted to one-five thousandth in 5% milk in PBS-Tween. After three PBS-Tween washes, membranes were soaked 1 h with antirabbit horseradish peroxidase-conjugated secondary antibody (Jackson Immuno-research, West Grove, PA, U.S.A.) diluted to one-five thousandth, and were revealed by chemiluminescence using a MultiGel-21 system (Top Bio Co., New Taipei City, Taiwan).

Western blotting.

Total protein from 0.5 g of untreated or agroinfiltrated *A. thaliana* leaves were extracted at 2 dpa by grinding the tissues (1:3 w:t/vol) in 3 $\times$ protein sample buffer (3% SDS, 30% glycerol, 0.01% bromophenol blue and 15% β -mercaptoethanol). Samples were incubated at 100°C for 5 min and then the supernatant was collected after short centrifugation (8,000 $\times$ g, 3 min). Proteins were resolved in a 12% sodium dodecyl sulphate-polyacrylamide gel and transferred to polyscreen PVDF membrane (PerkinElmer, Waltham, MA, USA). Membranes were probed with anti-CA1 polyclonal antibodies (as describe above) and the banding signal was quantified by chemiluminescence using a MultiGel-21 system (Top Bio Co.). Then, membranes were stained with Coomassie brilliant blue R250 and the amount of the large subunit of RuBisCO was used as loading control. Banding signal was quantified by Kodak Image system 4000MM (Eastman Kodak Co., Rochester, NY, U.S.A.).

Real time qRT-PCR experiments.

For gene expression experiments, leaf samples from agroinfiltrated or TuMV-infected *A. thaliana* were collected between 2 and 10 days posttreatment and total RNA was extracted using the Plant total RNA purification kit (GMBiolab Co. Ltd.). First-strand cDNA was synthesized from total RNA, using Superscript III reverse transcriptase (Invitrogen) with a poly_dT primer (Table 1). Real time qPCR was performed on a Rotor Gene Q system (Qiagen, Hilden, Germany) by using HOT FIREpol EvaGreen qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and one-tenth of the newly synthesized cDNAs. Transcript accumulation of AtCA1, TuMV HcPro, SID2, WIN3, PR1, and PR5 were amplified and detected, using their specific primer pairs (Table 1). PCR was performed using the following cycling conditions: 95°C for 15 min and 40 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 20 s, followed by a melting curve ramp from 72°C to 95°C for 10 s. All qRT-PCR experiments were conducted on three to four biological replicates for each sample and were repeated four times. For comparison of the data among experimental samples, the real-time PCR results were normalized using the levels of the housekeeping gene Actin8 amplified with Actin8_F/Actin8_R primer pair (Table 1).

HPLC-MS experiments.

To quantify total SA accumulation in *A. thaliana* wild-type (Col-0), knockout (*ca1*), and overexpression (CA1-OE) lines

during TuMV infection, leaf tissues were harvested 2 dpi and were weighed and ground in liquid nitrogen (N₂). SA extraction was performed as follows: 600 µl of methanol (90%) was added to the ground tissues and vortexed, 1 µg of SA diluted in water was added to each sample as recovery control, and the resulting mixture was sonicated for 20 min. After centrifugation at 7,500 × g for 10 min, the supernatant was collected and the pellet was re-extracted with 500 µl of methanol (100%) and recentrifuged at 7,500 × g for another 10 min. The supernatants from both extractions were combined and were air dried using a SpeedVac system (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The dried extract was resuspended in 50 µl of methanol (100%), was vortexed, was sonicated for 5 min, and was spun through a 0.22-µm nylon filter. To measure the amount of extracted SA, the samples were separated by HPLC through a COSMOSIL C18-AR-II column (Nacalai USA, San Diego, CA, U.S.A.), with a particle size of 5 µm and a pore size of 12 nm at a mobile phase flow rate of 0.5 ml/min with a linear gradient from 95% acetic acid (5%) and 5% methanol to 30% acetic acid (5%) and 70% methanol in 35 min, followed by an additional 15-min linear gradient to 90% acetic acid (5%) and 10% methanol. Mass detection was performed on an amaZon speed ion trap system (Bruker, Billerica, MA, U.S.A.). Standard curve made from serial dilution of pure SA in methanol (100%) allowed us to estimate the total SA amount in each sample.

SA treatments.

SA treatment was performed according to Naylor et al. (1998). SA (pH 6.5, neutralized with KOH) of 1 mM concentration was sprayed on the top leaves of plants 4 days prior to TuMV inoculation or agroinfiltration.

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