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# Production of Pokeweed Antiviral Proteins Nontoxic to Cells by Mutagenesis of PAP-cDNA

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**Pokeweed antiviral protein (PAP), one of ribosome inactivating proteins (RIPs), has very strong toxicity both to prokaryotic and eukaryotic cells. To produce mutant PAPs nontoxic to cells, the *PAP*-cDNA was inserted into a yeast-*E. coli* shuttle vector under the control of galactose promoter, mutagenized using hydroxylamine, and transformed into yeast cells. Transformed yeast cells were selected on the uracil-deficient plate containing glucose or raffinose, and the yeast cells producing mutant PAPs nontoxic to cells were then selected on the galactose plate. Eighteen mutants were obtained by immunoblot analyses of 1,000 transformants: among them, three, ten and five mutants produced unprocessed, mature and truncated PAPs, respectively. Fourteen PAP mutants among them did not inhibit the yeast cell growth, and showed no or less inhibition of protein synthesis *in vitro*. Six among fourteen mutants were able to protect TMV infection in coinoculation experiment. The mutant PAPs showing an antiviral activity either without or reduced RIP activity contain neither the active site mutation nor C-terminal deletion mutation. These results suggest that both the RIP activity and the antiviral activity will require other amino acid residue(s) besides the active site and that the antiviral acitivity of PAP can be dissociated from its toxicity.**

*Key words*: pokeweed antiviral protein, ribosome-inactivating protein, hydroxylamine mutagenesis, deletion mutant, antiviral activity

## INTRODUCTION

Pokeweed (*Phytolacca americana*) antiviral protein (PAP) is a ribosome inactivating protein (RIP) that inhibits the protein synthesis by catalytically removing a specific adenine residue from the large subunits of eukaryotic ribosomes as well as prokaryotic ones (Barbieri *et al.*, 1993). Pokeweed plants produce three types of PAPs; PAP or PAP-I in spring leaves or stems (Irvin, 1975), PAP-II in late summer leaves (Irvin *et al.*, 1980), and PAP-S in seeds (Barbieri *et al.*, 1982). All three types exhibit antiviral activities when coinoculated with viruses. PAP was demonstrated to be an effective RIP inhibiting the infection of various plant viruses (Tomlinson *et al.*, 1974; Chen *et al.*, 1991). Recently, PAP cDNA was cloned by several laboratories (Lin *et al.*, 1991; Lodge *et al.*, 1993). Lodge *et al.* (1993) showed that transgenic plants expressing PAP were resistant to a broad spectrum of viruses.

PAP also has a potent antiviral activity against animal viruses. PAP has been shown to inhibit infection and multiplication of influenza virus and poliovirus (Tolinson *et al.*, 1974; Ussery *et al.*, 1977; Aron and Irvin, 1988; Lee *et al.*, 1990). Furthermore, PAP inhibits infection and multiplication of herpes simples virus (HSV) in both Vero and HeLa cells (Aron *et al.*, 1980; Teltow *et al.*, 1983), and multiplication of human immunodeficiency virus (HIV) (Zarling *et al.*, 1990; Eric *et al.*, 1993). A number of recent studies have shown that conjugating PAP with monoclonal antibodies dramatically increases an antiviral activity against HIV- and human cytomegalovirus-infected cells (Irvin and Uckun, 1992). Due to the cell toxicity of PAP, it was also very effective to kill cancer cells (Barbieri *et al.*, 1993).

However, the application of PAP as a medicine or for the production of virus-resistant crop plants always causes side effects because of its strong

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toxicity (Barbieri *et al.*, 1993; Lodge *et al.*, 1993). It caused a capillary leaking when PAP was applied as a form of immunotoxin to cure cancer in an animal. Studying the molecular and biochemical mechanisms of PAP function might allow a better understanding of how we produce mutant PAP suitable for the application to cure human cancer and useful for curing human viral diseases as well as producing virus-resistant crop plants. Recently, the action mechanism of PAP in relation to its antiviral activity and RIP property has been reported by Dr. Tumer's lab; amino acid residues important for toxicity were identified (Hur *et al.*, 1995) and transgenic tobacco plants expressing those mutant PAPs inhibited the infection of potato virus X (PVX) (Tumer *et al.*, 1997). However, the production and analysis of more mutant PAPs are necessary to elucidate the mechanism associated with antiviral and RIP activities of PAP, and to use mutated *PAP* genes for practical applications.

The aims of this study are to produce and characterize PAP mutant proteins nontoxic or less toxic to cell, but still antiviral. Using a yeast-selection system, we obtained six mutated PAPs that had an antiviral activity without the RIP activity. Sequence analysis of three mutated *PAP* genes implied that the antiviral acitivity of PAP would be separated from its RIP activity and N-terminal amino acid sequence(s) as well as C-terminal sequence besides the active site would be required for the PAP's RIP activity.

## MATERIALS AND METHODS

### Plant Material

Tobacco (*Nicotinana tabacum* Samsun *n/n*), a susceptible line or a systemic infection line, seeds were obtained from Dr. Tumer (Rutgers U., USA) and grown in an environmental growth chamber for 3 weeks before a virus resistant test.

### *E. coli*-yeast Shuttle Vector Construction

*PAP*-cDNA was cloned into the yeast expression vector pAC55 under the control of the galactose-inducible promoter *pGAL1*, resulting in that the vector (pYK1) comprises $Amp^R$ for *E. coli* selection and *URA3* for yeast selelction. The vector DNA was amplified by transformation into *E. coli* DH5α cells.

### Mutagenesis and Mutant Selection

Hydroxylamine mutagenesis of pYK1 vector was performed as described by Rose *et al.* (1990). Ten micrograms of the plasmid were added in 500 μL of ice-cold hydroxylamine solution (7% hydroxylamine HCl, 0.45 M NaOH) and incubated at 37°C for 20 h. The reaction was stopped by addition of 10 μl of 5 M NaCl, 50 μL of 1 mg/mL BSA, and 1 mL of 100% EtOH. The plasmid DNA was precipitated and resuspended in 100 μL of TE, pH 8.0. The DNA was reprecipitated and resuspended in 100 μL TE again. The DNA was then transformed into yeast PSY strain (*Mata, ade2-1 trp1-1 ura3-1 leu2-3,112 his3-11,15 can1-100*) and plated on Ura$^-$ (DO: dropout) medium containing 2% glucose. Transformed yeast cells were tranferred on DO-2% raffinose plates in order to reduce the glucose suppression of the galactose promoter during further selections. The yeast cells containing mutations either in the *pGAL*-promoter or in the *PAP* gene were selected on DO-2% galactose plates, an inducible condition. To distinguish yeasts containing the mutagenized *PAP* cDNA from *pGAL*-promoter mutants, the colonies that grew on the DO-2% galactose plates were analyzed for PAP expression by western blot analysis using an anti-PAP antibodies (Hur *et al.*, 1997).

### Cell Toxicity and Translation Inhibition Assay

The effects of mutant PAPs in relation to cell toxicity or RIP activity were determined by both a growth test and the translation inhibition assay *in vitro*.

For the growth test, yeast cells containing pYK1 or the mutants derived from pYK1 were grown in DO-2% raffinose medium to a density of $2\times10^6$ cells per mL. At time 0, cells were harvested, washed with sterilized water, and resuspended in either DO-2% raffinose or DO-2% galactose to a density of $4\times10^5$ cell per mL. Cells were counted at the indicated times up to 48 h.

For *in vitro* translation inhibition assay, total yeast proteins were extracted as described by Rose *et al.* (1990) and PAP or mutated PAP content was determined by western blot analysis with purified PAP as a standard. One microgram of BMV RNA was translated in a rabbit reticulocyte lysate (50 μL total volume) (Promega) in the presence of yeast extracts (1-5 μg of total proteins, corresponding to

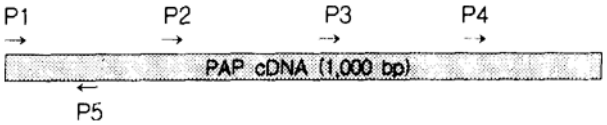


**Fig. 1.** Positions and orientation of designed primers for DNA sequencing.

2.3 ng PAP proteins) containing wild type or mutated PAPs, and in the presence of $^{35}$S-methionine. $^{35}$S-Met incorporation into BMV proteins was determined on SDS-PAGE. Ten microliters of the *in vitro* translation product were separated on the 10% SDS-PAGE, dried, and exposed to X-ray film.

### Antiviral Activity Test

Yeast proteins containing 5 ng of PAP or mutated PAPs were coinoculated with TMV (1 μg/mL) in $H_2PO_4$ buffer on a tobacco leaf. After 2 weeks, the second and third leaves from the inoculated leaf were sampled and stored at −20°C. Total proteins (10 μg) were extracted from the leaf samples and the immunoblot analysis was performed with TMV coat protein antibody (obtained from Dr. C. H. Lee, Korea Ginseng & Tobacco Research Institute, Taejeon, Korea).

### Nucleotide Sequence Analysis

Plasmid DNAs were isolated from the mutant yeast, transformed into *E. coli* DH5α (Robnzyk and Kassir, 1992), and purified using High Pure Plasmid Isolation Kit (Boehringer Mannheim). The mutated PAP-cDNAs were sequenced using the Sequenase 2.0 DNA sequencing kit (USB) (Sanger *et al.*, 1977). Positions and orientation of primers used in the DNA sequencing were shown in Fig. 1. Designed primer sequences are 18-20 mers;

P1: 5'-GATGAAGTCGATGCTTGTGG-3',
P2: 5'-GTGATGGGTTATTCTGATCC-3',
P3: 5'-CCGAAGCCGAATTCCTATTG-3',
P4: 5'-AACTACGTTGGTGGGAGCTG-3'
and P5: 5'-GCAGAGTCGTAATCACAC-3'.

## RESULTS

### Mutagenesis and Mutant Selection

We obtained 1,000 positive transformants from 2 sets of hydroxylamine-mutagenesis experiments. When these transformants were transferred to the DO-2% galactose plates, 587 transformants, called

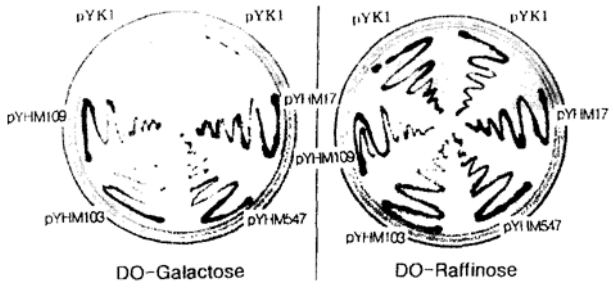


**Fig. 2.** Survival test of yeast cells expressing PAP or mutant PAP. pYK1 indicates yeast cells containing the vector with a wild type of PAP cDNA. pYHM represents yeast cells containing hydroxylamine-mutagenized vector. Both types of cells were streaked on a uracil-deficient 2% galactose (DO-galactose, an inducible condition) and 2% raffinose (DO-raffinose, a noninducible condition) plates, and incubated at 30°C for 48 h.

pYHM, grew in the inductive condition for 48 h. These cells are either to produce mutant PAP nontoxic to cells or not to produce PAP proteins due to the mutation in the *pGAL1* promoter. Fig. 2 showed results of the survival test of yeast cells expressing wild-type PAP and mutated PAP. All cells grew on the DO-raffinose medium, a noninductive condition, whereas only cells containing mutagenized plasmids could grow on the DO-galactose medium, an inductive condition.

To select yeast cells producing mutant PAP, we cultured 587 transformants in liquid medium, extracted total proteins, ran on SDS-PAGE, transferred to NC paper, and analyzed them by immunoblotting using PAP-IgG. Most transformants did not produce PAP protein, but only 18

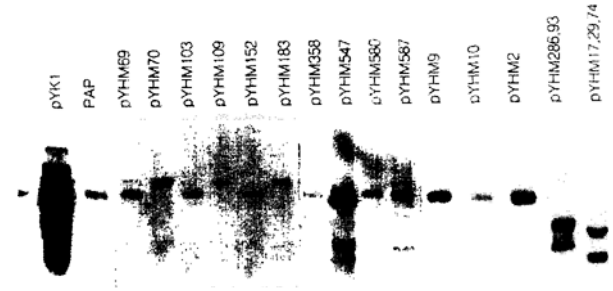


**Fig. 3.** Western blot analysis of PAP and mutated PAPs expressed in yeast cells. Proteins were isolated from the yeast cells, separated on the 10% SDS-PAGE, transferred to NC membrane and probed with PAP antibodies. pYK1 indicates yeast cells expressing PAP cDNA. PAP indicates the mature size of PAP protein purchased from Calbiochem. pYHMs indicate yeast cells expressing mutated *PAP* cDNAs. Arrow on the left indicates the mature size of PAP (30 kD).

transformants produced PAP proteins which would be nontoxic to cells (Fig. 3). pYHM70, pYHM109, and pYHM183 produced unprocessed type of PAP. pYHM69, pYHM103, pYHM152, pYHM358, pYHM547, pYHM580, pYHM587, pYHM10, pYHM9 and pYHM2 produced mutant PAPs that showed same size as the mature form of PAP. pYHM17, pYHM29, pYHM74, pYHM93, and pYHM286 produced truncated PAPs which are smaller than the mature PAP in size. pYHM93 and 286, pYHM17, 29 and 74 showed the same patterns, respectively. The pattern of the result was quite similar to the previous report (Hur *et al.*, 1995), but we obtained more numbers of mutants than in the report. This result would supply more chances to pinpoint the residues of antiviral activity.

## Cell Toxicity and Translation Inhibition Assay

Translation inhibition assay or survival test will provide important information on the RIP acitivity or its cell toxicity. Before mutant proteins are subjected to *in vitro* translation assay, growth of yeast cells was examined in the medium containing either raffinose (noninductive condition) or galactose (inductive condition). Cultures were started with $2\times 10^5$ cell/mL at 30°C and cells were counted every 4 h for 48 h (Fig. 4). The growth of cells containing *PAP* cDNA was arrested upon addition of the inducer galactose to the medium. However, yeast cells producing mutant PAPs grew better in galactose medium than in raffinose during the experimental times. The growth pattern can be divided into three groups: type-1 showing the same growth pattern both in the inductive and noninductive conditions, type-2 showing better growth in the inductive condition than noninductive one, and type-3 showing great reduction of growth in the noninductive condition. Type-1 includes pYHM183, pYHM152, pYHM547, pYHM580, pYHM29 and pYHM286. Type-2 includes pYHM 109, pYHM103, pYHM358, pYHM9, and pYHM17. Type-3 includes pYHM70, pYHM69 and pYHM93. These results indicate that mutant PAPs do not have cell toxicity or RIP activity, which is similar to the previous report (Hur *et al.*, 1995). However, the yeast cells expressing pYHM2, pYHM9, pYHM74 or pYHM587 could not grow in the galactose-

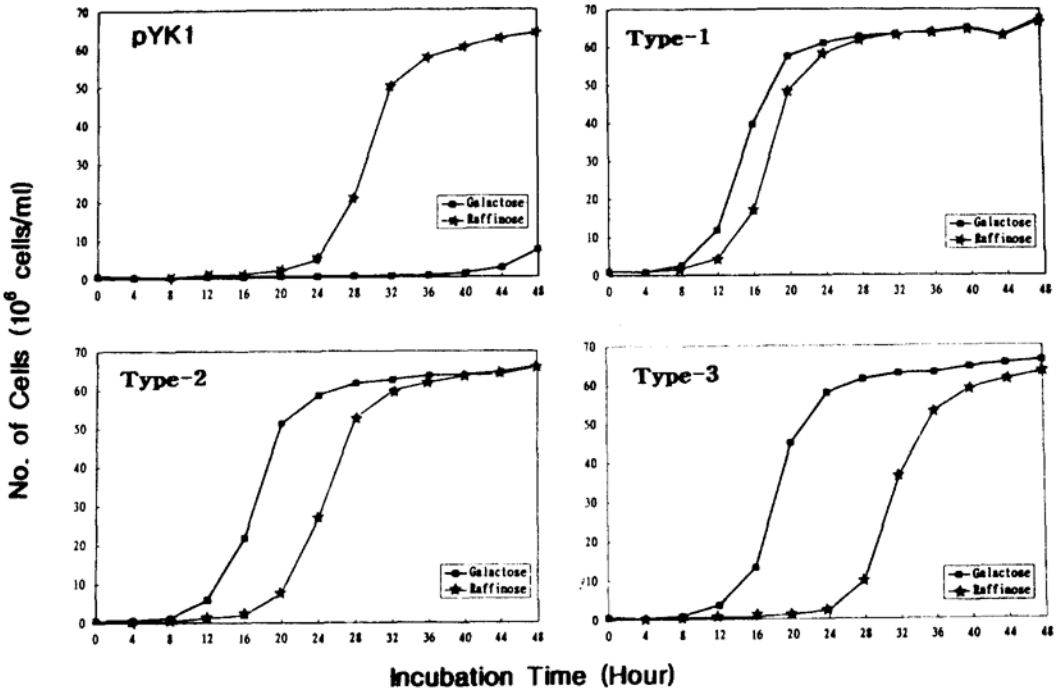


**Fig. 4.** Growth of yeasts expressing PAP or mutated PAPs under either the inductive or noninductive conditions. Cells ($4\times 10^5$ cells/mL) were grown in uracil-deficient medium containing 2% raffinose. At time 0, cells were harvested and transferred to either 2% raffinose- or 2% galactose-containing uracil-deficient medium, and cells were counted at the indicated times. pYK1 represents yeast cells expressing the wild type of PAP. Type-1, -2 and -3 are yeast cells expressing mutated PAPs grouped by their growth patterns.

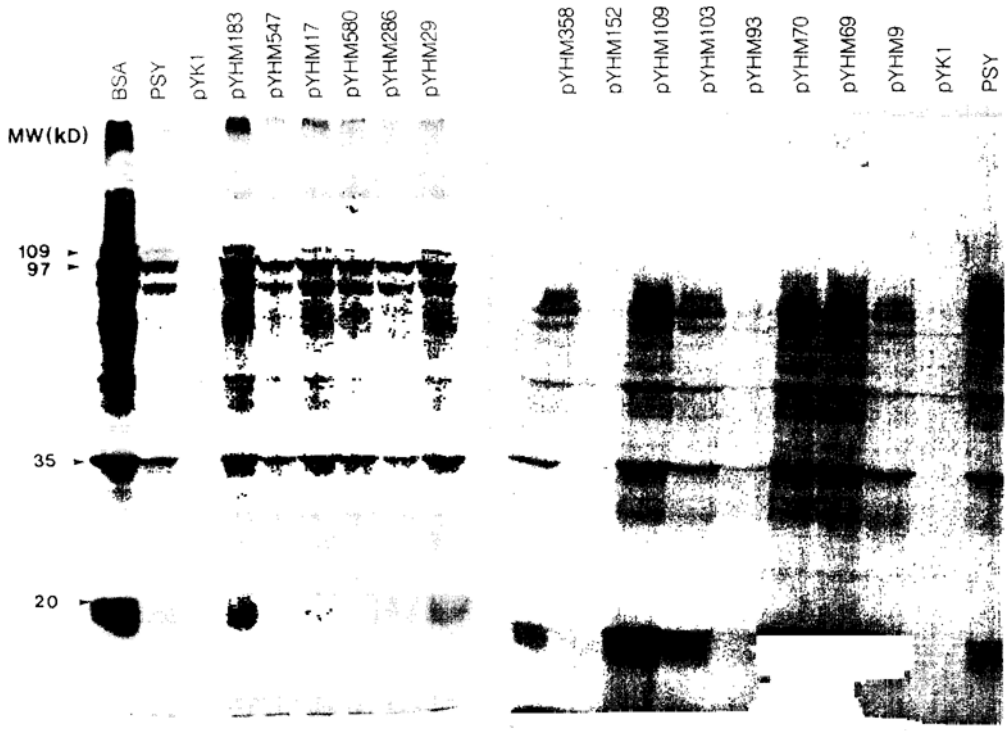


**Fig. 5.** SDS-PAGE analysis of inhibition of protein synthesis by wild-type and mutated PAPs. One microgam of BMV RNA was translated in a rabbit reticulocyte lysate (50 μL total volume) (Promega) in the presence of yeast extracts (1-5 μg of total proteins, corresponding to 2.3 ng PAP proteins) containing wild type or mutated PAPs, and in the presence of $^{35}$S-methionine. Ten microliters of the *in vitro* translation product were separated on the 10% SDS-PAGE, dried and exposed to X-ray film. 109 and 97 kD indicate BMV 1a and 2a protein, respectively. 35 kD protein indicates BMV 3a and BMV coat protein is 20 kD. BSA; control protein, PSY; proteins from non-transformant yeasts (negative control), pYK1; wild-type PAP expressing yeast proteins (positive control).

containing DO-liquid medium.

To confirm that mutant PAPs have no RIP enzyme activity, mutant PAP proteins were subjected to *in vitro* translation assay and analyzed by SDS-PAGE (Fig. 5). Brome mosaic virus (BMV) mRNA was translated in the rabbit reticulocyte lysate system in the presence of $^{35}$S-methionine and 2.3 ng of the mutant PAP. In this assay, BSA, proteins from nontransformed yeast, and wild type PAP from the transformed yeast are used as positive or negative control. As shown in Fig. 5, pYK1 expressing wild-type PAP completely inhibited the translation of BMV mRNA *in vitro*, while most mutated PAPs did not. However, yeast extracts both from nontransformant (PSY) and mutants inhibited somewhat the translation compared to BSA control, suggesting that partially purified yeast proteins seemed to contain the translation-inhibitory component(s). Compared with PSY, pYHM93 and pYHM152 exhibited an inhibitory effect, whereas rest of mutated PAPs had no inhibition on the translation.

## Antiviral Activity Test

We examined the antiviral activity of the mutant PAP proteins by coinoculating with TMV on tobacco leaves. Since the tobacco plant used in this experiment is a susceptiable line, TMV will be systemically spreaded to all leaves when it is infected. Since minor symptom could not be distinguishable, we perfomed the western blot analysis with antibodies against TMV coat protein. As shown in Fig. 6, wild type PAP (pYK1) perfectly protect from TMV infection. Six (pYHM103, pYHM93, pYHM69, pYHM29, pYHM17 and pYHM9) among fourteen tested-mutant PAPs showed the same antiviral activity as the wild type PAP. These six mutated PAPs would be the antiviral proteins nontoxic to cell or without RIP acitivity.

## Nucleotide Sequence Analysis

To determine which sequence(s) plays an

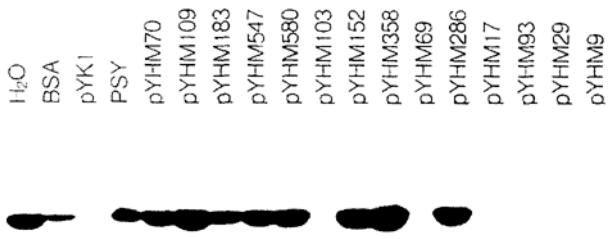


**Fig. 6.** Virus-resistant test by western blot analysis. TMV was coinoculated with 100 μg proteins (BSA, PSY) or yeast extract containing 5 ng of wild-type or mutated PAPs on a tobacco (Nicotiana toboccum Samsum n/n) leaf and western blot analysis was performed with TMV coat protein antibody on 2 weeks after the inoculation. pYK1 and pYHM represent wild type PAP and mutant PAP, respectively.

important role in relation to the antiviral activity and RIP activity of the PAP, we carried out sequence analyses with mutated PAPs selected by the antiviral test. Since nontoxic PAPs or PAPs with reduced toxicity resulted from the active site mutation or C-terminal deletion (Hur *et al.*, 1995), we focused on the mutants showing antiviral activity. We successfully back-transformed *E. coli* with three mutated *PAP*-containing plasmids, pYHM103, pYHM93 and pYHM17, and their full sequences were analyzed (Table 1). pYHM103 producing proteins as same size as the mature PAP contained a single point mutation, changing the valine at position 201 to isoleucine (V201I) outside the active site of RIP in PAP. On the other hand, other two mutants, pYHM93 and pYHM17, were premature terminated, resulting from change of arginine at position 68 to stop (R68Stop) and serine at position 157 to stop (S157Stop), respectively. On the contrary to the previous report (Hur *et al.*, 1995), all three mutants having the antiviral activity either without or reduced RIP activity were neither an active site mutant nor C-terminal deletion mutants.

**Table 1.** Mutations that abolish toxicity of PAP, but retain antiviral activity. The position is expressed based on the amino acid sequences of the mature PAP.

| Mutant Name | Mutation |
|---|---|
| pYHM103 | Val-201 (GTA) → Ile (ATA) |
| pYHM93 | Arg-68 (CGA) → stop (TGA) |
| pYHM17 | Ser-157 (TCA) → stop (TGA) |

providing additional informations on the action mechanism of PAP.

## DISCUSSION

PAP has been considered as a good candidate for the practical use in biotechnology because of its broad spectrum resistance to plant viruses as well as animal ones. However, there are some limitations in using PAP due to its strong cell toxicity or RIP activity. Moreover, it was questionable what biological functions of PAP *in vivo* are and how the protein confers the antiviral activity. It has long been a hypothesis that the antiviral activity of PAP appears to be due to its RIP activity (Barbieri *et al.*, 1993; Bonness *et al.*, 1994). We have, however, wondered about the hypothesis based on several facts. The facts are that PAP is localized in the cell wall matrix, it inhibited the viral replication of various animal viruses, and its antibody has uncommon binding properties to viral coat proteins. *PAP* cDNA was cloned and chracterized by Lin *et al.* (1991), and its active site seemed to be located between position 170 and 183: AIQMVSEAARFKYI (Lin *et al.*, 1991; Barbieri *et al.*, 1993). Specially, glutamic acid at position 176 (E176) is highly conserved among all RIPs sequenced to date and it is proposed to be at the active site cleft of PAP by an analysis of the three-dimesional structure (Monzingo *et al.*, 1993).

Nine mutant PAPs that abolished cell toxicity of the protein were first produced using the mutagenesis of PAP cDNA and a yeast-selection system (Hur *et al.*, 1995). Only one of them, a point mutation (E176V) in the active site, showed no RIP activity or no translation inhibition, whereas the rest including a C-terminal deletion mutant (W237Stop) inhibited *in vivo* translation of animal ribosomes. Both transgenic plants expressing the mutant PAP cDNAs, an active-site mutant and C-terminal deletion mutant, showed nomal phenotype (Tumer *et al.*, 1997), implying that the mutant PAPs are nontoxic to the plant. When protein extracted from both transgenic plants were coinoculated on tobacco leaves, however, C-terminal deletion mutant protected the plant from PVX infection while active site mutant did not. Similar results were obtained when PVX infected to the both transgenic plants; transgenic plants with C-terminal deletion mutant were resistant to PVX infection, whereas transgenic plants with active site mutant did not. These results lead a conclusion that an intact active site of PAP is

necessary for antiviral activity, toxicity, and *in vivo* depurination of tobacco ribosomes. However, an intact active site is not sufficient for all these activities (Table 2). An intact C-terminus is also required for toxicity and depurination of tobacco ribosomes *in vivo*, but not for antiviral activity, suggesting that antiviral acitivity of PAP can be dissociated from its toxicity. Recently, Chaddock *et al.* (1996) showed that sequences outside the active site of PAP are involved in recognition of prokaryotic ribosomes by making hybrids between different domains of PAP and the ricin A chain.

We obtained several new results in this study, compared to the previous results (Hur *et al.*, 1995; Tumer *et al.*, 1997) (Table 2). All three mutants analyzed in this work were found to be non-active site mutants, but they did not possess the cell toxicity for yeasts. In addition, pYHM103 and pYHM17 which have no translation inhibitory effect protected tobacco plants from TMV infection in the coinoculation experiment. Our results support an idea that the antiviral activity of PAP would be dissociated from its toxicity. However, a further analysis will be required for pYHM93 and pYHM17. As shown in Fig. 3, the translation of pYHM93 would be restarted at two different sites, resulting in the production of 10 and 23 kD polypeptides. The translation of pYHM17 would also produce 17 kD polypeptide and a smaller polypeptide. Polypeptides that did not contain a signal sequence could not be processed through ER, but stayed in the cytosol. The polypeptides in the cytosol seemed not to have a cell toxicity, but it is unclear whether they have the RIP activity. As a result of comparison of mutant PAPs (Table 2), N-terminal amino acid sequence(s) as well as C-terminal sequence besides the active site would be required for the PAP's RIP activity. Our results suggest that both the RIP activity and the antiviral activity will require other amino acid residue (s) besides the active site and that the antiviral acitivity of PAP can be dissociated from its toxicity. Now, we are trying to analyze the other mutant PAPs in order to completely dissect the relationship between the antiviral and RIP activities in a PAP.

**Table 2.** Comparison of mutant PAPs in relation to their mutations and activities.

| Mutant Name | Mutation | Cell Toxicity | Translation Inhibitory Effect | Protection Against Virus |
|---|---|---|---|---|
| NT123-2* | E176V | NO | NO | NO |
| NT123-6* | W237Stop | NO | YES | YES |
| pYHM103 | V201I | NO | NO | YES |
| pYHM93 | R68Stop | NO | YES | YES |
| pYHM17 | S157Stop | NO | NO | YES |

*Hur *et al.*, 1995

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