Pepino mosaic virus triple gene block protein 1 (TGBp1) interacts with and increases tomato catalase 1 activity to enhance virus accumulation

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SUMMARY

Various plant factors are co-opted by virus elements (RNA, proteins) and have been shown to act in pathways affecting virus accumulation and plant defence. Here, an interaction between *Pepino mosaic virus* (PepMV) triple gene block protein 1 (TGBp1; p26) and tomato catalase 1 (CAT1), a crucial enzyme in the decomposition of toxic hydrogen peroxide (H₂O₂), was identified using the yeast two-hybrid assay, and confirmed via an *in vitro* pull-down assay and bimolecular fluorescent complementation (BiFC) *in planta*. Each protein was independently localized within loci in the cytoplasm and nuclei, sites at which their interaction had been visualized by BiFC. Following PepMV inoculation, CAT mRNA and protein levels in leaves were unaltered at 0, 3 and 6 days (locally) and 8 days (systemically) post-inoculation; however, leaf extracts from the last two time points contained increased CAT activity and lower H₂O₂ levels. Overexpression of PepMV p26 *in vitro* and *in planta* conferred the same effect, suggesting an additional involvement of TGBp1 in potexvirus pathogenesis. The accumulation of PepMV genomic and subgenomic RNAs and the expression of viral coat protein in noninoculated (systemic) leaves were reduced significantly in CAT-silenced plants. It is postulated that, during PepMV infection, a p26–CAT1 interaction increases H₂O₂ scavenging, thus acting as a negative regulator of plant defence mechanisms to promote PepMV infections.

INTRODUCTION

Pepino mosaic virus (PepMV) is a highly infectious potexvirus, which has become a major pathogen of glasshouse tomato (*Solanum lycopersicum*) crops worldwide over the last decade

(Hanssen and Thomma, 2010). Apart from the original Peruvian PepMV isolate (LP), which does not induce symptoms in tomatoes, PepMV isolates are classified on the basis of their sequences into the European (EU), American (US1) and Chilean (CH2) groups (Hanssen and Thomma, 2010). Population dynamics studies have shown that the EU genotype is being gradually overtaken by CH2, and that EU and CH2 mixed infections may extend the host range beyond that of each single isolate (Gómez *et al.*, 2009).

The genome of PepMV is a 6.4-kb single-stranded sense RNA molecule (Aguilar *et al.*, 2002), encoding the RNA-dependent RNA polymerase (RdRp; 164 kDa), three triple gene block (TGB) proteins of 26 kDa (TGBp1), 14 kDa (TGBp2) and 9 kDa (TGBp3), and the coat protein (CP). To facilitate functional analysis of viral and plant genes during infections, full-length infectious clones and a PepMV-based vector expressing recombinant proteins in plants have been constructed (Hasiów-Jaroszewska and Borodynko, 2012; Hasiów-Jaroszewska *et al.*, 2009; Sempere *et al.*, 2011; Yu *et al.*, 2011). In addition, an *in vitro* template-dependent transcriptionally active PepMV RdRp system has been generated for the study of positive- and negative-strand PepMV RNA replication requirements (Osman *et al.*, 2012).

During infection, plant viruses affect the expression of host genes, and host factors have been shown to be required for the replication and spread of the virus to combat host defences and to induce symptoms (Ahlquist *et al.*, 2003; Diez *et al.*, 2000; Strauss and Strauss, 1999; Whitham and Wang, 2004). Transcriptome analyses of tomato seedlings inoculated with mild and aggressive PepMV isolates have revealed, in both cases, an overall repression of the photosynthesis-related genes, plus differential expression of plant defence pathway genes (Hanssen *et al.*, 2011). At the protein level, PepMV CP has been shown to induce and interact with a heat shock cognate protein 70 (Hsc70.3) homologue (Mathioudakis *et al.*, 2012). Moreover, a number of protein–protein and protein–RNA interactions have been reported for the potexvirus prototype *Potato virus X* (PVX) and for *Bamboo mosaic virus*, specifically for TGB1 in interactions with a remorin, a 26S

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proteasome, a CK2 kinase and ARGONAUTE1 (AGO1), affecting virus movement and phloem unloading, protein phosphorylation and RNA silencing, respectively (Chiu *et al.*, 2010; Fridborg *et al.*, 2003; Huang *et al.*, 2012; Mekuria *et al.*, 2008; Modena *et al.*, 2008; Park *et al.*, 2009; Prasanth *et al.*, 2011; Qiao *et al.*, 2009; Raffaele *et al.*, 2009).

Here, we have identified a PepMV TGBp1 (p26)–tomato catalase isozyme 1 (CAT1) interaction which increases CAT activity *in planta* and *in vitro* and corresponds to enhanced PepMV infection levels. A role for PepMV p26 in the regulation of the host oxidative stress-related defence mechanism is suggested.

RESULTS

PepMV p26 interacts with tomato CAT1 in the yeast two-hybrid assay

The expression of the LexA-p26 fusion protein in EGY48 yeast cells harbouring the pJG4-5/tomato cDNA library was confirmed on Western immunoblots using a specific anti-LexA antibody (data not shown). Seven positive clones (denoted p26E2, p26G3, p26I3, p26A3, p26J3, p26G1, p26G2) with the strongest LEU2⁺ phenotype were isolated and sequenced to reveal that all encoded putative polypeptides exhibiting 99%–100% identity to the C-terminal region of the *S. lycopersicum* CAT1 (Accession number P30264; Drory and Woodson, 1992; Fig. 1A). In order to verify the potential interaction in yeast, the complete tomato CAT1 was tested in a new set of two-hybrid experiments following its reverse transcription-polymerase chain reaction (RT-PCR) amplification and subcloning into the pJG4-5 vector. The p26–CAT1 interaction was confirmed by the growth of co-transformed yeast cells with pGILDA-p26/pJG4-5/CAT1 on the selective Gal-Raff-H,W,L plates (Fig. 1B). Co-transformed yeast cells with the combinations pGILDA-p26/pJG4-5, pGILDA/pJG4-5/CAT1 and pGILDA/pJG4-5 did not grow in this medium (Fig. 1B).

Using interactors of PepMV CP (CPJ1, CPG2; which had been identified previously in a yeast two-hybrid screen; Mathioudakis *et al.*, 2012), an assay was performed to further test the specificity of the p26–CAT1 interaction with four heterologous bait/interactor combinations. EGY48 yeast cells were transformed with pGILDA-p26/CPJ1, pGILDA-p26/CPG2, pGILDA-CP/p26G1 and pGILDA-CP/p26A3. As a positive control, EGY48 cells harbouring pGILDA-TGBp1 or pGILDA-CP constructs were transformed with each of the respective positive interactors. EGY48 cells harbouring 'empty' pGILDA were also transformed with each one of the interactors, and all transformants were tested for activation of the LEU2 reporter gene. Only yeast cells that contained the originally identified bait/interactor (homologous) combinations displayed a positive LEU2⁺ phenotype (growth), confirming the specificity of the interactions (data not shown).

In vitro co-immunoprecipitation assays

In a second *in vitro* experiment, PepMV p26 and tomato CAT1 were each overexpressed in *Escherichia coli* BL21 cells, the first as a maltose-binding fusion protein (MBP) to produce MBP-p26 and the second in frame with the S-epitope to produce CAT1-S.tag. Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of both induced and uninduced bacterial cell extracts indicated the production of a protein in the induced bacterial cultures of 71 or 53 kDa, which corresponded to MBP-p26 or CAT1-S.tag, respectively. MBP (negative control) and MBP-p26 fusions were affinity purified on amylose columns before their incubation with CAT1-S.tag. Associated matrix-bound proteins were eluted and subjected to analysis for the presence of MBP fusions and CAT1-S.Tag using α -MBP and α -S.tag antibodies. The CAT1-S.tag was exclusively detected in the eluate from the MBP-p26 column (Fig. 2; lane 3), suggesting the formation of a specific MBP-p26–CAT1-S.tag complex, whereas there was no interaction with MBP (Fig. 2; lane 2). These observations confirmed that, as in the yeast two-hybrid assay, a specific interaction between p26 and CAT1 could be detected *in vitro*.

In situ and *in planta* localization and visualization of p26–CAT1 interaction

Under electron microscopy, immunogold labelling (IGL) of ultra-thin sections of PepMV-infected tomato leaves using a polyclonal α -CAT antibody identified the protein as being predominantly associated with virions and viroplasm in various PepMV-infected tissues (Fig. 3A), but not in *Tomato chlorosis virus* (ToCV)-infected tissues (data not shown). In healthy plants, immunogold-labelled CAT was dispersed evenly throughout the cytoplasm and mitochondria, and, to a lesser extent, in the nucleus (data not shown). In contrast with the case of Hsp70 (Mathioudakis *et al.*, 2012), no CAT could be detected in the P-60 fraction of purified PepMV virions (data not shown).

The binary vector pROK2-GFP-HA was used to express p26 and CAT1 in fusion with the C-terminus of green fluorescent protein (GFP) and to facilitate their *in planta* subcellular tropism in *Agrobacterium*-infiltrated *Nicotiana benthamiana* leaves. The expression of the GFP-p26 and GFP-CAT1 fusions was initially confirmed by Western blot analysis using α -GFP antibodies (data not shown), and fluorescence signals for both protein constructs were observed in the cytoplasm and nucleus, but not in the nucleolus, of epidermal leaf cells of the agroinfiltrated plants (Fig. 3B). In the case of pGFP-p26, additional fluorescent inclusion spots were observed in a few epidermal cells (Fig. 3B).

Bimolecular fluorescent complementation (BiFC) assay was also used to verify the p26–CAT1 interaction *in planta* and to determine its subcellular location in agroinfiltrated *N. benthamiana* plants (Walter *et al.*, 2004). PepMV p26 and CAT1 were fused with

A)

B)

Fig. 1 Seven tomato cDNAs interacting with the *Pepino mosaic virus* (PepMV) triple gene block protein 1 (TGBp1) (p26) identified through the yeast two-hybrid assay using the full-length catalase isozyme 1 (CAT1). (A) Amino acid (aa) sequence alignment of the identified interactors (p26E2, p26G3, p26I3, p26A3, p26I3, p26G1, p26G2) from a tomato cDNA library and the C-termini of CAT1 (Accession number NP_001234827) and CAT2 (Accession number NM_001247257) from *Solanum lycopersicum* and CAT1 from other Solanaceae species (*Solanum tuberosum*, Accession number U27082; *Capsicum annum*, Accession number AF227952; *Nicotiana tabacum*, Accession number U93244; *Nicotiana benthamiana*, Accession number EU998969). The alignment was conducted using the CLUSTAL-X program. (B) Interaction between PepMV p26 and full-length CAT1 using the yeast two-hybrid assay. Co-transformed *Saccharomyces cerevisiae* EGY48 cells grown on nonselective glucose/complete medium lacking histidine and tryptophan (Glu/CM-H,W) (left) and selective galactose-raffinose/complete medium lacking histidine, tryptophan and leucine (Gal-Raff/CM-H,W,L) (right) of the pairwise combinations: I, pGILDA-p26/pJG4-5; II, pGILDA/pJG4-5/CAT1; III, pGILDA/pJG4-5; IV, pGILDA-p26/pJG4-5/CAT1.

the N-terminal and C-terminal fragments of yellow fluorescent protein (YFP) (NYFP, CYFP), respectively. The expression of PepMV p26 and CAT1 from the constructs within infiltrated tissue was confirmed by Western blot analysis using α -HA and α -c-myc antisera (data not shown). Co-expression of pNYFP-p26 and pCYFP-CAT1 induced the reconstitution of functional YFP, further confirming the p26–CAT interaction *in planta*. The YFP fluorescence was observed to be localized throughout the cytoplasm and

in the nucleus, but was absent from the nucleolus (Fig. 3C1–4). In pairwise co-expressions of pNYFP and pCYFP, or their combination with CAT1 or p26 fusions, no YFP fluorescence signal was observed (Fig. 3C5,6), whereas self-interaction of bZIP63 in the nucleus (positive control; Walter *et al.*, 2004) resulted in YFP fluorescence (Fig. 3C7). These results demonstrate a specific interaction in living epidermal cells for split YFP-tagged PepMV p26 and CAT1, and also the subcellular location of this interaction.

Fig. 2 Expression in *Escherichia coli* BL21 (DE3) cells of the maltose-binding fusion protein (MBP) and MBP-p26 fusions, and their *in vitro* interaction with the bacterially expressed CAT1-S.Tag. MBP alone (lane 2) or in fusion with p26 (MBP-p26; lane 3) were amylose affinity purified and incubated with the expressed catalase isozyme 1 (CAT1) labelled with S.Tag (CAT1-S.Tag; lanes 1–3). The eluates were analysed for the presence of the catalase and MBP fusions using α -S.Tag (A) and α -MBP (B) antibodies.

Increased CAT activity *in vitro* and *in planta*

The possibility of CAT1 induction in PepMV-infected tissue was investigated. PepMV-inoculated leaf samples were collected 3 and 6 days post-inoculation (dpi), and the upper systemic leaves at 8 dpi, and CAT1 mRNA expression was examined using semi-quantitative RT-PCR. In these experiments, no significant alteration of CAT1 expression in PepMV-infected leaves was observed, either in comparison with mock-inoculated plants, or between samples taken at different dpi (Fig. 4A). Complementary immunoblot experiments with extracts of the same plant samples probed with α -CAT antiserum also revealed no detectable differences (Fig. 4B). Here, as reported by Mathioudakis *et al.* (2012), PepMV CP levels were seen to progressively increase to significant levels post-inoculation, both locally and systemically; however, CAT expression levels remained unaffected. CAT expression levels in *Cucumber mosaic virus* (CMV)-infected *Arabidopsis* plants were also unaffected (Inaba *et al.*, 2011).

These results suggest that, despite evidence for CAT accumulation at the sites of infection, no induction of CAT occurs in PepMV-infected tomato leaves. In further experiments, CAT activity and H_2O_2 levels were examined in PepMV-infected inoculated (local) leaves at 0, 3 and 6 dpi and noninoculated young (systemic) leaves at 8 dpi. CAT activity was measured as the efficacy with which 100 μ g of total protein extract from PepMV-infected leaves catalysed H_2O_2 breakdown. The CAT activity in leaves collected at 6 and 8 dpi was 1.43- and 1.29-fold higher than in controls, respectively. Extracts from leaves collected at 0 and 3 dpi exhibited no significant difference in CAT activity in comparison with those from mock-inoculated leaves (Fig. 5A). H_2O_2 concentrations were also examined in the same leaf samples. As expected, no significant differences were detected between the H_2O_2 levels in the leaves of mock- or PepMV-inoculated tomato plants at 0 and 3 dpi. However, in leaves collected at 6 and 8 dpi from PepMV-infected plants, the H_2O_2 concentrations were 26% and 49% lower than those of mock-inoculated plants (Fig. 5B). In an additional experiment, a 66% diluted PepMV inoculum conferred a 1.3- and 1.21-fold increase in CAT activity at 6 and 8 dpi, corresponding to 19% and 38% reductions in H_2O_2 levels, respectively. Each of these experiments was performed three times, using three tomato plants for each treatment.

Overall, it was shown that, following PepMV infection, tomato leaves at 6 dpi locally and at 8 dpi systemically contained significantly lower H_2O_2 levels, and that this reduction corresponded to a dose (inoculum)-dependent increase in CAT activity. Subsequently, it was examined whether transient expression of p26 could also confer an increase in CAT activity in *N. benthamiana* leaves. pNYFP-p26 and pNYFP plasmid agroinfiltrated leaves were collected at 4 dpi and following the verification of p26 expression in the former by Western blot assays using α -HA antibody (data not shown), a 31% increase of CAT activity was observed in pNYFP-p26 agroinfiltrated leaves in comparison with the negative control. These data suggested that the presence of p26 alone could elevate CAT activity levels in *N. benthamiana* leaves (Fig. 5C).

PepMV p26 and a number of other plant virus proteins were expressed in *E. coli* BL21 cells to further examine the ability of the complex to increase CAT activity *in vitro*. First, quantification of the endogenous CAT activity in *E. coli* cells harbouring the S.Tag plasmid was carried out using equal amounts of total soluble cell extracts from bacteria expressing the S.Tag or the CAT1-S.Tag proteins. These experiments allowed the comparison of H_2O_2 decomposition rates and essentially indicated that the CAT activity detected originated from the expressed CAT1-S.Tag protein and not from endogenous CAT (data not shown). Identical amounts of soluble bacterial extracts expressing CAT1-S.Tag (140 μ g) were incubated with 1, 5, 10 or 25 μ g of purified MBP-p26 (Fig. 6), and the rate of H_2O_2 decomposition was estimated from each extract. CAT activity was shown to increase by 4%,

Fig. 3 Transmission electron microscopy and immunogold labelling (IGL) experiments in *Pepino mosaic virus* (PepMV)-infected plants using polyclonal catalase antisera, *in planta* subcellular localization of catalase and triple gene block protein 1 (TGBp1) (p26) in *Nicotiana benthamiana* cells and bimolecular fluorescent complementation (BiFC) interaction. (A) IGL of virus-like particles (VLPs) in ultra-thin sections from infected tomato leaves using catalase polyclonal antisera in mesophyll cells. Bar, 140 nm. Gold particle size, 10 nm (some indicated by arrows). Ch, chloroplast. (B) Subcellular localization of catalase and p26 fused with the C-terminus of green fluorescent protein (GFP). The fluorescence was visualized by confocal laser scanning microscopy in the cytoplasm and the nucleus, but not in the nucleolus. In the case of p26, inclusion spots were also observed. (C) Reconstitution of yellow fluorescent protein (YFP) fluorescence was visualized by confocal laser scanning microscopy after co-expression of the fusion proteins. (C1–C3) Co-infiltration of pCYFP-CAT1 and pNYFP-p26 revealed their interaction in the cytoplasm and nucleus, but not in the nucleolus [using YFP and 4',6-diamidino-2-phenylindole (DAPI) filter]. (C3) Overlay image of C1 and C2. (C4) Magnification of the rectangle in image C1. (C5, C6) Co-infiltrations of pCYFP-CAT1 with pNYFP, and pCYFP with pNYFP-p26 (negative controls), respectively. (C7) Co-infiltration of pCYFP-bZIP63 and pNYFP-bZIP63 (positive control) revealed the interaction in the nucleus. c, cytoplasm; i, inclusion spot; n, nucleus; nu, nucleolus. Bars: B, C1–C3 and C5–C7, 75 μ m; C4, 14 μ m.

16%, 22% and 26% respectively, whereas no significant variation in CAT activity was detected in bacterial extracts containing 1, 5, 10 and 25 μ g MBP or 10 μ g of purified MBP fusions made with *Cucurbit yellow stunting disorder virus* (CYSDV)-p25, CYSDV-p22, *Citrus tristeza virus* (CTV)-CP or with bovine serum albumin (BSA)

and lysozyme (data not shown). None of the MBP fusions or the commercially available proteins exhibited any CAT activity (data not shown). These observations allow the observed elevated levels of CAT activity in PepMV infection to be attributed to the p26–CAT-1 interaction.

Fig. 4 Gene expression and protein accumulation of catalase isozyme 1 (CAT1) in *Pepino mosaic virus* (PepMV) mechanically inoculated tomato plants. (A) CAT1 mRNA accumulation in PepMV-infected and mock-inoculated tomato leaves was investigated by semi-quantitative polymerase chain reaction (PCR) at 6 days (locally infected leaves) and 8 days (systemically infected leaves) post-inoculation (dpi). Elongation factor 1a (EF-1a) expression was used as an internal control for normalization. (B) Catalase Western blot analysis in PepMV-infected and mock-inoculated tomato plants at 3 and 6 dpi from locally infected leaves and at 8 dpi from systemically infected leaves. The small unit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein (LC) stained with Coomassie Brilliant Blue served as a protein loading control.

Increased CAT activity promotes PepMV infection

To evaluate the effect of CAT on PepMV RNA and protein accumulation, CAT mRNAs were silenced using the virus-induced gene silencing (VIGS) *Tobacco rattle virus* (TRV)-based agroinfiltration assay in *N. benthamiana* leaves (Hayward *et al.*, 2011). Two regions of *N. benthamiana* CAT1 isozyme (Accession number EU998969), which exhibit high degrees of homology with regions A and B, at the N- and C-termini of tomato CAT1 gene, were amplified and cloned into pTRV2 to generate the plasmids pTRV2-Nb-CatA and pTRV2-Nb-CatB. These constructs were co-agroinfiltrated together with the pTRV1 plasmid into *N. benthamiana* leaves, resulting in -CatA and -CatB plants, respectively (Fig. 7I). Co-agroinfiltration of pTRV1 and pTRV2-PDS (resulting in -PDS plants) was used as a positive control to evaluate the reliability of the VIGS assay, whereas co-agroinfiltration of pTRV1 and pTRV2 (resulting in -TRV plants) was used as a negative control. Six days post-agroinfiltration, when the phenotype in the -PDS plants was apparent (Burch-Smith *et al.*, 2004), the next set of upper leaves was mechanically inoculated with PepMV, and plant tissue collected from the inoculated (local) and next set of upper leaves (systemic) at 4 dpi was used to determine the mRNA and protein levels for CAT, CP and RdRp. Semi-quantitative PCR confirmed the silencing of the CAT genes and suppression of viral

Fig. 5 Catalase activity and hydrogen peroxide (H_2O_2) changes under *Pepino mosaic virus* (PepMV) infection and viral protein overexpression. (A) Changes in catalase activity (expressed as U/mg/min) in PepMV-infected tomato and mock-inoculated plants at 0, 3, 6 (local) and 8 (systemic) days post-inoculation (dpi). (B) Changes in H_2O_2 content [expressed as $\mu\text{mol/g}$ fresh weight (FW)] in PepMV-infected tomato and mock-inoculated plants in a time course experiment. The tested time points were immediately after inoculation (0L), at 3 and 6 dpi (3L, 6L) from locally inoculated leaves and at 8 dpi (8S) from systemically infected leaves. (C) Changes in catalase activity (expressed as U/mg/min) in *Nicotiana benthamiana* leaves with the PepMV p26 overexpressed at 4 days post-infiltration. As control, (mock) leaves infiltrated with the empty binary vector were used. In all the experiments (A–C), bars represent the mean values of three independent experiments $\pm$ SD. The values at 6 and 8 dpi were statistically significantly different from the control at $P < 0.05$ (A, B) and $P < 0.1$ (C).

Fig. 6 Catalase activity changes in the presence of p26 *in vitro*. The activity of 140 µg of bacterially expressed catalase isozyme 1 (CAT1-S.Tag) was estimated (white bar; internal control) and compared with the activity (expressed as U/mg/min) when the same amount of CAT1-S.tag was bound *in vitro* with 1, 5, 10 and 25 µg of purified maltose-binding fusion protein (MBP)-p26 fusion. As controls, 1, 5, 10 and 25 µg of MBP alone and 10 µg of MBP-*Cucurbit yellow stunting disorder virus* (CYSDV) p22, MBP-CYSDV p25, MBP-*Citrus tristeza virus* (CTV) CP, bovine serum albumin (BSA) and lysozyme were used. Bars represent the mean values of three independent experiments $\pm$ SD. The values of MBP-p26 were statistically significantly different from the control (140 µg CAT1-S.Tag) at $P < 0.05$.

replication. Specifically, CAT1 mRNA levels in -CatA and -CatB systemic leaves were 21-fold lower, being barely detectable following 30 PCR cycles (and only after gel overexposure) (Fig. 7II). Moreover, semi-quantitative RT-PCR determination of PepMV CP (sgRNA) and RdRp (gRNA) cDNA levels indicated reductions of 2.7- and 2.3-fold, respectively (Fig. 7III).

These data were further supported by Western blot analysis of CAT protein levels using specific monoclonal antibodies. Reductions in expression of 9% and 13% were indicated in the locally inoculated leaves of the -CatB and -CatA plants, respectively, whereas, in the systemic leaves, the reductions in the level of CAT expression were 92% for -CatB and 95% for -CatA plants, in comparison with the negative control (-TRV) plants (Fig. 7IV). Western blot analysis of PepMV CP levels in the systemic leaves revealed a strong correlation between CAT and PepMV CP levels. Specifically, detectable CP levels were reduced by 10%–12% (locally) and 56%–62% (systemically) when compared with those in the -TRV plants (Fig. 7IV). These data were the outcome of three experiments with two plants per treatment, and remarkably low levels of variability were observed among plants, within groups and between experiments. Moreover, no differences were recorded in the PepMV accumulation levels between -TRV and

wild-type (no TRV agroinfiltration) plants (data not shown), apparently confirming that mixed infection with PepMV and TRV did not affect the accumulation of the former.

DISCUSSION

Reactive oxygen species (ROS) are toxic byproducts of aerobic metabolism (Pitzschke *et al.*, 2006), and their production can be the result of plant/pathogen recognition as part of an oxidative burst with elevated levels of molecules such as H_2O_2 (Mittler, 2002). H_2O_2 molecules are toxic to both plants and pathogens, and typically increase plant defence through the strengthening of host cell walls (Bradley *et al.*, 1992; Lamb and Dixon, 1997) or the activation of defence responses (Alvarez *et al.*, 1998; Dangl and Jones, 2001; Levine *et al.*, 1994; Torres *et al.*, 2006). As a result, tight regulation of the temporal and spatial release of H_2O_2 is required, and this is achieved within plant cells via scavenging enzymes, such as CAT, to restrict damage and/or to fine tune the signalling response (Mhamdi *et al.*, 2012; Mittler *et al.*, 2004).

Here, a specific PepMV p26–tomato CAT1 interaction was identified by *in vitro* and *in vivo* experimentation. Seven clones were isolated using a yeast two-hybrid assay with PepMV p26 as bait,

Fig. 7 Knockdown of catalase plants by virus-induced gene silencing (VIGS) *Tobacco rattle virus* (TRV)-based assay affecting the *Pepino mosaic virus* (PepMV) accumulation in *Nicotiana benthamiana* plants. (I) Phenotypes of *N. benthamiana*-TRV plants (controls; A) and the silenced -CatA (B) and -CatB (C) after 10 days of agroinfiltration and 4 days of PepMV infection. The photographs were taken after the collection of plant tissue for mRNA and protein analysis. (1), (2) and (3) indicate the TRV-agroinfiltrated, PepMV-mechanically inoculated (local) and PepMV-systemically infected leaves, respectively. (II) Semi-quantitative polymerase chain reaction (PCR) confirmed the silencing of catalase isozyme 1 (CAT1) mRNA levels in both -CatA and -CatB plants. PCR was performed using the primers of CatB and CatA fragments for the mRNA analysis of CatA and CatB, respectively. (III) Semi-quantitative PCR revealed the reduction of the coat protein (CP) and RNA-dependent RNA polymerase (RdRp) subgenomic and genomic mRNA levels. (IV) Western blotting in systemic leaves of *N. benthamiana* plants agroinfiltrated with pTRV2-Nb-CatA or pTRV2-Nb-CatB for catalase gene silencing and TRV plants (control), 4 days after PepMV infection, using α -catalase (top panel) and α -PepMV CP (middle panel) antibodies. The small unit of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) protein (LC) stained with Coomassie Brilliant Blue served as protein loading control.

and all were shown to have 100% identity at the amino acid level with the C-terminus of tomato isozyme CAT1 and a 69% identity with CAT2 (Drory and Woodson, 1992; Accession number NM_001247257). Although a p26–CAT2 interaction cannot entirely be excluded, the CAT1 isozyme was selected and used in all downstream experimentation. Following PepMV infection, tomato CAT1 co-localized at the sites of infection with virions and viroplasm, as was seen for Hsc70 (Mathioudakis *et al.*, 2012). However, in this case, protein expression and mRNA levels were not affected, and CAT1 could not be detected in the purified virion fraction. Initially, it was assumed that a spatial regulation of stable CAT levels at the areas of virus accumulation was required. However, further investigation revealed that CAT activity was increased and H₂O₂ concentrations were decreased in tomato plants inoculated with PepMV in systemically infected leaves at 6 and 8 dpi, respectively. In follow-up experiments, both the transient expression of PepMV p26 in *N. benthamiana* and the bacterial overexpression of p26 *in vitro* elevated CAT activity, suggesting an involvement of the p26–CAT interaction in the reduction in H₂O₂ levels. Furthermore, the VIGS -Cat-A and -Cat-B plants, with suppressed CAT gene expression, revealed a negative correlation between CAT gene silencing and PepMV RdRp RNA and CP expression (54%–59% and 56%–62%, respectively) locally and systemically. These data further support the hypothesis that, in PepMV-infected plants, p26 plays an indirect role as a negative regulator of plant defence mechanisms by increasing the activity of CAT.

Among the cellular events that characterize plant defence are rapid and late oxidative bursts, cell wall strengthening, the induction of pathogenesis-related defence gene expression and resistance in the form of hypersensitive reaction (HR) necrosis. ROS induction by plant pathogen elicitors often occurs in two distinct phases: (i) short and early responses within minutes or hours (phase I), which do not always correlate with resistance; and (ii) later ROS production within hours or days (phase II), which is related directly to the resistance/susceptibility of the plant to the pathogen. Both the observed increase in CAT activity and the reduction in PepMV RNA and protein levels in -Cat-A and -Cat-B noninoculated (systemic) leaves were observed at a few dpi, further supporting an association with defence-related genes, possibly in conjunction with other transmitted signals (systemic acquired resistance) which may involve intermediates such as salicylic acid (SA) (Chen *et al.*, 1993; Durner and Klessig, 1996; Fukamatsu *et al.*, 2003). Interestingly, results by Malcuit *et al.* (1999) and Sánchez *et al.* (2010) have shown that the PVX TGBp1 (p25) protein, with the involvement of SA, is an elicitor of HR in *N. benthamiana*.

Similar to our observations, transgenic *N. benthamiana* plants with suppressed CAT exhibited reduced levels of pathogen infection and enhanced plant defence (resistance) reactions, typically associated with increased levels of ROS (Chamnongpol *et al.*,

1998; Willekens *et al.*, 1997), whereas plants that overproduced CAT were shown to have decreased resistance to pathogen infection (Polidoros *et al.*, 2001). It is generally considered to be in the interest of pathogen(s) to hinder ROS elevation and, consequently, downstream defence-associated phenomena (Torres *et al.*, 2006). However, some pathogens induce ROS, for example, necrotrophs, for which cell death facilitates subsequent infection (Govrin and Levine, 2000), and *Botrytis*, which inactivates the protective mechanism at advanced stages of infection. ROS may also be produced together with increased levels of ROS detoxification enzymes during compatible interactions with plant viruses (Allan *et al.*, 2001; Clarke *et al.*, 2002). Previously, the silencing suppressor CMV 2b has been reported to interfere with SA-mediated virus resistance (Ji and Ding, 2001) and, more recently, to represent a necrosis determinant, following interaction with and inactivation of *Arabidopsis* CAT-3 (Inaba *et al.*, 2011). The protein–protein interaction identified here furthers the knowledge concerning the multi-functional potexvirus TGBp1, unveiling its role in virus-induced regulation of plant homeostasis and defence system(s).

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

S. lycopersicum cv. Boludo (Seminis Vegetable Seeds Europe, Enkhuizen, the Netherlands) and *N. benthamiana* were used for the experiments. Tobacco seeds were sown on Murashige–Skoog medium to germinate for 10 days at 25 °C and 16 h : 8 h light/dark. Tomato and tobacco seedlings were grown under standard conditions in growth chambers.

Tomato cDNA library screening

A tomato cDNA library, cloned into the pJG4-5 vector (Zhou *et al.*, 1995; kindly provided by Professor G. Martin, Purdue University, West Lafayette, IN, USA), was used to screen for interacting proteins. The cDNAs were expressed as fusions to a portable transcriptional activation domain (the acid blob B42AD) and a haemagglutinin (HA) epitope under the control of the GAL1 promoter (Gyuris *et al.*, 1993). The full-length cDNA of TGBp1 was amplified from the pLMPepMV15 clone (PepMV-Sp13 isolate, kindly provided by M. Aranda, Centro de Edafología y Biología Aplicada del Segura, Murcia, Spain; Aguilar *et al.*, 2002) using PepMV p26-F and PepMV p26-R primers (Table 1), and cloned into the pGILDA vector (Clontech Laboratories, Inc., Palo Alto, CA, USA) via the *Bam*HI–*Xho*I sites to generate the construct pGILDA-p26, which was used as the screen 'bait'. pGILDA-p26 expresses a p26–LexA DNA-binding domain fusion under the control of the GAL1 promoter. The yeast reporter *Saccharomyces cerevisiae* strain EGY48 cells were co-transformed with pGILDA-p26 bait and pJG4-5/tomato cDNA library, as described previously (Mathioudakis *et al.*, 2012). Co-transformed pGILDA-p26 with pJG4-5 or pGILDA with pJG4-5/cDNA library were used as negative controls for the interaction of p26 or the library in the absence of the cDNA library and p26, respectively. The putative positive library plasmids were extracted, reintroduced back into

Table 1 List of oligonucleotide primers used in this study for plasmid construction.

Primer	Primer sequence (5'–3')*	Restriction site	Construct	Application assay
PepMV p26-F	GGATCCAAATGGAAGATCAACTCTGATTAA	<i>Bam</i> HI	pG-p26	Subcloning to pGILDA
PepMV p26-R	CTCGAGCTAGGAGTTAGACCTGGCATTAG	<i>Xho</i> I	pG-p26	Subcloning to pGILDA
Catalase-F	GAATTCGATCCCTCTAAGTATCGC	<i>Eco</i> RI	pG-Catalase	Yeast two-hybrid
Catalase-R	GTCGACCATTGTAGGCTTCACAGT	<i>Sal</i> I	pG-Catalase	Yeast two-hybrid
Catalase-BiFC-F	GGCGGCCCATGGATCCCTCTAAGTATCGC	<i>Asc</i> I	pG-YFP-Catalase(A/S)	BiFC
Catalase-BiFC-R	GTCGACCATTGTAGGCTTCACAGT	<i>Sal</i> I	pG-YFP-Catalase(A/S)	BiFC
Catalase-GFP-F	CCCGGGTATGGATCCCTCTAAGTATCGC	<i>Sma</i> I	pG-GFP-Catalase(S/A)	Subcellular location
Catalase-GFP-R	GGCGGCCCATGGATGGCTTCACAGT	<i>Asc</i> I	pG-GFP-Catalase(S/A)	Subcellular location
Catalase-EXP-F	CCATGGCCGATCCCTCTAAGTATCGC	<i>Nco</i> I	pG-Exp-Catalase(N/S)	<i>In vitro</i> expression
Catalase-EXP-R	GTCGACCATTGTAGGCTTCACAGTGAGACG	<i>Sal</i> I	pG-Exp-Catalase(N/S)	<i>In vitro</i> expression
PepMV p26-BiFC-F	ACTAGTATGGAAGATCAACTCTGATT	<i>Spe</i> I	pG-YFP-p26(S/S)	BiFC
PepMV p26-BiFC-R	GTCGACGGAGTTAGACCTGGCATTAG	<i>Sal</i> I	pG-YFP-p26(S/S)	BiFC
PepMV p26-MBP-F	GGATCCATGGAAGATCAACTCTGATTAA	<i>Bam</i> HI	pG-Mal-p26(B/S)	<i>In vitro</i> expression
PepMV p26-MBP-R	GTCGACCTAGGAGTTAGACCTGGCATTAG	<i>Sal</i> I	pG-Mal-p26(B/S)	<i>In vitro</i> expression
PepMV p26-GFP-F	GGATCCATGGAAGATCAACTCTGATT	<i>Bam</i> HI	pG-GFP-p26(B/A)	Subcellular location
PepMV p26-GFP-R	GGCGGCCCGGAGTTAGACCTGGCATTAG	<i>Asc</i> I	pG-GFP-p26(B/A)	Subcellular location
sqCatalase-F	GCAGCTCCCAGTTAATGCTC	n.i.	–	Semi-quantitative PCR
sqCatalase-R	CAAACCTCGAGGGCAAATAA	n.i.	–	Semi-quantitative PCR
sqEF-1a-F†	GATTGGTGGTATTGGAAGTCTC	n.i.	–	Semi-quantitative PCR
sqEF-1a-R†	AGCTTCGTGGTGATCTC	n.i.	–	Semi-quantitative PCR

BiFC, bimolecular fluorescent complementation; PCR, polymerase chain reaction.

*Restriction enzymes used in the primers are shown in italic type; n.i., not included.

†Primers used in Rotenberg *et al.* (2006).

the original reporter strain in the presence or absence of LexA-TGBp1, and retested to examine the reproducibility of the interactions. The isolated positive clone inserts were identified by sequencing.

Yeast two-hybrid assay

A yeast two-hybrid assay using the full-length tomato CAT1 and the PepMV p26 (TGBp1) cDNAs was performed. To create the prey plasmid pJG4-5/CAT1, total RNAs were extracted from tomato plants using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and full-length cDNAs of CAT1 were prepared by RT-PCR using the primer set Catalase-F and Catalase-R (Table 1), and cloned into pGEM-T easy vector (pG-Catalase). DNA inserts were subcloned into pJG4-5 vector via their restriction sites and in frame with the GAL1 activation domain. EGY48 cells were co-transformed for each CAT1 construct with the bait pGILDA-p26 and grown on selective agar plates, as described previously (Mathioudakis *et al.*, 2012). The co-transformed pJG4-5 and pGILDA empty plasmids with pGILDA-p26 and pJG4-5/CAT1, respectively, were used as negative controls.

In vitro protein binding pull-down assay

To obtain bacterially expressed proteins, the open reading frames (ORFs) of CAT and p26 were PCR amplified and cloned into the pRSF1b (Novagen, Madison, WI, USA) and pMAL-c2x (New England BioLabs, Ipswich, MA, USA) vectors via *Nco*I-*Sal*I and *Bam*HI-*Sal*I sites, respectively. The CAT1-S.Tag and MBP-p26 fusion proteins were expressed in *E. coli* BL21-(DE3) cells. Cell lysis was performed in column buffer [CB: 10 mM Tris, pH 8.0, 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 25% glycerol] by sonication (10 s for 1.5 min with intermittent incubation on ice). The soluble fraction was collected and purified using S.Tag/amylose resin, according to the manufacturer's instructions.

For the *in vitro* binding assay, 200 µg of MBP (negative control) or the MBP-p26 amylose-attached samples were washed five times with 2.5 mL of CB and incubated with 50 µg of CAT1-S.Tag at room temperature for 1 h with gentle rotation. The unbound proteins were removed by six washes (3 mL of CB each) and the protein complexes captured on the amylose matrix were eluted with 10 mM maltose in 8 × 250-µL fractions. The proteins were resuspended in 2 × Laemmli buffer, subjected to SDS-PAGE and either stained with Coomassie Brilliant Blue or blotted onto polyvinylidene fluoride (PVDF) membranes, as described previously (Mathioudakis *et al.*, 2012). For protein detection, anti-MBP (rabbit polyclonal; New England BioLabs) and anti-S.Tag (mouse monoclonal; Novagen), conjugated to alkaline phosphatase, were used.

BiFC assay

N. benthamiana fully expanded leaves were co-agroinfiltrated with pCYFP-CAT1 and pNYFP-p26 constructs, together with the *Tomato bushy stunt virus* (TBSV) p19 RNA-silencing suppressor, to study their *in planta* interaction following the protocol of Mathioudakis *et al.* (2012). The plant binary vectors used in BiFC experiments were pSPYNE-35S and pSPYCE-35S, allowing the expression of the proteins of interest as fusions to the N-(NYFP) or C-terminal half (CYFP) of YFP, together with a c-myc (pSPYNE-35S) or HA (pSPYCE-35S) affinity tag (Walter *et al.*, 2004). To generate the above constructs, the full-length coding sequences of the tomato CAT1 and p26 genes were PCR amplified using the pG-Catalase and pLM-PepMV15 constructs as templates and the primers listed in Table 1. DNAs were inserted, in frame, into the binary vectors pSPYCE-35S and pSPYNE-35S via *Asc*I-*Sal*I and *Spe*I-*Sal*I sites, respectively. The bZIP63 transcription factor was used as a positive control (Walter *et al.*, 2004), and combinations of pSPYNE-35S (pNYFP) and pSPYCE-35S (pCYFP) plasmids with the plant and viral constructs, respectively, were used as negative controls. The

images were recorded on SP2 and SP5 (Leica Microsystems, Heidelberg, Germany) confocal fluorescence laser scanning microscopes, and the YFP fluorescence was visualized with excitation at 514 nm and emission at 535–545 nm from the epidermal cell layers at 3–4 dpi.

Protein subcellular localization in *N. benthamiana* epidermal cells

To generate the sGFP fusions with CAT1 (pGFP-CAT1) or TGBp1 (pGFP-p26), the full-length coding fragments were PCR amplified using the primers listed in Table 1, and cloned into the binary vector pROK2-GFP-HA (kindly provided by Dr T. Canto, Centro de Investigaciones Biológicas, Madrid, Spain) via *Sma*I-*Asc*I and *Bam*HI-*Asc*I sites, respectively. The constructs were introduced into *Agrobacterium* C58C1 cells and co-infiltrated, together with the TBSV p19 suppressor, into *N. benthamiana* leaves, as for the BiFC assay. On days 2 and 3 post-infiltration, the epidermal cells were observed with excitation at 488 nm and emission at 500–530 nm for the GFP fluorescence, as described for the BiFC method.

Plant inoculation and virus purification

The Spanish PepMV-Sp13 isolate (EU type) was used to inoculate tomato and *N. benthamiana* plants (Mathioudakis *et al.*, 2012). For virus purification, a modified protocol was applied to systemically infected tomato leaves at 12 dpi (Mathioudakis *et al.*, 2012). The fractions which were analysed were the pellet from a low-speed centrifugation (LS-12), and the supernatant (Sup-60) and resuspended virus pellets (P-60) collected following a high-speed centrifugation (253 000 *g* for 3 h). The purified virion concentration was estimated based on known concentrations of BSA. The same experimental procedure was also followed in healthy tomato plants.

IGL and transmission electron microscopy (TEM) analysis

Leaves from healthy and PepMV mechanically inoculated tomato plants (cv. Boludo) were harvested 2 weeks post-inoculation, together with those from ToCV-infected tomatoes (control). Small pieces (0.1 × 1 cm²) of leaves were processed as described by Medina *et al.* (1998). IGL analysis followed the protocol of Pinner *et al.* (1993) on ultra-thin sections (70 nm) mounted on Formvar® carbon-coated gold grids (200 mesh). The analysis was performed using an antiserum raised against PepMV CP (rabbit polyclonal) and four antisera raised against CAT (three mouse monoclonal and one rabbit polyclonal; AbFrontier, Seoul, Korea), as described previously (Mathioudakis *et al.*, 2012). Secondary antibodies, goat-anti-rabbit immunoglobulin G (IgG) (Electron Microscopy Sciences, Hatfield, PA, USA) and goat-anti-mouse IgG (Sigma, St. Louis, MO, USA), conjugated with 25- and 10-nm gold, were used for CP and CAT.

Time course protein analysis and semi-quantitative PCR

Tomato plants were inoculated with identical volumes of virus inocula, whereas mock plants were inoculated with a phosphate buffer (0.5 M,

pH 7.0). Immunoblot analysis was used to study CAT accumulation in PepMV-infected and mock-inoculated plants using CAT-specific monoclonal antiserum (kindly provided by Dr D. Klessing, Boyce Thompson Institute for Plant Research, New York, USA). Three different time periods (3 and 6 dpi for locally infected leaves and 8 dpi for systemically infected leaves) were compared. In all experiments, leaves from three different plants were used at each of the different time periods tested. Viral infection was confirmed by immunoblot analysis with anti-PepMV CP antibodies (Neogen, Scotland, UK) as in Mathioudakis *et al.* (2012).

A semi-quantitative PCR was conducted in PepMV-infected and mock-inoculated tomato plants at 6 and 8 dpi, as described previously (Mathioudakis *et al.*, 2012), using specific primer pairs (Table 1) to amplify a partial fragment of the gene. Elongation factor 1a (EF-1a) gene was used as an internal (normalization) control for transcription stability (Rotenberg *et al.*, 2006). The PCR DNA bands were visualized using the Gel Doc™ XR imager system, and their intensities were quantified as absorbance units using Quantity One analysis software (Bio-Rad Laboratories, Hemel Hempstead, UK).

Detection of H₂O₂ and CAT activity in infected plants

Plant tissue (0.1 g) from PepMV-infected and mock-inoculated plants, collected in the previous time course analysis, was used for the determination of H₂O₂ content and CAT activity. The time point 0 dpi (immediately after plant inoculation) was also included in this experiment. For the H₂O₂ concentration determination, the protocol of Alexieva *et al.* (2001) was applied, with measurements taken at A₃₉₀ and expressed as μmol/g fresh weight (FW). The calculations were based on a standard curve created using standard dilutions of 30% H₂O₂ (Sigma).

The method used for the determination of CAT activity was that of Aebi (1984), with slight modifications. Total proteins were extracted from plant tissue chilled in liquid nitrogen and homogenized in 1 mL of extraction buffer [50 mM KH₂PO₄, pH 7.0, 1:9 Triton 0.1%, 1 mM phenylmethylsulfonylfluoride (PMSF), 2% polyvinylpyrrolidone (PVP)]. Following centrifugation at 15 000 *g* for 20 min at 4 °C, the protein content of the supernatant was determined by the Bradford assay (Bradford, 1976; Bio-Rad Laboratories). The reaction mixture included 100 μg of total proteins, 10 mM H₂O₂ and phosphate buffer to 3 mL. As a blank, equal amounts of proteins were used without H₂O₂. The degradation of H₂O₂ was recorded every 20 s as A₂₉₀, and the CAT activity was expressed as U/mg/min of total protein extract.

In planta and in vitro determination of CAT activity in the presence of p26

The pNYFP-p26 construct was used for transient overexpression of TGBp1 in *N. benthamiana* leaves, together with the TBSV p19 suppressor, as for the BiFC method. At 4 dpi, the agroinfiltrated tissue was subjected to CAT assay, as described in the previous section. *N. benthamiana* leaves co-infiltrated with pNYFP and TBSV p19 were used as negative control.

The activity of the bacterially expressed CAT1 from the *in vitro* binding experiment was estimated. The reaction mixture was as described in the above section for the plants, but using 140 μg of total bacterial protein extract. To study the effect on CAT1 activity after binding with the p26 fusion protein, 140 μg of CAT1-S.Tag were incubated with 1, 5, 10 and

25 µg of MBP-p26 for 1 h. As negative controls for the *E. coli* endogenous CAT activity, the S.Tag expressed fusion was used, whereas the incubation of CAT1-S.tag with the same amount of MBP fusion was also used as a control. Combinations of CAT1-S.Tag with 10 µg of the MBP-fused viral proteins MBP-CYSDV p22, MBP-CYSDV p25, MBP-CTV CP and commercially prepared BSA and lysozyme were used to test the specificity of the results.

Silencing of CAT by a VIGS TRV-based assay

For the CAT gene silencing, the vectors pTRV1 and pTRV2 (Liu *et al.*, 2002) were used (kindly provided by Professor S. Dinesh-Kumar, University of California, Davis, CA, USA). Two fragments from the homologous *N. benthamiana* CAT1 gene, one from the N- (CatA) and one from the C-terminus (CatB), of 305 and 439 bp in size, respectively, were PCR amplified from total RNA extracts. The primers are listed in Table 1. The DNA amplicons were cloned into the pTRV2 vector via *EcoRI-XhoI* and *BamHI-XhoI*, respectively, to generate the plasmids pTRV2-Nb-CatA and pTRV2-Nb-CatB. The construct pTRV2-PDS was used as a silencing control (kindly provided by S. Dinesh-Kumar). All of the constructs were introduced into the *Agrobacterium* GV2260 strain by electroporation, and infiltrated into young third to fourth leaves of *N. benthamiana* as described previously (Hayward *et al.*, 2011).

Six days after the TRV agro-infiltration, the same leaves were mechanically inoculated with PepMV. Total RNAs and protein extracts were obtained from the inoculated and systemically infected leaves, 4 days after PepMV inoculation, to permit the estimation of the protein and mRNA levels for CAT, CP and RdRp using Western blotting and semi-quantitative PCR, employing the primers listed in Table 1.

Protein extraction and immunoblot analysis

For the protein extraction, for yeast two-hybrid experiments, 20 mL of a Gal-Raf/CM-H culture were used, whereas, for *in planta* (BiFC, GFP constructs) and time course experiments, 0.1 g of plant tissue was used as described previously (Mathioudakis *et al.*, 2012). Specific proteins were detected by Western blotting (Mathioudakis *et al.*, 2012) using the anti-LexA rabbit polyclonal (pGILDA construct; kindly provided by Dr E. Golemis, Fox Chase Cancer Center, Philadelphia, PA, USA), anti-HA rat polyclonal (Roche Diagnostics, Burgess Hill, UK; pJG4-5 and pSPYCE-35S constructs), anti-c-myc mouse monoclonal (Roche; pSPYNE-35S constructs) and anti-GFP (Abcam, Cambridge, UK; GFP constructs) antibodies, all conjugated with alkaline phosphatase. The products were Western blotted and visualized using a nitroblue tetrazolium/5-bromo-4-chloroindol-3-yl phosphate (NBT/BCIP) substrate (Promega, Madison, WI, USA).

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