Salicylic Acid Inhibits the Replication of *Tomato bushy stunt virus* by Directly Targeting a Host Component in the Replication Complex

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Although the plant hormone salicylic acid (SA) plays a central role in signaling resistance to viral infection, the underlying mechanisms are only partially understood. Identification and characterization of SA's direct targets have been shown to be an effective strategy for dissecting the complex SA-mediated defense signaling network. In search of additional SA targets, we previously developed two sensitive approaches that utilize SA analogs in conjunction with either a photoaffinity labeling technique or surface plasmon resonance-based technology to identify and evaluate candidate SA-binding proteins (SABPs) from *Arabidopsis*. Using these approaches, we have now identified several members of the *Arabidopsis* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein family, including two chloroplast-localized and two cytosolic isoforms, as SABPs. Cytosolic GAPDH is a well-known glycolytic enzyme; it also is an important host factor involved in the replication of *Tomato bushy stunt virus* (TBSV), a single-stranded RNA virus. Using a yeast cell-free extract, an *in vivo* yeast replication system, and plant protoplasts, we demonstrate that SA inhibits TBSV replication. SA does so by inhibiting the binding of cytosolic GAPDH to the negative (–)RNA strand of TBSV. Thus, this study reveals a novel molecular mechanism through which SA regulates virus replication.

To defend against fungal, bacterial, and oomycete pathogens, plants have evolved a multilayer immune system comprising pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI), effector-triggered immunity (ETI), and a whole-plant immunity termed systemic acquired resistance (SAR) (Chisholm et al. 2006; Dempsey and Klessig 2012; Durrant and Dong 2004; Jones and Dangl 2006). Many studies have shown that salicylic acid (SA) serves as a key signal molecule for activating PTI, ETI, and SAR following infection by these diverse types of pathogens (An and Mou 2011; Vlot et al. 2009). In comparison, the mechanism through which plants resist viral infection is less well understood. Because viruses are intracellular

pathogens and do not generate extracellular PAMPs, they may not trigger PTI, which is activated via PAMP recognition receptors with extracellular recognition or binding domains (Carr et al. 2010). However, a number of plant resistance (R) proteins that recognize specific viruses have been identified. Like many of the R proteins that recognize bacterial, fungal, and oomycete pathogens, the antiviral R proteins belong to the nucleotide-binding site leucine-rich repeat (NBS-LRR) class, and they trigger ETI and SAR via SA-dependent pathways that appear identical to those activated by nonviral pathogens (Baebler et al. 2014; Sánchez et al. 2010; Soosaar et al. 2005; Takahashi et al. 2002; Vlot et al. 2009).

In addition to these R protein-mediated resistance responses, plants employ RNA silencing to combat viral infection. Activation of this cellular process by viral-derived double-stranded RNAs leads to the targeted destruction of transcripts generated by both RNA and DNA viruses (Carr et al. 2010; Soosaar et al. 2005). The combined observations that (i) expression of RNA-dependent RNA polymerase 1, a critical component of the RNA silencing machinery, is induced by SA treatment in a variety of plants (Liu et al. 2009; Quilis et al. 2008; Xie et al. 2001; Yang et al. 2004; Yu et al. 2003) and (ii) SA-induced gene expression is suppressed by certain viral silencing suppressor proteins (Alamillo et al. 2006; Ji and Ding 2001) suggest that there is at least some level of cross-talk between RNA silencing and the SA-inducible resistance pathways. Other studies have suggested that resistance to various viruses but not bacteria or fungi is mediated via SA-induced activation of the mitochondrial alternative respiratory pathway (Carr et al. 2010; Chivasa et al. 1997; Murphy et al. 1999; Singh et al. 2004), although the mechanism remains to be determined (Carr et al. 2010; Singh et al. 2004). Together, these findings suggest that SA's ability to suppress all three major stages of virus infection, including replication, cell-to-cell movement, and long-distance movement (Murphy et al. 1999; Singh et al. 2004), is dependent on its parallel activation of several antiviral resistance mechanisms.

To gain insights into how SA exerts its many functions, a variety of SA effectors and target proteins have been identified. In addition to catalase (Chen et al. 1993), ascorbate peroxidase (Durner and Klessig 1995), chloroplast carbonic anhydrase (Slaymaker et al. 2002), methyl salicylate esterase (Kumar and Klessig 2003; Vlot et al. 2008), NPR1 (Wu et al. 2012), and its homologs NPR3 and NPR4 (Fu et al. 2012), two new high-throughput screens have identified several more SA-binding proteins (SABPs), including the E2 subunit of α -ketoglutarate dehydrogenase (Tian et al. 2012); the glutathione *S*-transferases GSTF2, GSTF8, GSTF10, and GSTF11 (Tian et al. 2012); and

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the oligopeptidases TOP1 and TOP2 (Moreau et al. 2013). In addition, here, we report that several isoforms of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an abundant enzyme conserved in all organisms (Sirover 1999), are SABPs. Although GAPDH was first recognized for its enzymatic role in glycolysis, it was subsequently found to be involved in diverse nonmetabolic processes such as transcription activation, apoptosis, membrane fusion, DNA replication and repair, and nuclear RNA export (Sirover 1999, 2005; Tristan et al. 2011). In addition, GAPDH was shown to bind RNAs from a range of viruses (De et al. 1996; Petrik et al. 1999; Schultz et al. 1996; Wang and Nagy 2008; Zang et al. 1998).

Tomato bushy stunt virus (TBSV) is a single, positive (+)-strand RNA virus that infects a wide range of plants (Huang and Nagy 2011; Wang and Nagy 2008; White and Nagy 2004). Like other (+)RNA viruses, TBSV replication occurs in the cytosol and is directed by a membrane-bound replicase complex consisting of both viral-encoded proteins and co-opted host proteins (Huang and Nagy 2011; Nagy and Pogany 2012). A proteomics analysis of the TBSV replicase revealed that one of these co-opted host proteins is GAPDH (Serva and Nagy 2006). Subsequent

studies indicated that GAPDH binds the (–)RNA strand of TBSV with greater efficiency than the (+) strand, and this binding is required for asymmetric TBSV replication, in which higher levels of (+)RNA progeny are synthesized by the viral replicase compared with the (–)RNA replication intermediate (Huang and Nagy 2011; Wang and Nagy 2008). Given GAPDH's role in TBSV replication and its identification as an SABP, we tested whether SA affects TBSV replication via its interaction with GAPDH. Here, we demonstrate that GAPDH binds SA and this binding suppresses TBSV replication in vitro and in vivo in yeast cells and *Nicotiana benthamiana* protoplasts by inhibiting the binding of GAPDH to the viral (–)RNA strand, thereby revealing a novel molecular mechanism through which SA mediates viral replication.

RESULTS

Identification and characterization

of *Arabidopsis* GAPDH isoforms as SA-binding proteins.

We previously developed a sensitive photoaffinity labeling approach using the photoreactive SA analog 4-azido SA (4-AzSA) as a probe to identify candidate SABPs (cSABPs) from *Arabidopsis* (Tian et al. 2012). From a total of four pull-down assays, several dozen cSABPs were identified, including several members of the GAPDH family. In *Arabidopsis*, this family is comprised of seven members (The *Arabidopsis* Information Resource) (Muñoz-Bertomeu et al. 2009), among which we identified AtGAPA-1 (At3g26650), AtGAPA-2 (At1g12900), AtGAPB (At1g42970), AtGAPC-1 (At3g04120), and AtGAPC-2 (At1g13440) as cSABPs. AtGAPA-1, AtGAPA-2, and AtGAPB are chloroplast localized and involved in the photosynthetic reductive carbon cycle, while AtGAPC-1 and AtGAPC-2 are cytosolic glycolytic enzymes.

To validate the SA-binding activity of these five AtGAPDHs, we expressed and purified their recombinant, his-tagged proteins from *Escherichia coli*. Two sensitive approaches that utilize SA analogs in conjunction with either a photoaffinity labeling technique or a surface plasmon resonance (SPR)-based technology (Tian et al. 2012) were used for the validation. For the photoaffinity labeling approach, recombinant AtGAPDHs were crosslinked with 4-AzSA by UV irradiation in the presence or absence of SA (adjusted to pH 7.4), followed by immunoblot analysis with anti-SA antibody (α -SA Ab). AtGAPA-1 and AtGAPA-2 crosslinked with 4-AzSA, and the crosslinking was inhibited in the presence of excess SA, confirming their SA-binding activity (Fig. 1A and B). AtGAPC-1 and AtGAPC-2 also crosslinked with 4-AzSA (Fig. 1C and D). Interestingly, crosslinking was enhanced in the presence of increasing concentrations of SA in a dose-dependent manner. This result may suggest that AtGAPC-1 and AtGAPC-2 contain two or more SA-binding sites which interact allosterically, such that binding of SA at one site enhances subsequent binding at the other site or sites. Although AtGAPB also crosslinked with 4-AzSA, this crosslinking was not altered by the presence of SA (data not shown). In summary, crosslinking of AtGAPA-1, AtGAPA-2, AtGAPC-1, and AtGAPC-2 to 4-AzSA was either suppressed or enhanced by the presence of SA, suggesting that their interaction with 4-AzSA represents authentic SA-binding activity.

SA-binding activity was further confirmed for these four GAPDH isoforms using the SPR-based approach developed previously (Tian et al. 2012). When AtGAPA-1 and AtGAPA-2 were passed over a CM5 sensor chip surface to which the SA analog 3-aminoethyl SA (3-AESA) was immobilized, binding signals were observed (Fig. 2). Preincubation with SA reduced these signals in a dose-dependent manner, indicating that the ability of these proteins to bind 3-AESA was inhibited (Fig. 2A and C). Preincubation with the biologically inactive SA analog

Fig. 1. Salicylic acid (SA)-binding activity of several AtGAPDH isoforms was detected by photoaffinity labeling with 4-AzSA. Purified recombinant proteins **A**, AtGAPA-1; **B**, AtGAPA-2; **C**, AtGAPC-1; and **D**, AtGAPC-2 were incubated with (+) or without (–) 4-AzSA in the absence or presence of the indicated concentrations of SA on ice for 1 h and then exposed to UV light (50 mJ). Detection of 4-AzSA-crosslinked proteins was performed using immunoblot analysis with an α -SA antibody. Proteins stained with Coomassie Brilliant Blue R-250 (CBB) served as a loading control.

4-hydroxybenzoic acid (4-HBA) either failed to reduce the binding signal or reduced it to a lesser extent than comparable levels of SA (Fig. 2B and D), further suggesting that binding of AtGAPA-1 and AtGAPA-2 to the chip is a true reflection of their SA-binding activity. SPR analysis also detected binding when AtGAPC-1 or AtGAPC-2 was passed over the CM5 sensor chip surface, but the effect of SA on this binding was more complex (Fig. 3). When these proteins were preincubated with SA, binding to 3-AESA was enhanced in the presence of increasing concentrations of SA (Fig. 3A and D); this result is consistent with those obtained using the photoaffinity labeling approach. However, when the preincubation step was skipped and AtGAPC-1 or AtGAPC-2 was passed over the chip in the presence of various concentrations of SA, the binding signal decreased as the SA concentration increased (Fig. 3B and E). Under this assay condition, 4-HBA was a less effective competitor than SA for 3-AESA binding (Fig. 3C and F). The results from these latter assays lacking the preincubation step are consistent with the hypothesis that AtGAPC-1 contains multiple SA binding sites but that SA binding to the first site is slow, whereas SA binding to an additional site or sites is fast. Thus, in the absence of a preincubation step, SA is able to compete with 3-AESA for binding to the “fast” binding site in AtGAPC-1. By contrast, a preincubation with SA fills the initial binding site and, thereby, enhances binding of 3-AESA in the second, “fast” binding site.

SA inhibits the replication of TBSV.

Cytosolic GAPDHs in yeast and *N. benthamiana* were shown to be required for efficient TBSV replication (Huang and Nagy 2011; Wang and Nagy 2008). Because several isoforms of AtGAPDH are SABPs, we tested whether SA affects TBSV replication using a yeast in vivo replication system, plant protoplasts, and a yeast cell-free extract (CFE) replication assay established previously (Huang and Nagy 2011; Pogany et al. 2008). In the yeast in vivo assay, 300 μ M SA (Fig. 4B) and 1 mM SA (Fig. 4A) inhibited the accumulation of TBSV DI-72 replicon RNA (repRNA) by 50 and 70%, respectively, whereas little to no inhibition was observed in the presence of 4-HBA (Fig. 4). Similarly, the accumulation of full-length TBSV genomic RNA and subgenomic RNAs was inhibited by SA up to approximately 90% in *N. benthamiana* protoplasts (Fig. 5). SA also inhibited repRNA accumulation in the yeast CFE replication assay. In the presence of 2 mM SA, TBSV replication was strongly suppressed; greater SA concentrations led to an almost complete loss of TBSV replication (Fig. 6). By contrast, 2 mM 4-HBA did not substantially affect TBSV replication (Fig. 6).

SA inhibits the binding of AtGAPC-1 to the (–)RNA strand of the TBSV DI-72 repRNA.

It was previously proposed that GAPDH enhances TBSV replication by selectively binding the (–)RNA and retaining it

Fig. 2. Analysis of salicylic acid (SA)-binding activities of AtGAPA-1 and AtGAPA-2 by surface plasmon resonance. Sensograms of **A** and **B**, AtGAPA-1 (1 μ M) and **C** and **D**, AtGAPA-2 (1 μ M) preincubated with the indicated concentrations of SA, 4-hydroxybenzoic acid (4-HBA), or equivalent volumes of 1 $\times$ phosphate-buffered saline buffer on ice for 1 h before being passed over the 3-AESA-immobilized sensor chip surface. The specific binding signal was determined by subtracting the signal generated with the control flow cell from the signal generated with the 3-AESA-bound flow cell.

in the replication complex, thereby promoting multiple rounds of genomic (+)RNA synthesis (Wang and Nagy 2008).

Thus, one possible mechanism through which SA might suppress TBSV replication in the yeast *in vivo* and CFE assays is by disrupting the binding between GAPDH and the (–)repRNA. To assess this possibility, SPR analysis was performed using His-tagged AtGAPC-1 immobilized on an NTA chip. The validity of this approach was first tested by monitoring binding when either the (–) or (+)RNA strand of DI-72 was passed over the sensor chip surface containing immobilized His-AtGAPC-1. Consistent with prior results (Wang and Nagy 2008), the binding of AtGAPC-1 to DI-72(–) was much stronger than its binding to DI-72(+) (Fig. 7A). To test whether SA affects the binding of AtGAPC-1 to DI-72(–), 1 μ M of DI-72(–) was passed over the AtGAPC-1-immobilized NTA chip in the presence of increasing concentrations of SA or 4-HBA. SA suppressed GAPDH binding to DI-72(–) in a dose-dependent manner (Fig. 7B), while 4-HBA was less effective than SA at disrupting this binding (Fig. 7C).

DISCUSSION

SA has been proposed to mediate plant resistance to viral infection by activating multiple defense pathways in parallel (including signaling ETI and SAR), promoting RNA silencing, and activating the mitochondrial alternative respiration pathway (Carr et al. 2010; Singh et al. 2004; Soosaar et al. 2005; Vlot et al. 2009). In this study, we report a novel molecular mechanism through which SA confers virus resistance; it directly targets cytosolic GAPDH, a host factor that is required for TBSV replication. Plus-stranded RNA viruses rely heavily on multiple host factors to support their infection and replication (Nagy and Pogany 2012). Thus, the ability of plants to prevent the co-opting of these proteins could be a valuable

antiviral strategy. Indeed, the observation that replication of *Cucumber necrosis virus*, a tombusvirus closely related to TBSV, was reduced in *GAPDH-C*-silenced *N. benthamiana* (Wang and Nagy 2008), is consistent with the possibility that SA targeting of GAPDH-C helps mediate resistance to this family of viruses. It should be noted, however, that *GAPDH-C*-silenced *N. benthamiana* plants support wild-type levels of replication for *Tobacco mosaic virus*, which belongs to a different super group of (+)RNA viruses and is unrelated to tombusviruses (Wang and Nagy 2008). Thus, modulation of GAPDH-C by SA does not appear to be a global antiviral mechanism. However, SA interacts with many diverse targets (Tian et al. 2012), and it is possible that SA mediates resistance to different subgroups of viruses by targeting other host factors required for their replication. More research will be needed to establish whether modulation of host factors represents yet another common mechanism through which SA mediates antiviral defenses.

The function of cytosolic GAPDHs in glycolysis is conserved from prokaryotes to eukaryotes. In addition, cytosolic GAPDH's RNA binding activity and its function in TBSV replication appear to be conserved. Using a yeast *in vivo* system, Wang and Nagy (2008) found that TBSV replication was suppressed in yeast deficient in the cytosolic GAPDHs TDH2 and TDH3. Expression of AtGAPDH-C in these TDH2- or TDH3-deficient yeast restored TBSV replication, suggesting that the cytosolic GAPDHs from yeast and *Arabidopsis* are interchangeable in supporting TBSV replication. The cytosolic GAPDH promotes TBSV replication through binding to an AU-rich sequence of a 3' proximal region in the (–)-strand RNA and brings the TBSV p92^{pol} replication protein to the promoter region to facilitate initiation of (+)-strand synthesis (Huang and Nagy 2011; Kovalev et al. 2012; Wang and Nagy 2008). Thus, the ability of the yeast TDH2 protein to

Fig. 3. Analysis of salicylic acid (SA)-binding activities of AtGAPC-1 and AtGAPC-2 via surface plasmon resonance. Sensograms of **A** and **C**, AtGAPC-1 (3 μ M) and **D** and **F**, AtGAPC-2 (2 μ M) in the presence of the indicated concentrations of SA, 4-hydroxybenzoic acid (4-HBA), or equivalent volumes of 1 $\times$ phosphate-buffered saline buffer. The mixture was either preincubated on ice for 1 h before being passed over the 3-AESA-immobilized sensor chip surface (**A** and **D**) or passed directly over the chip without preincubation (**B**, **C**, **E**, and **F**). The signal from the mock-immobilized surface was subtracted.

preferentially bind viral (–)RNA is critical for the robust production of (+)RNA progeny (Wang and Nagy 2008). In this report, we demonstrate that AtGAPC-1, like its yeast ortholog, binds the (–)-strand of the DI-72 repRNA with high efficiency, whereas its interaction with the (+)-strand is weak. Although *Arabidopsis* is not a host for TBSV, the above findings strongly suggest that cytosolic GAPDHs from TBSV host plants also will exhibit conserved features with their *Arabidopsis* ortholog, including SA-binding activity, which could regulate their

ability to promote TBSV replication. Our results are consistent with a previous report showing that acetylsalicylic acid induces resistance to TBSV in *Gomphrena globosa* plants (Pennazio and Redolfi 1980).

In addition to conferring TBSV resistance, the interaction between SA and GAPDHs may regulate other plant cellular processes. For example, SA binding might modulate the glycolytic activity of AtGAPC-1 and AtGAPC-2 or the carbon cycle activity of AtGAPC-1 and AtGAPC-2, thereby affecting carbon metabolism. Furthermore, the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate during glycolysis involves the concomitant reduction of NAD⁺ to NADH (Rius et al. 2008), whereas the reverse reaction in the carbon cycle uses NADPH and generates NADP⁺ (Baalmann et al. 1995). Thus, SA binding to GAPDH might alter the NAD⁺/NADH ratio and, thereby, influence cellular redox. Because NADP⁺ buffers free radical formation by accepting electrons generated during photosynthesis (Price et al. 1995), inhibition of the GAPC isoforms by SA also might lead to increased production of reactive oxygen species (ROS). Interestingly, stress conditions that induce the accumulation of ROS, such as drought, cold, salinity, and elicitors of plant defense responses, inhibit the glycolytic activity of cytoplasmic GAPDH (GAPC) (Zaffagnini et al. 2013). Instead, these conditions promote various non-metabolic (moonlighting) functions of GAPC, similar to those observed in animal homologs, via various redox-regulated posttranslational modifications such as sulfenylation, glutathionylation, and S-nitrosylation, as well as nuclear localization (Zaffagnini et al. 2013). Recently, oxidation of GAPC also was shown to promote its ability to bind and stimulate the activity of a novel protein partner, the plasma membrane-associated phospholipase D; this interaction appears to be involved in transducing the ROS signal initiated by drought stress (Guo et al. 2012). Whether SA binds and modulates these other moonlighting activities of GAPC is currently unknown.

In summary, this study has identified several members of the GAPDH protein family as SABPs. Although elucidating the physiological effects exerted when different GAPDH isoforms bind SA clearly will require further study, here we demonstrate that SA binding to AtGAPC-1 disrupts its ability to preferentially bind the (–)RNA of TBSV. Because this secondary activity has been co-opted to promote TBSV replication, our results reveal a novel molecular mechanism through which SA's antiviral activity can be mediated.

Fig. 4. Salicylic acid (SA) inhibits the replication of TBSV in vivo in yeast. Total RNA and protein extracts were prepared from yeast cells 24 h after induction of repRNA replication in the absence (*) or presence of **A**, 1 mM or **B**, 300 μM of SA or its biologically inactive analog 4-hydroxybenzoic acid (4-HBA). Northern blot analysis of total RNA extracts was done with a probe for DI-72(+), the TBSV replicon RNA (repRNA). Ribosomal RNA (rRNA) served as the loading control. The accumulation level of repRNA was normalized based on the rRNA. Immunoblot analysis with anti-His₆ antibody was done to detect the level of His₆-tagged p33, a protein required for tombusvirus replication. The assay confirmed that translation of p33 is not inhibited by SA. Total protein is shown as a loading control. Assays were done in triplicate, with the average level of viral RNA ± standard deviation presented as a percentage of the replication observed in the absence of SA, which was set at 100%.

Fig. 5. Salicylic acid (SA) inhibits full-length TBSV RNA accumulation in *Nicotiana benthamiana* protoplasts. Total RNA was obtained from protoplasts 24 h after electroporation with infectious TBSV RNA present in total RNA prepared from TBSV-infected *N. benthamiana*. Electroporation of protoplasts was performed in the presence of 0, 600 μM, 1 mM, or 2 mM SA or 4-HBA. Top images: Northern blot analysis of total RNA extracts was done with a TBSV (+)RNA-specific probe. Bottom images: Ribosomal RNA (rRNA) served as the loading control. The accumulation level of TBSV RNAs was normalized based on the rRNA.

MATERIALS AND METHODS

Identification of putative SA-binding proteins from *Arabidopsis*.

A photoaffinity labeling approach using the photoreactive SA analog 4-AzSA as a probe and anti-SA antibodies (AbD Serotec) (Tian et al. 2012) was utilized to identify cSABPs from *Arabidopsis*. Total proteins were extracted from the uninoculated distal leaves of *Arabidopsis* Col-0 plants 48 h postinoculation after primary infection with *Pseudomonas syringae* pv. *maculicola* ES4326 expressing AvrRpt2, and cross-linked with 0.5 mM 4-AzSA. The cross-linked proteins were then pulled down with anti-SA antibodies after they were denatured in 4 M urea to expose SA moiety of 4-AzSA from their binding pockets and make it fully accessible to the α -SA Ab, followed by elution in 5 mM SA. The isolated 4-AzSA-linked proteins were identified with tandem mass spectrometry, as described previously (Tian et al. 2012). A label-free semiquantitative proteomics approach was utilized to analyze all proteins from both the 4-AzSA cross-linked sample and the negative control sample. Proteins with a minimum of two spectral counts identified in the 4-AzSA cross-linked sample that were at least twofold more abundant than in the negative control or absent in the negative control were chosen as cSABPs.

Plasmid construction, protein expression, and purification.

The chloroplast transit peptides of AtGAPA-1, AtGAPA-2, and AtGAPB were predicted by ChloroP 1.1 and their coding segments were excluded from the expression plasmids. The

plasmids pET28a-AtGAPA-1(46-396), pET28a-AtGAPA-2(49-399), pET28a-AtGAPB(46-447), and pET28a-AtGAPC-2 were constructed by cloning polymerase chain reaction (PCR)-amplified protein-encoding sequences minus the predicted transit peptides coding segment into *Bam*HI and *Sall* sites of pET28a to express N-terminally His-tagged proteins in *E. coli*. pET28a-AtGAPC-1 plasmids were constructed by PCR-amplified protein-encoding sequences into *Bam*HI and *Hind*III sites of pET28a. The primers used were AtGAPA-1(46-396) (F: 5'-gcgggatccGC AATGGGAAGCAGTGGTGGATACAG-3' and R: 5'-gcggatcgac TCACTTCCAGTTGTTGGCAACAATG-3'), AtGAPA-2(49-399) (F: 5'-gcgggatccGCAATGAGAAGCAATGGTGGATAC-3' and R: 5'-gcggatcgacTCACTTCCAGTTATTGGCAAC-3'), AtGAPB(46-447) (F: 5'-gcgggatccATGAGTAGTATCGGTGGGGA AG-3' and R: 5'-gcggatcgacTCAGTCATAGACTTTGCATTCC T-3'), AtGAPC-1 (F: 5'-gcgggatccATGGCTGACAAGAAGAT TAGGATC-3' and R: 5'-gcgaagcctTTAGGCCTTTGACATGT GGACGATC-3'), and AtGAPC-2 (F: 5'-gcgggatccATGGCTG ACAAGAAGATCAGAATC-3' and R: 5'-gcggatcgacTTAGGC CTTTGACATGTGAAC-3'). Gene-specific sequences are in uppercase letters and the restriction sites are underlined. The His-tagged recombinant proteins were expressed and purified in *E. coli*, as described previously (Kumar and Klessig 2003).

SA-binding activity analysis.

SA-binding activity analyses by photoaffinity labeling and SPR were performed as described previously (Tian et al. 2012).

Analysis of the effect of SA on replication of TBSV DI-72 replicon RNA.

Saccharomyces cerevisiae strain BY4741 (*MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*) was transformed with plasmids pGBK-CUP1-6xHis33/ADH1-DI-72 (*his3* selection) (Mendu et al. 2010) and pGAD-CUP1-6xHis92 (*leu2* selection) (Serviene et al. 2006). Cells were grown at 23°C overnight in SC-LH⁺ media containing bathocuproinedisulphonate copper chelator at the concentration of 100 μ M to inhibit the expression of the *CUP1* promoter-driven viral proteins. The media also contained 1 mM or 300 μ M SA or 4-HBA. The control samples contained only the ethanol solvent. Then, BCS was washed out and CuSO₄ was added to a final concentration of 50 μ M to induce the expression of the viral proteins. The cells were harvested after 24 h and total RNA was extracted and phenol/chloroform purified and precipitated. TBSV DI-72 replicon RNA(repRNA) accumulation was detected by Northern blot analysis with 3'-specific ³²P-labeled probe to repRNA (Panavas and Nagy 2003). The expression of His₆-p33 viral

Fig. 6. Salicylic acid (SA) but not 4-hydroxybenzoic acid (4-HBA) inhibits the replication of TBSV in a yeast cell-free extract (CFE)-based assay. Purified recombinant MBP-p33, MBP-p92, and T7 transcripts of TBSV DI-72 (+) repRNA were added to yeast CFE together with isotope-labeled and unlabeled ribonucleotides. An increasing amount of SA or 4-HBA was added to the assay as indicated. The ³²P-labeled repRNA products were analyzed on denaturing polyacrylamide gel. Assays were done in duplicate.

Fig. 7. Salicylic acid (SA) inhibits the binding of AtGAPC-1 to TBSV DI-72(-) repRNA. **A**, Sensorgrams were generated by passing DI-72(-) or DI-72(+) (1 μ M), in the absence or presence of the indicated concentrations of **B**, SA or **C**, 4-HBA, over an NTA sensor chip surface containing immobilized AtGAPC-1. The specific binding signal was determined by subtracting the signal generated with the control flow cell from the signal generated with the AtGAPC-1-bound flow cell.

protein was analyzed by Western blot with His₆-specific antibody.

Yeast CFEs capable of supporting TBSV replication *in vitro* were prepared as previously described (Pogany et al. 2008). SA or 4-HBA was added to the reactions to the final concentrations of 0, 2, 2.5, and 3.3 mM. The assays were performed at 25°C for 3 h. The newly synthesized ³²P-labeled RNA products were separated by electrophoresis in a 5% polyacrylamide gel with 8 M urea, containing 0.5× Tris-borate-EDTA buffer.

Measuring the effect of SA on replication of TBSV genomic RNA in *N. benthamiana* protoplasts.

Isolation of protoplasts from *N. benthamiana* callus by treatment with 1 g of Cellulysin and 0.2 g of Macerace (Calbiochem) was performed as described previously (Panaviene et al. 2003). Protoplasts were electroporated with 2 µg of a total RNA from TBSV-infected *N. benthamiana* obtained by the phenol-chloroform method (Nagy et al. 2001). SA or 4-HBA were dissolved in dimethyl sulfoxide (DMSO) and were added at a final concentration of 0.6, 1, or 2 mM a few seconds prior to electroporation. DMSO was used as a control applied in the same concentration as for SA or 4-HBA treatment. After electroporation, samples were kept on ice for 30 min, followed by adding 1.7 ml of protoplast culture medium (Kong et al. 1997) to each sample. Protoplasts were incubated in 35-by-10-mm petri dishes in the dark for 24 h at room temperature.

For extraction of total RNA, protoplasts were harvested by centrifugation with no breaks to avoid premature rupture. Cells were broken by vortexing for approximately 30 s with equal volumes of RNA extraction buffer (50 mM NaAc [pH 5.2], 10 mM EDTA, and 1% sodium dodecyl sulfate) and water-saturated phenol followed by a 5-min centrifugation at maximum rpm at 4°C. The aqueous phase was transferred to saturated phenol-chloroform (approximately 250 µl in volume) mixed in a 1:1 ratio, vortexed, and centrifuged again for 5 min at maximum rpm at 4°C. RNA was precipitated from the final aqueous phase in absolute ethanol at -20°C, washed with 70% ethanol, and dissolved in sterile water. The obtained RNA samples were separated on a 1.5% agarose gel and transferred to a Hybond-XL membrane (Amersham) before hybridization with a ³²P-labeled TBSV DI-72-specific probe (Panavas and Nagy 2003). Viral RNA accumulation was normalized based on rRNA using the ImageQuant software and a Typhoon scanner (General Electric).

In vitro transcription of DI-72(+) and DI-72(-) RNAs.

DI-72(+) and DI-72(-) were synthesized by *in vitro* transcription using T7 RNA polymerase (New England Biolabs) following the manufacturer's instructions. DNA templates in the reaction mix were removed using RQ1 DNase (Promega). The synthesized RNAs were purified with a SurePrep RNA cleanup and concentration kit (Fisher Scientific).

Binding analyses of AtGAPC-1 with DI-72(-) and DI-72(+).

The binding analyses of AtGAPC-1 with DI-72(-) and DI-72(+) were performed by SPR using a Biacore 3000 instrument. His-tagged AtGAPC-1 was immobilized on an NTA sensor chip (GE Healthcare), essentially following the manufacturer's instructions. HBS-P buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, and 0.005% [vol/vol] Surfactant P20) supplemented with 50 µM EDTA was used as the running buffer, and HBS-EP buffer (0.01 M HEPES [pH 7.4], 0.15 M NaCl, 3 mM EDTA, and 0.005% [vol/vol] Surfactant P20) was used as dispenser buffer. To immobilize His-AtGAPC-1, 10 µl of 0.5 mM NiCl₂ (NTA reagent kit; GE Healthcare) was flowed over the NTA sensor chip surface, followed by 25 µl of 0.5 µM of His-AtGAPC-1. The immobilization level was stable under this

condition. To test the binding of His-AtGAPC-1 with RNAs of DI-72(-) or DI-72(+) and the effect of SA on the binding, 20 µl of 1 µM RNAs in the absence or presence of various concentrations of SA or 4-HBA was flowed over both the AtGAPC-1-immobilized flow cell and the nonimmobilized control flow cell. The specific binding signal was determined by subtracting the signal generated with the control flow cell from the signal generated with the AtGAPC-1-bound flow cell. To reuse the chip, bound proteins together with RNAs were stripped off by injecting 30 µl of 350 mM EDTA (NTA reagent kit, GE Healthcare). The flow rate used throughout the experiment was set at 10 µl min⁻¹.

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 AbD Serotec: www.abdserotec.com/
 ChloroP 1.1: www.cbs.dtu.dk/services/ChloroP/