

## Pokeweed (*Phytolacca americana* L.) antiviral protein inhibits Zucchini yellow mosaic virus infection in a dose-dependent manner in squash plants

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**Abstract:** Pokeweed antiviral protein (PAP) of *Phytolacca americana* L. (pokeweed) is a single-chain ribosome-inactivating protein (RIP) characterized by its ability to depurinate plant ribosomes. Here, we isolated, cloned, and expressed the ribosome inactivating protein (RIP) gene, designated as pokeweed antiviral protein type 1 (PAP I), from the summer leaves of pokeweed collected from the Black Sea region (Turkey). Our findings presented here provide direct evidence that exogenous application of PAP I causes concentration-dependent inhibition of *Zucchini yellow mosaic virus* (ZYMV) infection on squash plants. Squash plants were exposed to PAP I protein with and without DMSO for four consecutive days. Regular spraying of approximately 30 kDa recombinant PAP I at 2 µg mL<sup>-1</sup> concentration prevented treated plants from mechanical virus infection. PAP I showed antiviral activity in 9 plants out of 15 inoculated plants. Remarkably, simultaneous application of PAP, DMSO, and ZYMV did not prevent virus infection, suggesting that PAP did not have any effect on viral RNA. In the absence of ZYMV the purified peptide was not cytotoxic for squash plants, although a reduction of plant size, possibly caused by host ribosome depurination, was observed.

**Key words:** *Phytolacca americana*, antiviral protein, expression, inhibition, *Zucchini yellow mosaic virus*, squash

### 1. Introduction

Many plants contain ribosome-inactivating proteins (RIPs) catalytically inactivating prokaryotic and eukaryotic ribosomes (Domashevskiy and Goss, 2015). These enzymes remove a single adenine residue from the large rRNA through RNA N-glycosidase activity. To date, different RIPs have been reported from 17 families covering 50 plant species (Girbés et al., 2004). Some important RIP-producing plant families belong to the superorder *Caryophyllales* (Stirpe and Barbieri, 1986; Kwon et al., 2000; Girbés et al., 2004). Based on their physical properties, the number of polypeptide chains, and posttranscriptional modifications, RIPs are divided into three categories. Type I RIPs are single-chained proteins with a molecular mass of approximately 30 kDa. Type II RIPs contain two functionally distinct subunits: a catalytic subunit (A chain) and a lectin subunit (B chain) (Mehta and Boston, 1998; Tumer et al., 1999; Nielsen and Boston, 2001). The type III RIPs are less frequent and have been identified in barley (*Hordeum vulgare* L.) (Reinbothe et al., 1994) and maize (*Zea mays*) (Bass et al., 1992).

Pokeweed antiviral protein (PAP) has been isolated from the leaves of the pokeweed (*Phytolacca americana*) plant as a single-chain ribosome-inactivating protein (Irvin and Uckun, 1992). Since PAP is a site-specific RNA N-glycosidase, it catalytically removes a single adenine base from a highly conserved “a-sarcin/ricin” (SR) loop of the larger RNA species, resulting in an irreversible inhibition of protein synthesis (Endo et al., 1987; Bolognesi et al., 2000; Qi et al., 2004).

Unlike coat protein-mediated resistance, RIPs have shown broad-spectrum antiviral activity against plant RNA and DNA viruses (Battelli and Stirpe, 1995; Wang and Tumer, 2000). It has been demonstrated that the RIP isolated from *Mirabilis jalapa* has antiviral activity against several plant viruses and viroids including *Tobacco mosaic virus* (TMV), *Potato virus X* (PVX), *Potato virus Y* (PVY), and *Potato spindle tuber viroid* (PSTVd) (Kubo et al., 1990; Kataoka et al., 1991; Vivanco, 1997). A few type I RIPs have also been reported to inhibit bacterial and fungal growth (Roberts and Selitrennikof, 1986; Vivanco et al., 1999; Park et al., 2002).

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In the present report, we expand on our initial *in vitro* studies to show evidence that purified PAP preparations inhibit the infection of *Zucchini yellow mosaic virus* (ZYMV) in squash plants in a dose-dependent manner. A slight size reduction was observed in plants treated with PAP, very likely caused by PAP by host ribosome depurination.

## 2. Materials and methods

### 2.1. Source of plants

Summer leaves of pokeweed (*Phytolacca americana*) plants (Figure 1) were collected from Trabzon Province located on the Black Sea coast (Turkey). Mature leaves were harvested at the development stage and rapidly extracted in a cooled mortar.

### 2.2. Virus isolate and test plants

The severe isolate of ZYMV used in this study was obtained from the Department of Plant Protection, Yüzüncü Yıl University. *Cucurbita pepo* L. 'Sakiz' was used as the host plant for virus propagation and a source of inoculum. Its seeds were obtained from regional sources and were sown in turf pots.

### 2.3. Preparation of crude virus inoculum

Virus inoculum was prepared by grinding 1 g of fresh infected squash leaves in a sterile mortar and pestle in 5 mL of 20 mM sodium phosphate buffer (pH 7.2). The plant debris was squeezed through two layers of Miracloth and centrifuged at 5000 rpm for 5 min. The supernatant was collected and used as virus inoculum on squash cotyledons.

### 2.4. Isolation and construction of full-length PAP gene

RNA was extracted with a silica-based method from the mature leaves of *P. americana* as described by Foissac et al. (2001). The reverse transcription was carried out with RNA using the RevertAid First Strand cDNA Kit



**Figure 1.** Summer leaves of *Phytolacca americana* L. collected from Trabzon Province.

as per the manufacturer's protocol (Thermo-Fermentas, Vilnius, Lithuania). The gene-specific forward and reverse primers included restriction sites (PAP BamHI-F 5'-CAGTGGATCCATGAAGTCGATGCTTGTGGT-3' and PAP-HindIII-R 5'-CAGTAAGCTTTCAGAATCCTTCAAATAGATCACC-3') for cloning into the pETDuet-1 Vector (Novagen, Darmstadt, Germany). The complete PAP gene was amplified by PCR with the thermal cycling scheme and the ratios described by Sipahioğlu et al. (2012). The PCR product was separated on 1% agarose gel and recovered with a gel extraction kit (Isolate II PCR and Gel Kit, Bionline, Luckenwalde, Germany). The PCR fragments of the PAP gene and the pET Duet 1 vector were digested with *Bam*HI and *Hind*III and ligated to produce PAP-bearing clones. The DNA sequence of the final construct was verified using sequencing analysis.

### 2.5. Expression and purification of recombinant His-tagged PAP protein and determination of PAP yield

Recombinant plasmids were transformed into competent cells of *E. coli* BL21(DE3)pLysS cells by micropulser (Bio-Rad, Hercules, CA, USA) and were plated on LB agar containing ampicillin at 37 °C overnight. Colonies of *E. coli* cells transformed with the expression plasmid were grown in liquid LB medium at 37 °C for 16 h supplemented with 50 mg mL<sup>-1</sup> ampicillin until the OD<sub>600</sub> reached 0.5–0.7. Once this density was reached, protein expression was induced by adding isopropyl β-D thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM followed by incubation with constant shaking at 25 °C for 16 h. The total broth medium was centrifuged to collect the cells and pelleted at 6000 rpm for 5 min. The pelleted cells were resuspended in the presence of proteinase inhibitor (Roche, Penzberg, Germany). After adding 0.5 mL of Tris-HCl (pH 7.5), 1 mL of NP40 (10%), 25 μL of MgCl<sub>2</sub> (1 M), 7 μL of 2-mercaptoethanol, and 20 μL of DNase I (10 U μL<sup>-1</sup>), the suspension was sonicated four times (3–5 s) in ice with 2-s intervals and then the mixture was incubated at 4 °C for 45 min on a shaker. After adding 0.3 g of NaCl (final concentration: 0.5 M), the protein extract was ultracentrifuged at 30,000 rpm for 30 min (4 °C). The protein concentrations were determined according to the method of Bradford (Bradford, 1976).

### 2.6. In vitro assays of anti-ZYMV activity

All experiments were run using the climate chamber of the Department of Plant Protection, Yüzüncü Yıl University. To test the viral inhibitory activity of PAP I two basic methods were implemented. For each method, the tested plants were divided in five groups.

Method 1: Plants were simultaneously treated with three different concentrations (1, 2, and 4 μg mL<sup>-1</sup>) of PAP I with an equal volume of virus inoculum (1:1, v v<sup>-1</sup>) in 10% DMSO solution. The controls consisted of "only DMSO 10%" and "only virus-inoculated plants (positive

control)". Untreated plants were also used as negative controls (Table). For each treatment, the cotyledons of five *C. pepo* plants were used. All were tested in pentaplicate in each run of the experiments.

**Method 2:** In the second method, to determine the mode of antiviral action of PAP I, squash plants were pretreated with PAP I protein before virus inoculation. PAP I was applied exogenously in 10% DMSO to cotyledons of squash plants in order to facilitate its penetration into the host cells with three different concentrations (1, 2, and 4  $\mu\text{g mL}^{-1}$ ) for 4 days before viral inoculation. The other three groups of plants were established for each method as "negative (noninoculated plants)", "only DMSO 10%", and "positive (virus-inoculated plants)" controls. All experiments were performed in pentaplicate. In order to exclude any influence of DMSO, positive controls contained the same concentration of DMSO as the plants treated with PAP I. The inoculations were performed on the cotyledons using carborundum powder. In Method 2, 4 days after PAP I was applied plants were treated with carborundum and the cotyledon leaves of each plant were inoculated with ZYMV in 20 mM phosphate buffer, pH 7.2. Inoculated plants were kept in a growth chamber with a 16-h photoperiod. The same controls of Method 1 were created for Method 2. In both methods, RT-PCR analyses were performed 3 weeks after virus inoculation.

## 2.7. RNA extraction and RT-PCR detection of ZYMV

Leaf samples from each group were pooled and ground together in a cooled mortar before total nucleic acid extraction. The nucleic acids were extracted from squash leaves (100 mg) according to the silica capture method as described by Foissac et al. (2001). Reverse transcription and PCR were done by a two-step RT-PCR commercial kit (Fermentas). Specific primers were designed using Primer3 (<http://frodo.wi.mit.edu/primer3/>) targeting the coat proteingene(CP)(Z-F-5'-TCAGGCACTCAGCCAACT-3' and Z-R-5'-CTGCATTGTATTACACCTAGT-3').

Molecular diagnosis of ZYMV from squash plants was performed as described by Özer et al. (2012).

## 3. Results

The full-length cDNAs corresponding to PAP I were cloned into the *E. coli* expression vector under the control of the IPTG-inducible promoter. As previously demonstrated by our group (Sipahioğlu et al., 2012), the antiviral protein gene (PAP I) studied in this paper had an amino acid sequence identical to that determined by others in databases, except for a single substitution of Leu 273 to Phe. In order to facilitate purification of the expressed protein, a His-tag sequence was added in the C-terminal region of PAP I and was separated by 12.5% SDS-PAGE gel. As shown in Figure 2, PAGE analysis detected a single band of approximately 30 kDa in polyacrylamide gel expressed in the *E. coli* expression system, which agrees with the known size of the PAP I protein. The measured yield was 25 mg  $\text{mL}^{-1}$  of total starting material.

In the present study, we explored the biological significance of the enzymatic activity of PAP, isolated from *P. americana*, on the infection and replication ability of a specific plant pathogenic virus with two experimental studies.

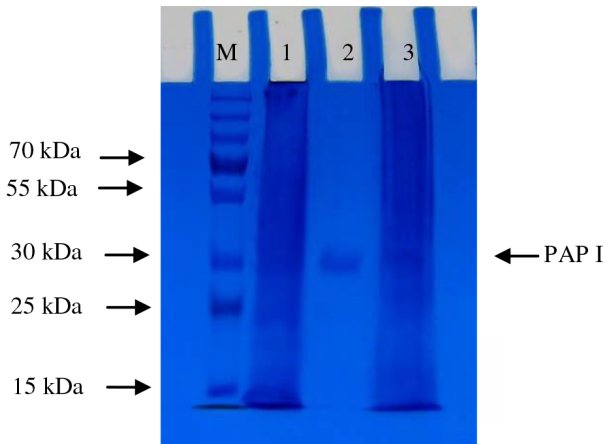
In the first experiment the purified PAP I was mixed with an equal volume of purified ZYMV and the infectivity of the virus/PAP I mixture was compared with that of the negative, 10% DMSO solution, and positive controls. ZYMV infectivity was determined by inoculating the cotyledons of *C. pepo* and observing the systemic symptoms that formed subsequently. The RT-PCR results showed that coinoculation of purified PAP I at different concentrations with ZYMV did not inhibit virus infection. Uninoculated squash plants (controls) reacted negatively in RT-PCR (Figure 3; Table).

In the second experiment, a study was conducted to analyze the dose-dependent effect of PAP protein

**Table.** Antiviral activity of various concentrations of PAP I on ZYMV infection.

| No. of replicate | Results of coinoculation of purified PAP I preparation with ZYMV extract |                         |                         | Results of exogenous application of PAP I for 4 days before ZYMV inoculation |                         |                         |
|------------------|--------------------------------------------------------------------------|-------------------------|-------------------------|------------------------------------------------------------------------------|-------------------------|-------------------------|
|                  | 1 <sup>a</sup> $\mu\text{g mL}^{-1}$                                     | 2 $\mu\text{g mL}^{-1}$ | 4 $\mu\text{g mL}^{-1}$ | 1 $\mu\text{g mL}^{-1}$                                                      | 2 $\mu\text{g mL}^{-1}$ | 4 $\mu\text{g mL}^{-1}$ |
| 1                | –                                                                        | –                       | –                       | +                                                                            | +                       | –                       |
| 2                | –                                                                        | –                       | –                       | +                                                                            | +                       | –                       |
| 3                | –                                                                        | –                       | –                       | –                                                                            | +                       | –                       |
| 4                | –                                                                        | –                       | –                       | –                                                                            | +                       | +                       |
| 5                | –                                                                        | –                       | –                       | –                                                                            | +                       | +                       |

<sup>a</sup>: Concentration of PAP I, +: inhibition of ZYMV inoculation, –: no inhibition of ZYMV inoculation.



**Figure 2.** Purification and separation of 10  $\mu\text{g}$  of PAP I, IPTG-induced, and noninduced proteins on 12.5% SDS-PAGE gel. The gel was stained with Coomassie blue. The PAP gene was expressed in *E. coli* and affinity-purified using a Ni-NTA affinity column. Total protein was obtained from precipitated bacterial cells (lanes 1 and 3). The cells were cultivated either in the absence (lane 1) or in the presence of IPTG (lane 3). The purified PAP I is pure because a single band of approximately 30 kDa is seen after Coomassie staining (lane 2).

at three different concentrations (1, 2, and 4  $\mu\text{g mL}^{-1}$ ) after viral infection. For that purpose, a range of PAP I concentrations of purified preparations were exogenously applied to the cotyledons of squash plants for 4 days before ZYMV inoculation. The infectivity of the virus was compared with the same controls as in the first experiment. All pretreated plants with three different concentrations of PAP I were subjected to RT-PCR with two repetitions to test the presence of ZYMV inoculation. Figure 4 shows an ethidium bromide-stained gel of RT-

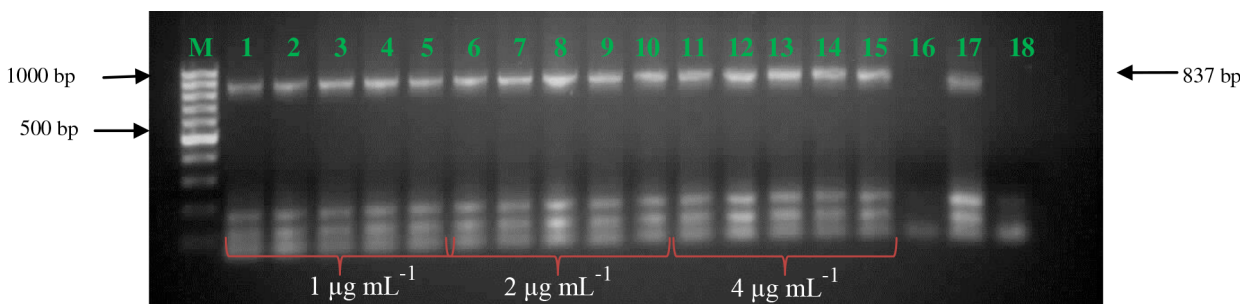
PCR products amplified from squash plants to investigate the presence of the virus in ZYMV-inoculated plants. To determine if PAP I permanently inactivated ZYMV, PAP I was first administrated with purified preparations (or with buffer) for 4 days as described. The results obtained clearly showed that the infectivity of ZYMV with purified PAP I had permanent effects. The results clearly showed that pretreatment of purified PAP I at specific concentrations with ZYMV inhibited virus infection (Table).

PAP I exhibited antiviral activity preventing the ZYMV infection when tested at a concentration of 2  $\mu\text{g mL}^{-1}$  (Table). At concentrations of 1 and 4  $\mu\text{g mL}^{-1}$ , there were limited inhibition effects of PAP I (Table). At both concentrations, an average of 40% inhibition was recorded against ZYMV infectivity. Based on these results, it was concluded that the inhibition activity of the purified preparations was dose-dependent (Figure 4).

The application of PAP I in the presence of 10% DMSO resulted in a severe reduction in control plant size. The controls involving only 10% DMSO showed no significant changes in plant size as compared to the control group (NC).

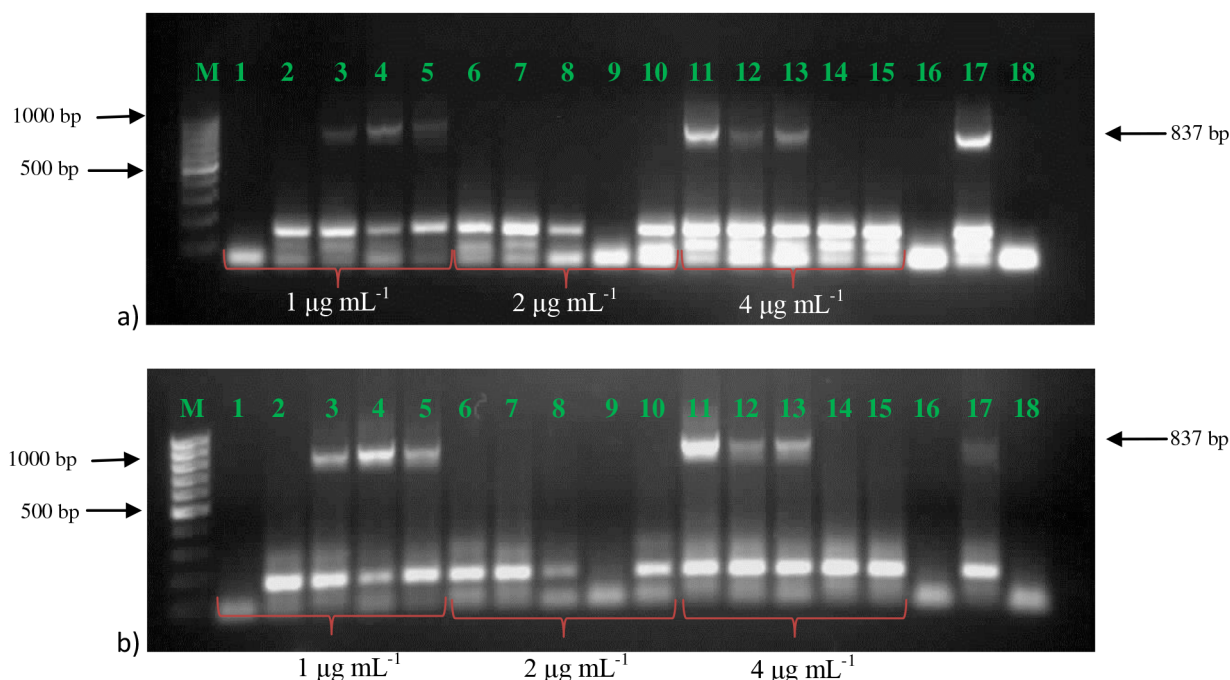
#### 4. Discussion

In the present study, the inhibitory effect of PAP I protein against ZYMV infection was analyzed. The results provide evidence that the infection of squash plants by ZYMV can be inhibited by continuous application of PAP I protein from *P. americana*. In the literature, the molecular weight ranges from 29 kDa to 30 kDa for PAP (Poyet and Hoeveler, 1997). On SDS-PAGE gel, a protein band with mobility corresponding to approximately MW 30,000 was present in the purified preparation. The preparations were electrophoretically homogeneous and concentrated. The



**Figure 3.** RT-PCR analysis of ZYMV in plants inoculated with the virus in the presence of purified PAP I preparations. PAP I was applied simultaneously in 10% DMSO solution to cotyledons of squash plants with three different concentrations (1, 2, and 4  $\mu\text{g mL}^{-1}$ ). Lanes 1–5 are plants for which 1  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was mechanically inoculated. Lanes 6–10 are plants for which 2  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was mechanically inoculated, and lanes 11–15 are plants for which 4  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was mechanically inoculated. Lane 16 is a negative control (noninoculated plant), lane 17 is a positive control (virus-inoculated plant), and lane 18 is the application of only 10% DMSO solution; M: 1000-bp molecular size marker. Note that PAP I did not protect the squash seedlings from ZYMV infection at any concentrations tested. All the coinoculated plants reacted positively for ZYMV. No PCR band was observed in the negative control (lane 16) or in the sample treated only with 10% DMSO solution (lane 18).





**Figure 4.** Two replicates of RT-PCR results of Method 2 (a and b). PAP I was applied exogenously in 10% DMSO solution to cotyledons of squash plants with three different concentrations (1, 2, and 4  $\mu\text{g mL}^{-1}$ ) for 4 days before viral inoculation. Lanes 1–5 are the plants for which 1  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was exogenously applied. Lanes 6–10 are the plants for which 2  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was exogenously applied, and lanes 11–15 are the plants for which 4  $\mu\text{g mL}^{-1}$  of purified PAP I preparation was exogenously applied. Lane 16 is a negative control (noninoculated plant), lane 17 is a positive control (virus-inoculated plant), and lane 18 is the application of only 10% DMSO solution; M: 1000-bp molecular size marker. Notice that there are no PCR bands in tested plants for which the 2  $\mu\text{g mL}^{-1}$  concentration of PAP I preparation was exogenously applied. No PCR band was observed in the negative control (lane 16) or in the sample treated with only 10% DMSO solution (lane 18).

inhibition of ZYMV by the recombinant protein appears to occur after adsorption of protein by the cells but not after penetration of the virus into the cells. Experiments to assess the inhibition effects of PAP I protein in squash seedlings indicated a high level of antiviral activity against ZYMV when 2  $\mu\text{g mL}^{-1}$  PAP I was applied for 4 days. This observation suggests that the mechanism of the antiviral activity of the peptide involves its transport into intact cells by the virus followed by inactivation of host cell ribosomes. It remains to be determined whether the inhibitory effect of 2  $\mu\text{g mL}^{-1}$  PAP I is due to better absorption of the protein with DMSO into host cells. Based on our observations, a good correlation was seen between the level of PAP concentration and the level of resistance to viral infection. The present study has demonstrated that PAP I protein possesses antiviral activity and might be used as a potential antiviral agent against ZYMV infection. The lack of an inhibitory effect of PAP I at concentrations of 1 and 4  $\mu\text{g mL}^{-1}$  is probably due to insufficient biological activity of the protein at these concentrations.

Xu et al. (1998) measured the antiviral activity of PAP by a local lesion assay using tobacco (*Nicotiana glutinosa*) and TMV. They adjusted the PAP concentration to be 62.5

ng  $\text{mL}^{-1}$ , 125 ng  $\text{mL}^{-1}$ , 0.25  $\mu\text{g mL}^{-1}$ , 0.5  $\mu\text{g mL}^{-1}$ , 1.0  $\mu\text{g mL}^{-1}$ , 2  $\mu\text{g mL}^{-1}$ , and 4  $\mu\text{g mL}^{-1}$  in 0.1 M phosphate buffer. Their experiments showed that PAP at its lowest concentration (62.5 ng  $\text{mL}^{-1}$ ) caused a 58% reduction in the number of local lesions. Song et al. (2000) reported that when plants were inoculated with TMV along with recombinant PIP from *Phytolacca insularis* at the concentration of 6  $\mu\text{g mL}^{-1}$  or 12  $\mu\text{g mL}^{-1}$ , the infected leaves showed no local lesions.

Coinoculation of squash cells with ZYMV and PAP I had no inhibition effect on virus infection. Simultaneously applied PAP I did not protect squash plants against mechanical transmission of ZYMV (Table). It has been proposed that simultaneously applied PAP I enters damaged cells along with the invading virus and does not inhibit the translation of viral gene products. This result contrasts with that found by Iglesias et al. (2005), who observed that the simultaneous application of a type I RIP from sugar beet together with viral particles of the *Artichoke mottle crinkle virus* (AMCV) strongly prevented the infective process as assessed by visual inspection. These apparently contradictory results could be explained by considering the different nature of the utilized viruses. AMCV is a *Tombusvirus*, whereas ZYMV belongs to

the genus *Potyvirus*, whose members are characterized by having a VPg protein at the 5' end of their genome. Recently it was shown that the VPg of a potyvirus binds PAP with high affinity, being a potent inhibitor of PAP depurination (Domashevskiy et al., 2012), which can explain the lack of antiviral effects observed at the local level. However, pretreatment with PAP before inoculation could prepare the plant to avoid viral infection as observed in our experiments where the virus was inoculated 4 days after PAP treatment. A similar result was recently reported by Zhu et al. (2016), who observed that PAP increases plant systemic resistance to TMV infection in *Nicotiana benthamiana*.

Since the type 1 RIPs are not able to cross the cell membrane on their own, they are not as cytotoxic as type 2 RIPs. However, we have found that PAP I treatment along with 10% DMSO causes a severe size reduction in squash plants. Similar results were obtained by our group in another study with the bouganin antiviral protein (BAP) gene isolated from *Bougainvillea spectabilis* Willd. (unpublished data). As in the case of the previous study with BAP, it has been hypothesized that once the PAP

reaches the plant cells, it may depurinate the host plant ribosomes and arrest the protein synthesis necessary for growth. It has been reported that the ribosome-inactivating property of RIPs is responsible for the inhibition of protein synthesis in eukaryotes (Gessner and Irvin, 1980; Irvin, 1995). To date, the potential size reduction effect of RIPs in plants has not been investigated adequately. RIPs act on ribosomes to inhibit polypeptide chain elongation (Barbieri and Stirpe, 1982; Olsnes and Pihl, 1982; Irvin, 1983), thereby arresting protein synthesis and causing cell death (Endo and Tsurugi, 1988). For a better understanding of the molecular mechanisms of PAP and BAP blockage of cellular functions would be the focus in future research.

Here we report the dose-dependent antiviral properties of a PAP isoform (PAP I). This may provide a basis for novel and effective plant virus control approaches using PAP.

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