Interaction between *Cucumber mosaic virus* 2b protein and plant catalase induces a specific necrosis in association with proteasome activity

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Abstract

Key message *Cucumber mosaic virus* (CMV) can induce a specific necrosis on *Arabidopsis* through the interaction between the CMV 2b protein and host catalase, in which the ubiquitin–proteasome pathway may be involved.

Abstract We previously reported that the CMV 2b protein, the viral RNA silencing suppressor, interacted with the H$_2$O$_2$ scavenger catalase (CAT3), leading to necrosis on CMV-inoculated *Arabidopsis* leaves. We here confirmed that CMV could more abundantly accumulate in the CAT3-knockout mutant (cat3), and that CAT3 makes host plants a little more tolerant to CMV. We also found that the necrosis severity is not simply explained by a high level of H$_2$O$_2$ given by the lack of CAT3, because the recombinant CMV, CMV-N, induced much milder necrosis in cat3 than in the wild type, suggesting some specific mechanism for the necrosis induction. To further characterize the 2b-inducing necrosis in relation to its binding to CAT3, we conducted the agroinfiltration experiments to overexpress CAT3 and 2b in *N. benthamiana* leaves. The accumulation levels of CAT3 were higher when co-expressed with the CMV-N 2b (N2b) than with CMV-Y 2b (Y2b). We infer that N2b made a more stable complex with CAT3 than Y2b did, and the longevity of the 2b–CAT3 complex seemed to be important to induce necrosis. By immunoprecipitation (IP) with an anti-ubiquitin antibody followed by the detection with anti-CAT3 antibodies, we detected a higher molecular-weight smear and several breakdown products of CAT3 among the IP-proteins. In addition, the proteasome inhibitor MG132 treatment could actually increase the accumulation levels of CAT3. This study suggests that the host proteasome pathway is, at least partially, responsible for the degradation of CAT3, which is manifested in CMV-infected tissues.

Keywords *Cucumber mosaic virus* · Catalase · Ubiquitin–proteasome · 2b Protein *Arabidopsis thaliana* · Necrosis

Abbreviations

CAT Catalase
CMV Cucumber mosaic virus
H$_2$O$_2$ Hydrogen peroxide
HR Hypersensitive reaction
PCD Programmed cell death
ROS Reactive oxygen species

Introduction

Reactive oxygen species (ROS), such as hydrogen peroxide (H$_2$O$_2$) and O$_2^-$, are generated during numerous physiological processes, including photosynthesis, plant development, and resistance responses against pathogens. H$_2$O$_2$ serves as an important molecular messenger to induce a form of programmed cell death (PCD) and especially...
called as the hypersensitive reaction (HR) in plant–pathogen interactions. Catalase is one of the most important antioxidant enzymes that catalyze the decomposition of H$_2$O$_2$, thus playing a role in protecting cells from H$_2$O$_2$ toxicity. *Arabidopsis* has three catalase enzymes (Frugoli et al. 1996), and catalase 3 (CAT3) is the most abundantly expressed and controlled by a circadian rhythm. CAT3 expression is enhanced with plant age and is accompanied by H$_2$O$_2$ accumulation in vascular bundles (Zimmermann et al. 2006; Hu et al. 2010). CAT3 has been found to interact with several proteins, such as nucleoside diphosphate kinase, NDK1 (Fukamatsu et al. 2003), class 3 sucrose-nonfermenting 1-related kinase, SOS2 (Verslues et al. 2007), and LESION SIMULATING DISEASE1, LSD1 (Li et al. 2013), and even a viral protein, the 2b protein (2b) of *Cucumber mosaic virus* (CMV) (Inaba et al. 2011). The interactions between catalase and other proteins may cause the diverse effects on catalase’s function. For example, Zou et al. (2015) demonstrated that the interaction between CAT3 and calcium-dependent protein kinase 8 (CPK8) enhanced CAT3 activity to maintain H$_2$O$_2$ homeostasis in response to drought stress. On the other hand, some interactions cause functional disturbance of catalase resulting in the accumulation of H$_2$O$_2$ and subsequent cell death (i.e., necrosis); the interactions of CAT3 with LSD1 and with 2b have been reported to be involved in necrosis on *Arabidopsis* (Inaba et al. 2011; Li et al. 2013).

CMV, the type member of the genus *Cucumovirus*, has a broad host range of more than 1000 plant species. It has a tripartite genome consisting of RNAs 1–3. RNAs 1 and 2 encode viral helicase and replicase, respectively, for viral replication, and RNA 3 encodes the viral movement protein 3a. RNA 4, a subgenomic RNA derived from the 3’ half of RNA 3, is the mRNA for the coat protein, while RNA 4A, a subgenomic RNA from RNA 2, encodes 2b (Ding et al. 1994). 2b is known as an RNA silencing suppressor (RSS) and also functions in viral cell-to-cell and long-distance movement (Ding et al. 1995; Ji and Ding 2001; Soards et al. 2002; Shi et al. 2003; Goto et al. 2007). In addition, 2b contains nuclear localization signals (NLSs) that are required for the manifestation of viral symptoms and for RSS activity (Lucy et al. 2000; Lewsey et al. 2009). We previously found a protein–protein interaction between 2b and *Arabidopsis* CAT3, which apparently causes H$_2$O$_2$ accumulation and subsequent necrosis in infected *Arabidopsis* leaves. The interaction between 2b and CAT3 also dramatically changes the localization of CAT3, which is normally localized in the cytoplasm; CAT3 was translocated to the nucleus in the presence of 2b (Inaba et al. 2011; Masuta et al. 2012).

Although catalase is well known to play an important role in regulating HR through the decomposition of H$_2$O$_2$ during plant–pathogen interactions, there are not many reports that describe the molecular details of the catalase-mediated pathways against viruses. For CMV in pepper plants, catalase activity was important for determining the degree of host susceptibility to CMV (Petrova et al. 2009). In addition, it was shown that CMV infection significantly induced catalase expression in squash plants (Havelda and Maule 2000). We also observed that 2b’s RSS activities were cancelled by a high level of CAT3 expression in the protoplast experiment (Inaba et al. 2011), and that CAT3-overexpressing transgenic Col-0 lines showed the suppression of CMV multiplication until 7 dpi, although the levels of CMV reached those of the nontransgenic control plants at 14 dpi. Therefore, CAT3 seems to play an antiviral role in CMV infection, but a role of CAT3 in CMV tolerance of *Arabidopsis* still remains unknown. For the necrosis induction, we reasoned that CAT3 and 2b were important, but we did not have any answer to explain the phenomenon that the necrosis severity greatly varied depending on the CMV strains and *Arabidopsis* ecotypes. Here, we further investigated the mechanism underlying the manifestation of necrosis symptoms observed in *Arabidopsis* infected with CMV. Our results of this study suggest that the stability of the CAT3-2b complex is important for the necrosis, and that the proteasome system is involved in degrading CAT and regulating the induction of necrosis in *Arabidopsis*.

**Materials and methods**

**Plant materials and viruses**

For *Arabidopsis thaliana*, ecotype Col-0 and the CAT3-knockout Col-0 mutant (cat3) were used in this study. *Arabidopsis* was grown in a growth chamber at 21°C with 12 h photoperiod (150 µmol/m$^2$/s). *Nicotiana benthamiana*, which were used for agroinfiltration, was grown at 23°C with 16 h light/8 h dark. To create CAT3-complemented plants (CAT3/cat3), the homozygous cat3 plant (cat3/cat3, T-DNA insertion line) was transformed with the wild-type CAT3 cDNA. We first PCR-amplified the cDNA covering the ORF using a primer pair (the forward primer, 5’-GGACTAGTATGGATCCTTACAAGTATCGTCC-3’ and the reverse primer, 5’-GCGGAGCTCCTAGATGCTTGCCCTGACGTTCAG-3’) based on the sequence in GenBank (accession no. NM_001035996). The cDNA was then inserted in the plant expression vector, pIG121-Hm, to create pIG21-CAT3. cat3 plants were transformed with pIG21-CAT3 by the conventional floral dip method. T1 plants were selected for resistance to hygromycin and used for the subsequent inoculation experiments. CMV-Y infectious clones (pCY1, pCY2, and pCY3) (Suzuki et al. 2012) were selected for resistance to hygromycin and used for the subsequent inoculation experiments.
1991) and the CMV-Y-based vectors of CMV-A1 (Otagaki et al. 2006) and CMV-H1 (Matsuo et al. 2007) were previously constructed. A1Ds and H1Ds were created by inserting the *Ds-Red2* gene into CMV-A1 or CMV-H1, respectively (Takeshita et al. 2012). CMV-N has the CMV-Y backbone, but it contains a different C-terminal of the 2b protein; CMV-N was coincidentally created by inserting a 100-bp DNA fragment into the CMV-A1 vector.

**Viral inoculation and fluorescence microscopy**

The plasmids containing the full-length cDNAs of RNAs of CMV were transcribed in vitro. Leaves of 6-week-old plants of *N. benthamiana* were dusted with carborundum and rub-inoculated with the in vitro-transcribed RNAs. For *Arabidopsis*, 4-week-old plants were inoculated with the sap of infected tissues. *Ds-Red2* fluorescence images were taken essentially according to Takeshita et al. (2012). In brief, red fluorescence of inoculated leaves of *Arabidopsis Col-0* and cat3 was acquired using SMZ1500 (Nikon) with *Ds-Red2* filter sets. For each inoculum, a set of four plants was used. Leaves of different plants were removed and used for imaging at 11 dpi.

**BiFC assay**

The BiFC plasmid vectors for transient expression (Singh et al. 2009) were kindly supplied by Dr. S. Mano, National Institute for Basic Biology, Japan. The full-length cDNA of the *CAT3* gene of *Arabidopsis* was cloned in either pGWnG or pGWcG. All constructs were created by the Gateway Technology (Invitrogen). To amplify the designed fusion genes from the constructs containing the inserts, PCR was conducted using the forward primer (T7 promoter sequence + the 5' end sequence of the ORF for the N-terminal protein) and the reverse primer (oligo-dT of 66 T residues + the sequence of the 3' nontranslated region just before the terminator). Capped RNAs were then in vitro-transcribed from the PCR products and subsequently co-transfected into *N. benthamiana* protoplasts as essentially described before (Shimura et al. 2008a, b).

**MG132 treatment**

For the MG132 treatment, either healthy Col-0 leaves or CMV-Y-inoculated leaves at 2 dpi were detached from the basal part of the petiole, and the leaves were then transferred to glass tubes containing 50 μM MG132 (Sigma), which was originally dissolved in DMSO, and incubated at 21 °C with 12 h photoperiod for 3 days before protein extraction.

**Quantitative RT-PCR**

Total RNA was isolated using the Trizol reagent (Invitrogen) essentially as described before (Kim et al. 2008). Total RNA (100 ng) was used for the first-strand cDNA synthesis by AMV reverse transcriptase (Nippon Gene). Quantitative PCR was performed using Universal SYBR Select Master Mix (Applied Biosystems) in a StepOne Real-Time PCR System (Applied Biosystems). The *Arabidopsis* tubulin gene (*AtTub*) was used as an internal control. Primer sets for each gene amplification were as follows: 5'-GAGGGGACCTTGACAACATCTT-3' and 5'-GCGAACAGTTCAACAGCTATGCTTA-3' (for *AtTub*), 5'-GCAGTCGACGTTGACGTCGACCCACACCC-3' and 5'-CCATACATTTGCTCTTCTTTGGAGGCC-3' (for CMV).

**Agroinfiltration experiments**

The plasmid construct of pBE2113:*CAT3*-FLAG with a FLAG tag sequence at the 3' end has been already described (Inaba et al. 2011). In addition, the FLAG-*CAT3* with a FLAG tag at the 5' end and the 2b gene of CMV-N (N2b) were inserted in the Ti plasmid vector pBE2113 in this study. The agroinfiltration was conducted according to Goto et al. (2007). *Agrobacterium* (KYRT1) culture containing each construct (FLAG-*CAT3*, CAT3-FLAG, Y2b, and N2b) was prepared to an optimal density (OD) at 600 of 1.0 and infiltrated in *N. benthamiana* leaves using a 1-ml syringe. Total proteins were extracted 3 days postinfiltration (dpi) and then subjected to Western blot analysis.

**Immunoprecipitation and Western blot analysis**

Total protein was extracted from the inoculated leaves essentially as described before (Masuta et al. 1995). Immunoprecipitation was performed using Dynabeads protein G (Life Technologies) with anti-ubiquitin antibody (Abcam) according to the method essentially described by He and Kermode (2010). Western blots were probed using either anti-FLAG (Sigma) antibody or anti-*CAT3* antibodies (Inaba et al. 2011). The anti-*CAT3* antibodies can recognize the catalase(s) in *N. benthamiana* (at least NbCAT1) as well as *Arabidopsis* *CAT3*, because *CAT3* and NbCAT1 share 94% amino-acid sequence similarity.

**Statistical analysis**

Data were evaluated using Student’s *t* test. A *P* value of <0.05 was considered to be significant.
Results

CMV symptom, accumulation, and movement in CAT3-knockout mutant (cat3)

We previously reported that the interaction between CAT3 and CMV 2b causes necrosis accompanied with H\textsubscript{2}O\textsubscript{2} accumulation, suggesting that the malfunction of CAT3 induced H\textsubscript{2}O\textsubscript{2}-mediated cell death. We here examined the effects of depletion of CAT3 from Arabidopsis on CMV symptom and accumulation using CAT3-knockout plants of Col-0 (cat3). The T-DNA insertion knockout mutant (SALK-092911) was obtained from the Arabidopsis Biological Resource Center (ABRC). We identified homozygous T-DNA insertion line from T4 plants using PCR. Compared with the wild-type control plants, the cat3 plants infected with CMV-Y at 14 dpi were somewhat more stunted (Fig. 1a) and showed weak necrosis; there was a little difference in visible necrotic appearance between wild type (Col-0) and cat3. We examined the catalase activities and found that the activities were one-third of the levels in the control even after CMV infection (Supplementary Fig. 1). These results suggest that the depletion of CAT3 did not facilitate necrosis symptoms, and that 2b alone has an ability to essentially induce weak necrosis on Arabidopsis leaves.

We previously reported that CAT3 can weaken the RSS activity of 2b, and that CMV levels were reduced at least until 7 dpi in CAT3-overexpressing Col-0 (Inaba et al. 2011). In this study, we observed that CMV accumulation levels were significantly higher in cat3 plants at 14 dpi (Fig. 1b). To confirm that the susceptibility of cat3 to CMV was due to disruption of the CAT3 gene, we complemented cat3/cat3 with the wild-type CAT3 gene. We selected several hygromycin-resistant T1 plants, which produce certain levels of the CAT3 gene transcript (Supplementary Fig. 2). When those T1 transformants were inoculated with CMV, there was no difference in CMV accumulation between WT and the CAT3-complemented cat3/cat3 (CAT3/cat3) (Fig. 1c). We also analyzed viral movement in cat3 plants by monitoring fluorescence in leaves inoculated with a recombinant CMV expressing the DS-Red2 protein (A1Ds) (Supplementary Fig. 3a; Takeshita et al. 2012). A1Ds induced weak necrotic spots on both Col-0 and cat3, and spread more rapidly in the inoculated leaves of cat3 than in the wild-type control (Fig. 2a). On the other hand, for the CMV-H1 vector (H1Ds) that does not have 2b, we did not find any difference in viral spread between WT and cat3, and any necrotic spots on the inoculated leaves (Fig. 2b). These results suggest that 2b is indispensable for necrosis induced by CMV, and that the presence of 2b can...
make CMV move more rapidly in cat3 than in wild-type. Taken together, to some extent, CAT3 contributes to inhibiting CMV movement.

Specific induction of necrosis through the interaction between 2b and CAT3

In our previous experiments, we found that the recombinant CMV, Y1H2Y3, which has the CMV-Y backbone but contains HL2b derived from CMV-HL (Inaba et al. 2011), induced necrotic spots on infected Arabidopsis plants, which are more discrete than those induced by CMV-Y. In this study, we found that another recombinant CMV (CMV-N) could induce very severe necrosis that spread rapidly over the inoculated leaves. The 2b protein of CMV-N (N2b) has a nonviral C-terminal sequence (56 amino acids) (Supplementary Fig. 3b), which was coincidentally created by the insertion of a
100 bp-foreign fragment in the CMV-A1 vector. For Arabidopsis, CMV-N induced a very distinct, extensive necrosis on inoculated Col-0 leaves (Fig. 3a, b). However, when we inoculated CMV-N onto cat3 plants, we observed much milder necrosis (Fig. 3a), suggesting that the necrosis severity on CMV-inoculated Arabidopsis leaves is determined by the presence of CAT3; CAT3 would be a modulator of the 2b-inducing necrosis. In addition to Arabidopsis, CMV-N induced not only severe necrosis on the inoculated leaves but also systemic lethal necrosis in N. benthamiana (Fig. 3c).

To artificially reproduce such a symptom by co-expressing 2b and CAT3, we conducted overexpression experiments of the two proteins in N. benthamiana by the Agrobacterium-mediated transient expression assay. However, we eventually found that CAT3 did not accumulate to an expected high level, but seemed to be degraded to some extent. We also detected a distinct higher molecular-weight smear above the 57-kDa band of CAT3 using anti-FLAG antibody against FLAG-CAT3 in the infiltrated tissues but not in the Mock (Fig. 5a). Because we suspected that the high molecular-weight smear might be due to ubiquitination of CAT3, we conducted immunoprecipitation (IP) with anti-ubiquitin antibodies and
then detected CAT3 with anti-CAT3 antibodies. Our Western blot analysis revealed that the anti-ubiquitin IP fraction indeed contained CAT3, whose molecular weights are classified into three size groups (1–3) (Fig. 5b). Size 1 contained a smear, which should be the higher molecular weight ubiquitinated CAT proteins, and size 2 and 3 contained several bands for possible breakdown products of CAT3. In this Western blot, we were not able to detect any protein signals over 100 kDa in the IP fraction, while the input and flow-through lanes contained a strong smear. Heavily ubiquitinated, high molecular-weight CAT proteins may not have efficiently been precipitated with the anti-ubiquitin antibody under the non-denaturing conditions used.

Because ubiquitination of protein is a signal for degradation through the proteasome, we inferred that CAT3 might be degraded by the ubiquitin–proteasome pathway. To confirm the involvement of proteasome in the degradation of CAT3, we treated Col-0 leaves for 3 days with a proteasome inhibitor MG132 and then examined CAT3 levels by Western blot (Fig. 6). The CAT3 band intensity was normalized by the Rubisco band intensity. When healthy leaves were treated with MG132, the CAT levels were approximately 1.3-fold increased (Fig. 6). When Col-0 leaves were inoculated with CMV-Y and those leaves were treated with MG132 at 2 dpi, a twofold increase in CAT3 levels was detected compared with the untreated control (Fig. 6, Supplementary Fig. 4). These results leaves agroinfiltrated with the FLAG-CAT3. Protein extracts were prepared from the agroinfiltrated leaves expressing the FLAG-CAT3 construct at 3 dpi. Immunoprecipitation was performed with anti-ubiquitin antibodies, and precipitated FLAG-CAT3 proteins were detected with anti-FLAG antibody. Three size groups (brackets 1–3) of eluted proteins were detected; the strongest band is ~30 kDa. Arrow indicates FLAG-CAT3 (~60 kDa).

**Fig. 5** CAT3 accumulation in leaves of *N. benthamiana* agroinfiltrated with the FLAG-CAT3 construct. **a** Western blot analysis of protein extracts from agroinfiltrated leaves at 2 and 3 dpi using anti-FLAG antibody to detect CAT3. Coomassie brilliant blue (CBB) staining was used to monitor the equivalence of protein loading. Bracket shows FLAG-CAT3 proteins with higher molecular weights. Mock, buffer-infiltrated. **b** Ubiquitination of the CAT3 protein in

**Fig. 6** CAT3 accumulation levels in Col-0 or CMV-Y-infected Col-0 after MG132 treatment. CMV-Y was inoculated 2 days before MG132 treatment. Leaves were treated for 3 days by MG132, and CAT3 was detected by Western blots using anti-CAT3 antibodies. The band intensity of CAT3 was normalized by that of Rubisco large subunit visualized by Coomassie brilliant blue (CBB) staining. The CAT3 level in nontreated control leaves (Control) was set to 1.0. Data are means of 3–4 independent replicates with SD. Differences in means were evaluated by *t* test; one asterisk and two asterisk indicate significance at the 0.05 and 0.01 levels, respectively. A representative image of a Western blot is shown in Supplementary Fig. 4.
suggest that proteasome activity is, at least partially, responsible for the CAT3 degradation, which is promoted by CMV infection.

We next investigated whether 2b can affect CAT3 degradation by the Western blot analysis following the transient overexpression of CAT3 and 2b by agroinfiltration. The overexpression of CAT3-FLAG itself caused a slight decrease in the CAT3 level compared with the control (Fig. 7, lanes 1, 3). In contrast, the coexpression of CAT3 and Y2b markedly reduced the CAT3 levels (lanes 5, 6). On the other hand, the overexpression of N2b did not reduce the CAT3 level as efficiently as Y2b did (lanes 7, 8). In this experiment, we also observed that Y2b was barely detected compared with the distinct band of N2b in the Agrobacterium-infiltrated tissues (Fig. 7), suggesting that Y2b was less stable than N2b. These results suggest that the degradation of Y2b and CAT3 was simultaneously taken place when they were co-expressed but that in the combination of N2b and CAT3, they were more slowly degraded.

**Discussion**

To analyze the interaction between 2b and CAT3, we previously produced transgenic Col-0 plants that overexpress the CAT3 gene under the 35S promoter (Inaba et al. 2011). With difficulty, we finally obtained several CAT3-overexpressing transgenic lines, but they produced CAT3 at most ~two-fold more than in the wild type (Inaba et al. 2011). We assumed that when the accumulation levels of CAT3 exceed a certain threshold, the CAT3 protein levels must be lowered by a well-regulated mechanism, because the CAT3 levels should be very critical to maintain the cellular redox balance. Based on the detection of the higher molecular weight, ubiquitinated smear band (above the 57 kDa) in the agroinfiltration experiment, we considered that the proteasome pathway regulated CAT3 degradation. CAT3 expression levels are drastically controlled by a circadian rhythm, in which the amplitude of the oscillations in CAT3 mRNA accumulation is ~fivefold; the lowest peak is in the early morning (Zhong and McClung 1996). If CAT3 functions according to the circadian rhythm and is regulated at the mRNA level, the synthesized protein should be quickly degraded. We thus consider that even in healthy Col-0 plants, CAT3 levels must be reset by protein degradation along with the circadian rhythm. In fact, a proteasome inhibitor, MG132, treatment increased 1.3-fold CAT3 accumulation compared to the untreated control, while the CAT3 levels were increased ca. twofold by MG132 when plants were infected with CMV-Y (Fig. 6). These results provide the evidence that the accumulation levels of CAT3 in the presence of CMV 2b are significantly affected by the ubiquitin–proteasome pathway.

As for the involvement of the proteasome in the regulation of cellular CAT3 levels and association of programmed cell death with the generation of ROS, this study is not the first one. He and Kermode (2010) have already demonstrated that white spruce plants actually used the proteasome to control the levels of CAT (a homolog of Arabidopsis CAT3) during the seedling development. One important observation that we share with them is the finding of several proteins with lower molecular weights as breakdown products of CAT after MG132 treatment. As shown in Fig. 5, we also detected several lower molecular weights bands (20–30 kDa) in our Western blots, suggesting that those breakdown products of CAT may have been generated through the same ubiquitin–proteasome pathway.
pathway, as is the case for the white spruce CAT. When these results are considered together, it is likely that the proteasome-mediated *Arabidopsis* CAT3 degradation plays an important role in viral symptom expression and host defense in CMV-infected plants.

Although we here described that interaction between 2b and CAT3 promoted CAT3 degradation probably by the ubiquitin–proteasome pathway, the phenomena that viral proteins can promote degradation of host factors through the proteasome system are not rare events (reviewed by Verchet 2014). As for the viral RSS-mediated degradation of a host factor, Chiu et al. (2010) demonstrated that P25, the RSS of PVX, could induce the degradation of host AGO1 via proteasomes by agroinfiltration in *N. benthamiana*. The P0 proteins of Polerovirus and Enamovirus have been also shown to target AGO1 for degradation (Baumberger et al. 2007; Fusaro et al. 2012). On the other hand, it is noteworthy that many viral RSSs are ubiquitinated to be targeted for proteasome degradation (reviewed by Alcaide-Loridan and Jupin 2012). Conversely, the HC-Pro protein, the RSS of *Papaya ringspot virus* (PRSV), can inhibit host proteasomes, enhancing viral infection (Sahan et al. 2012). Therefore, viral RSSs appear to be deeply involved in the ubiquitin–proteasome pathway during plant–virus interactions whether viruses use it to degrade cellular proteins for their own benefit or inhibit it to prevent them from being a target.

In this study, we demonstrated that CAT3 would work as a modulator of the 2b-inducing necrosis, and that the catalase binding to 2b would be degraded through the proteasome pathway. Considering the catalase activity to erase H$_2$O$_2$ triggering HR, it is conceivable that the observed necrosis may be a simple result of catalase consumption by the interaction between 2b and CAT3. However, there are also some other explanations. For example, Li et al. (2013) previously demonstrated that the interaction between LSD (an important negative regulator of PCD) and *Arabidopsis* catalases (CATs) played an important role in pathogen-induced PCD, which requires the accumulation of salicylic acid. This LSD1-mediated PCD may be involved in the CMV-induced necrosis. Alternatively, based on the observation by He and Ker-mode (2010) that CAT was ubiquitinated and degraded by the proteasome, in white spruce just before extensive PCD occurred, we may raise another possibility that the 2b’s binding to CAT3 can induce necrosis by a certain specific mechanism, which is not necessarily explained only by the catalase activity. This idea is consistent with our finding that the necrosis severity and development depend on the 2b’s sequences; we here assume the involvement of resistance gene (*R* gene)-mediated HR. Because the necrosis induced by CMV infection accompanied H$_2$O$_2$ generation and expression of the PR genes (Inaba et al. 2011), this necrosis could be regarded as an HR-like defense response. In addition, N2b could induce very severe necrosis (even lethal necrosis) not only in *Arabidopsis* but also in *N. benthamiana*, suggesting the involvement of systemic HR-like necrosis driven by an R gene, depending on the nature of the interaction between 2b and catalase. Several reports, indeed, suggest that the ubiquitin–proteasome pathway is deeply involved in HR-like cell death in host defense responses (Liu et al. 2002; Kim et al. 2003; Yang et al. 2006; Sadanandom et al. 2012). For example, gene silencing of the COP9 signalosome, a multiprotein complex involved in protein degradation via the ubiquitin–proteasome pathway compromised the R gene (*N* gene)-mediated HR on tobacco infected with *Tobacco mosaic virus* (TMV) (Liu et al. 2002). Similarly, silencing of an ACRE276, E3-ubiquitin ligase in the proteasome pathway resulted in loss of two HRs in tobacco: N-mediated HR to the Avr protein p50 of TMV and also Cf9-mediated HR to the Avr protein Avr9(4) of *Cladosporium fulvum* (Yang et al. 2006). Although any R gene that can recognize CMV 2b has not been identified, we rather prefer the idea that a CAT3-2b complex may induce HR-like cell death in a putative R gene-mediated resistance, which is associated with the ubiquitin–proteasome pathway.

**Author contribution statement** KM, HS, and CM designed the experiments and wrote the paper. MT conducted the inoculation experiments using the CMV vector expressing Ds-Red.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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