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Expression Characteristics of Pokeweed Antiviral Proteins (PAPs): Two Distinct Types of Proteins

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Pokeweed antiviral proteins (PAPs) become novel therapeutic agents in relation to application in human viral diseases and cancer, as well as potent tools in plant system for defending viral infection. We have studied the expression characteristics of PAPs in pokeweed plants by western blot analysis. PAP-I was constitutively expressed in leaves, stems and roots of the pokeweed plant, while PAP-II was not expressed in roots. The expression of PAP-II began in May and then gradually increased with development of the plants. The PAP-II expression was induced and/or stimulated not only by biotic stresses, such as insect pests and viral infection, but also by abiotic stresses, like drought. Interestingly, low-light intensity was found to be more effective than high-light in the expression of both PAP-I and PAP-II. Our results suggest that PAP-II appears to have an additive effect in terms of protection of the plant against pathogens during summer-time when the plant actively grows and is attacked by various pathogens.

Keywords: pokeweed antiviral proteins, tissue-specific expression, seasonal variations, stress-induced expression, photoperiod.

The pokeweed (*Phytolacca americana*) plant produces at least three types of pokeweed antiviral proteins (PAPs) in different tissues and at various stages of its development. PAP-I, PAP-II and PAP-S appear in spring leaves (Irvin, 1975; Irvin, 1983), summer leaves (Irvin *et al.*, 1980; Irvin, 1983; Poyet *et al.*, 1994) and seeds (Barbieri *et al.*, 1982; Houston *et al.*, 1983; Kung *et al.*, 1990), respectively. PAPs seemed to be synthesized as precursor forms and localized in the cell wall (Ready *et al.*, 1986; Hur *et al.*, 1995). All PAPs belong to a ribosome-inactivating protein (RIP) that inhibits the protein synthesis by catalytically removing a specific adenine residue from the large subunits of eukaryotic ribosomes (Endo *et al.*, 1988; Chen *et al.*, 1993; Bonness *et al.*, 1994) as well as prokaryotic ones (Hartley *et al.*, 1991) and show similar activities on the inactivation of ribosomes (Irvin and Ucken, 1992).

Since Duggar and Armstrong (1925) first reported that the pokeweed plant contained an antiviral agent which was capable of reducing the infectivity of tobacco mosaic virus (TMV), PAPs coinoculated with viruses have been considered as an effective inhibitor against the transmission of a number of other plant viruses (Tomlinson *et al.*, 1974; Chen *et al.*, 1991). Transgenic plants expressing PAP-I cDNA showed the broad-spectrum resistance to infection of various viruses (Lodge *et al.*, 1993). PAP also inhibited the infection of both Vero and HeLa cells by herpes simplex virus (HSV) (Aron and Irvin, 1980), and inhibited the replication of human immunodeficiency virus 1 (HIV-1) in T-cells and macrophages (Zarling *et al.*, 1990) and of poliovirus (Ussery *et al.*, 1977). In this case, use of immunotoxin by conjugating with a monoclonal antibody was more effective than PAP alone (Zarling *et al.*, 1990; Irvin and Ucken, 1992; Jansen *et al.*, 1993). All these researches imply that PAPs are very important proteins in relation to application both in crop protection against pathogens and development of antiviral and cancer therapeutic agents.

The action mechanism of PAP with respect to its antiviral activity has been suggested that when a virus infects to a cell, PAP enters the cells with

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virus, disrupts ribosome and thus inhibits protein synthesis, finally kills the infected cells, and thereby prevents viral multiplication and propagation (Chen *et al.*, 1993; Bonness *et al.*, 1994). Hur *et al.* (1995) reported important residues of PAP conferring the cytotoxic effect or RIP activity. Recently, cDNAs encoding PAP-I and PAP-II proteins were cloned and the full-length sequences were characterized (Lin *et al.*, 1991; Poyet *et al.*, 1994). However, we do not know what the physiological roles of PAPs in a pokeweed plant are and why pokeweed plants produce three types of antiviral proteins. At the first step to answer the important questions, one should elucidate the expression characteristics of PAPs. Therefore, the aims of this research are to examine the tissue specific expression of PAPs and to identify how the expression of PAP-II, a summer form, is induced.

MATERIALS AND METHODS

Plant materials

Pokeweed (*Phytolacca americana*) seeds were imbibed overnight, sowed in 7.6×7.6 cm pots and kept in a greenhouse or an environmental growth chamber for approximately 3 weeks until germinated. Temperature in the greenhouse was kept at 20-25°C. In the growth chamber, the irradiance from mixture of fluorescence lamps and incandescent bulbs was adjusted at 15 $W.m^{-2}$, and the temperature was kept at 25±1°C. All plants were grown with Hoagland nutrient solution.

Pokeweed plants in a field are perennial and shooting (sprouting) in the early May at Chungnam university campus. The second leaf from the apex was sampled at three sites in the campus during the experimental period. We also sowed pokeweed seeds on the field in March and harvested cotyledons in May.

Light illumination

To examine the effects of photoperiod on the expression of PAPs, plants were exposed to continuous light (15 $W.m^{-2}$), continuous darkness, short-day (SD; 8 h light-16 h dark) and long-day (LD; 16 h light-8 h dark) conditions. Plants grown under the LD condition for a month were also subjected to different irradiance levels (57, 15 and 5 $W.m^{-2}$) for 10 days in order to test the effect of light intensity.

Stress treatments

Pokeweed plants grown in a greenhouse were treated with different abiotic and biotic stresses; nutrient deficiency, drought stress, insect pests and viral infections. To induce the nutrient-deficient pokeweed, young plants, less than one month old, were grown in a pot containing the mixture of vermiculite and perlite without nutrient supply. To induce the drought stress (wilt), a very small amount of water was provided to 3-months-old mature plants for 2 weeks. Insect pests were transferred to healthy leaves from old leaves having many different types of insects. The leaves were kept for 2 weeks, harvested, and then washed with deionized water before protein extraction.

To induce viral infection, the virus-containing solution (50 μg/ml of PBS: Phosphate-Buffered Saline) was gently rubbed on a leaf of pokeweed plant in the presence of carborundum. Five classes of RNA virus used for the infection were cucumber mosaic virus (CMV), alfalfa mosaic virus (AMV), potato virus Y (PVY), tobacco mosaic virus (TMV), and potato virus X (PVX). Instead of virus, bovine serum albumin (BSA) was used in the control plant. The second leaf from the inoculated leaf was sampled 2 weeks after the inoculation and total proteins were extracted.

Preparation of antisera of PAPs

PAP polyclonal antibodies were prepared with PAP-I, PAP-II and PAP-S purchased from Cal-Biochem Co. Fifty micrograms of each PAP were diluted with 100 μl PBS, combined with an equal volume of complete Freund's adjuvant, thoroughly mixed, and injected into each pretested rabbit. One time injection provided enough antiserum titer in 4 weeks.

Protein extraction

Samples were ground into fine powder with liquid nitrogen. The powder was transferred into a microfuge tube, well suspended in an extraction buffer (10 mM sodium phosphate buffer, pH 7.5 plus 0.5 mM phenylmethylsulfonyl fluoride), and microfuged for 10 min. at 4°C. Supernatant was transferred into a new tube and spun again to eliminate any debris. Protein concentration was measured by BCA protein assay method (PIERCE) (Bradford, 1976) using BSA supplied with the kit as standard.

Immunoblot analysis

Equal amounts of protein were separated on 10% SDS-PAGE at a constant current of 15 mA until the bromophenol blue reached the bottom of the gel, as

described by Laemmli (1970). Gels were rinsed with 1X SDS-gel running buffer (25 mM Tris-Cl, pH 8.3, 192 mM glycine and 0.1% SDS) containing 20% methanol and set for blotting to prewet nitrocellulose membrane (0.1 μm, S&S). Proteins were electroblotted for 14-20 h at 6-8 V/cm at 4°C. The following steps were performed at room temperature. After protein transfer, the membrane was blocked with 5% non-fat dry milk (NFDM) in TBS-T, pH 7.4 (10 mM Tris, 150 mM NaCl and 0.1% Tween 20) for more than 1 h at room temperature. The membrane was briefly washed three times with TBS-T for 30 min. and incubated with 1:1,500 or 1:1,000 dilution of the antiserum in the TBS-T containing 2.5% NFDM for 1 h. The blot was washed as described above and incubated with TBS-T containing diluted HRP (Horseradish Peroxidase)-conjugated secondary antibody (1:3,500 dilution, PIERCE) for 1 h. The blot was washed with TBS-T for 50 min with 5 times of change, and developed and exposed to X-ray film by the method as described in the manufacturer's manual (ECL method, Amersham).

RESULTS

Cross-reactivities of PAP antisera with three types of PAPs

The antisera raised against three different PAPs were tested with three PAP proteins, PAP-I, PAP-II and PAP-S (Fig. 1). Anti-PAP-I IgG weakly cross-reacted with PAP-S, but not with PAP-II. This result is consistent with a previous report that PAP-II failed to cross-react with the PAP-I antibody (Irvin *et al.*, 1980), but is discrepant to another report that PAP-I antibody partially cross-reacted with PAP-S (Barbieri *et al*, 1982). Anti-PAP-II IgG weakly cross-reacted with PAP-I, but not with PAP-S. Anti-PAP-S IgG strongly cross-reacted with PAP-I, but not with PAP-II. These results suggest that in addition to their size differences the examination of cross-reactivities could provide a clue to distinguish three types of PAPs.

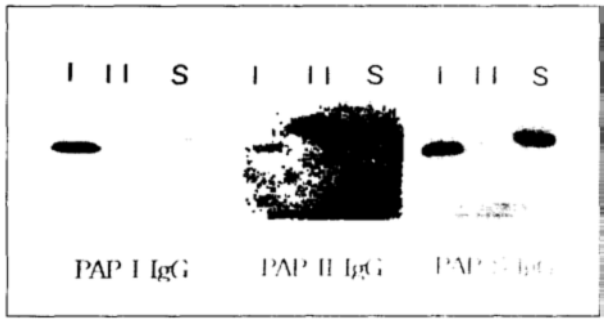

Fig. 1. Immunoassay of PAP antibodies raised against three PAPs. Fifty micrograms of PAP-I, PAP-II and PAP-S were once injected to each rabbit and the antisera were collected after 4 weeks. PAP proteins (10 ng/ea) purchased from Calbiochem Co. were fractionated on 10% SDS-PAGE, transferred to NC membrane (S&S) and detected by ECL method. I, II and S indicate PAP-I, PAP-II and PAP-S, respectively.

Tissue-specific expression of PAPs

Fig. 2 showed the tissue-specific and developemental expression of PAP-I and PAP-II. PAP-I was constitutively expressed in all tissues tested, except fruits and seeds (Fig. 2A), while roots did not express PAP-II (Fig. 2B). The expression of PAP-II increased as the plant aged, which corresponds to a previous report (Houston *et al.*, 1993). As shown in Fig. 1, we expected that the intensity of PAP-I bands should be faint when reacted with PAP-II antiserum. However, the bands were thicker than PAP-II one, implying that PAP-I expression in pokeweed could extremely prevail over PAP-II. Therefore, we have used more total proteins and less diluted anti-PAP-II IgG in following experiments. Molecular mass of PAP-I, PAP-II and PAP-S estimated by SDS-PAGE was 29, 30 and 29.5 kD, respectively.

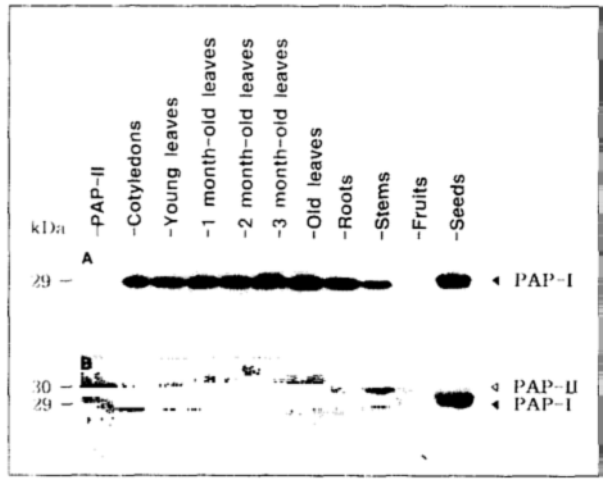

Fig. 2. Tissue-specific expression of PAPs determined by immunoblot analysis. Greenhouse-grown pokeweed plants were harvested in May and total proteins were extracted. Five-micrograms of total proteins were separated on 10% SDS-PAGE, transferred to NC membrane, and blotted with antibodies raised against PAP-I (A) and PAP-II (B). PAP-II (15 ng) was loaded on the 1st lane as a control. Protein molecular mass markers were represented on the left as kDa.

Expression characteristics of PAP in greenhouse- and field-grown plants

Since PAP-II could be expressed in the late summer leaves (Irvin *et al.*, 1980), we examined when PAP-II begins to be expressed by analyzing PAP proteins extracted from the pokeweed leaves grown in greenhouse in March (Fig. 3) and May (Fig. 4). PAP-II seemed not to be expressed in March (Fig. 3B), but large amounts of the proteins were expressed in May (Fig. 4B).

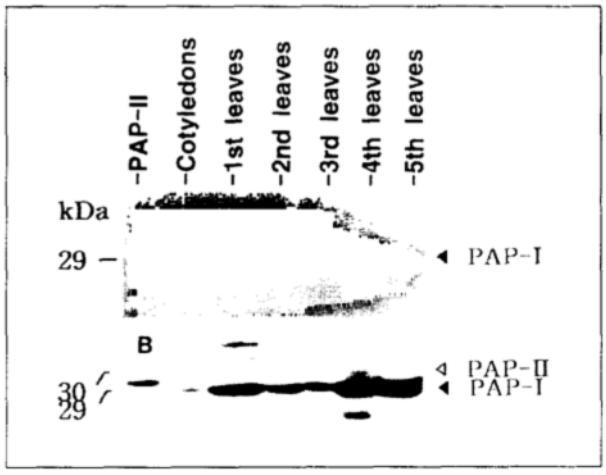

Fig. 3. Western blot analysis of PAPs extracted from greenhouse-grown pokeweed plants in March. Equal amounts (5 μg for A and 10 μg for B) of total proteins were fractionated on 10% SDS-PAGE, transferred to NC membrane (S&S) and detected by ECL method. A: cross-reactivity with PAP-I antiserum, B: cross-reactivity with PAP-II antiserum. PAP-II (10 ng) was loaded on the 1st lane as control. Protein molecular mass markers were represented on the left as kDa.

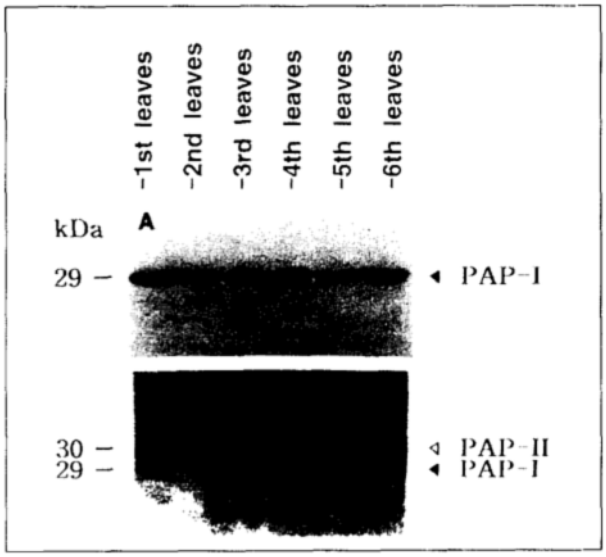

Fig. 4. Western blot analysis of PAPs extracted from greenhouse-grown pokeweed leaves in May. Western blot analysis was performed as described in Fig. 3. A and B are same as described in Fig. 3. Protein molecular mass markers were represented on the left as kDa.

To clarify if PAP-II begins to be expressed at the early stage of development, we performed western blot analysis during cotyledon development in the field-grown pokeweed in May when the plant was shooting at Chungnam university campus (Fig. 5). As shown in Fig. 5, the amounts of PAP-I and PAP-II were progressively increased with the aging of the cotyledons. Interestingly, PAP-II started to be expressed even at the germination time (Fig. 5B).

To examine the seasonal variations of PAP-II in pokweed leaves grown in field, we harvested samples at 1 week interval from May 9, a week after shooting, to June 27, and performed western blot analysis. As shown in Fig. 6, both PAP-I and PAP-II were expressed from the beginning of shooting time and then gradually increased by June.

Effects of light on the expression of PAPs

The results of Figs. 3 and 4 naturally lead to con-

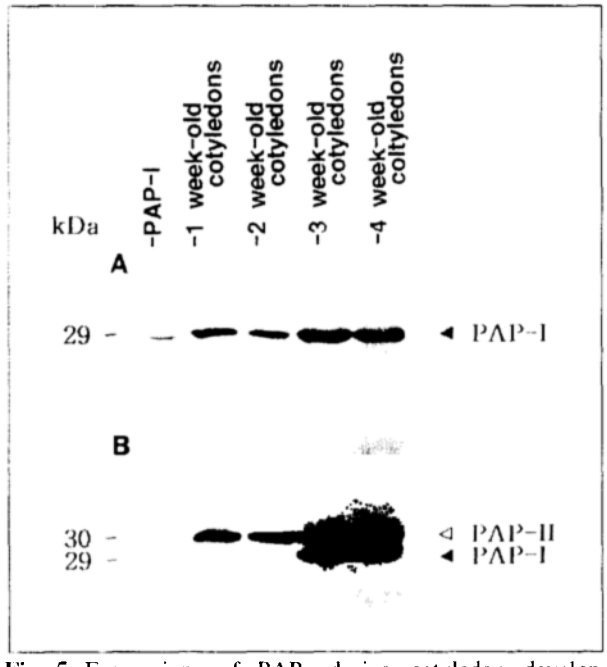

Fig. 5. Expressions of PAPs during cotyledon development from field-grown pokeweed plants in May. Proteins were extracted from 1-week- to 4-week old cotyldons. Western blot analysis was performed as described in Fig. 3. A and B are same as described in Fig. 3. PAP-I (5 ng) was loaded on the 1st lane as a control. Protein molecular mass markers were represented on the left as kDa.

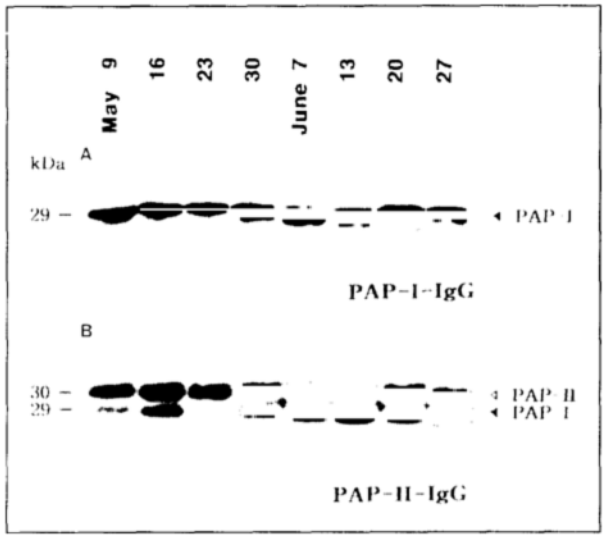

Fig. 6. Expressions of PAPs from field-grown pokeweed leaves. Samples were harvested and total proteins were extracted from May 9 to June 27. Western blot analysis was performed as described in Fig. 3. A and B are same as described in Fig. 3. Protein molecular mass markers were represented on the left as kDa.

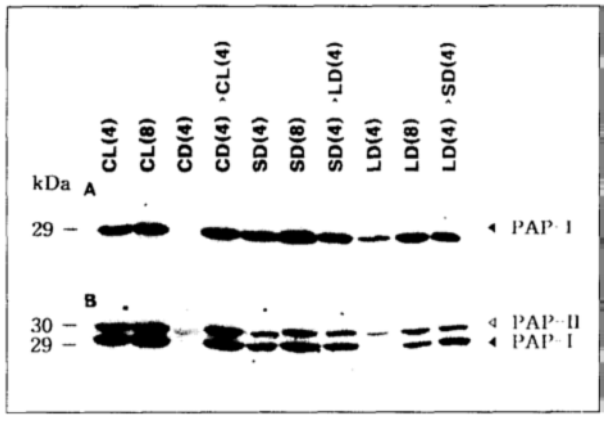

Fig. 7. Effects of photoperiods on the expression of PAPs in pokeweed cotyledons. Western blot analysis was performed as described in Fig. 3. A and B are same as described in Fig. 3. CL(4); 4 days in the continuous light, CD (4); 4 days in the continuous darkness, SD(4): 4 days in short day, LD(4); 4 days in long day. Protein molecular mass markers were represented on the left as kDa.

clude that the expression of PAP-II will be induced and/or stimulated by the length of light period and not by temperature since the temperature was fixed constant during the experiments. Therefore, we tested the expression of PAP-II on the different light periods and intensities (Figs. 7 and 8). Fig. 7 showed several unexpected results: the plant leaves exposed to short-day (SD) condition (8-h light-16 h

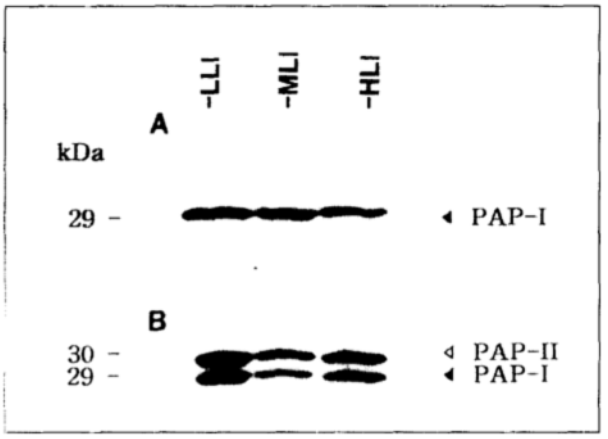

Fig. 8. Effects of light intensities on the expression of PAPs in pokeweed leaves. One-month old pokeweed plants grown in an environmental growth chamber under 16 h light period were exposed to three different intensities of light for 10 days. Western blot analysis was performed as described in Fig. 3. A and B are same as described in Fig. 3. LLI; low light intensity (5 $W.m^{-2}$), MLI; medium light intensity (15 $W.m^{-2}$), and HLI; high light intensity (57 $W.m^{-2}$). Protein molecular mass markers were represented on the left as kDa.

dark) expressed more PAP-II than those exposed to long-day (LD) condition (16 h light-8 h dark); SD/LD or LD/SD transition did not affect the expression. However, we found that both PAP-I and PAP-II were not expressed in the continuous darkness (or in etiolated plants), while the continuous light was particularly effective in the PAP-II expression.

We assumed that the light intensity could affect the expression of PAP-II because the intensity gradually increases in the growing seasons of the plant, from spring to summer. One-month old pokeweed plants were exposed to three different light intensities under 16 h photoperiod for 10 days; low light intensity (5 $W.m^{-2}$), medium light intensity (15 $W.m^{-2}$), and high light intensity (57 $W.m^{-2}$). The highest expression of both PAP-I and PAP-II was observed in the leaves exposed to the low light intensity, and less amounts of PAPs were detected under the medium and high light intensities (Fig. 8).

Stress-induced or stimulated expression of PAP-II

In addition to photoperiod, light intensity and developmental stage, various stresses might be important factors affecting the expression of PAP-II, a summer form. Pokeweed plants were subjected to abiotic stresses, such as depletion of nutrients and water-stress (drought stress), and to biotic stresses,

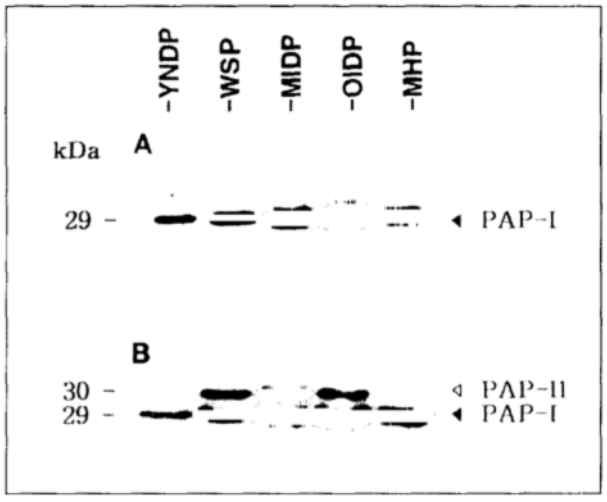

Fig. 9. Immunoblot analysis of PAPs, showing the stress-induced expression of PAP-II. Young (1 month old), mature (3 months old), and old (over 6 months old) pokeweed plants were subjected to nutrient deficient (YNDP), water-stress (WSP), insect-damaged (MIDP and OIDP) conditions. MHP represents mature healthy plants, a control. Total proteins were extracted, separated on 10%-SDS-PAGE, transferred to NC membrane, and blotted with antibodies raised against PAP-I (A) and PAP-II (B). Protein molecular mass markers were represented on the left as kDa.

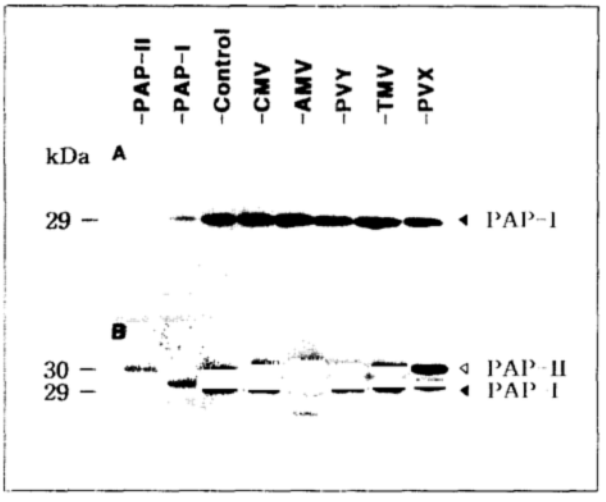

Fig. 10. Immunoblot analysis of PAPs expressed in the virus infection conditions. Greenhouse-grown-2-month old pokeweed plants were infected to various viruses by coinoculation method, and unincoculated leaf was sampled in 2 weeks after the inoculation. Total proteins were extracted, separated on 10% SDS-PAGE, transferred to NC membrane, and blotted with antibodies raised against PAP-I (A) and PAP-II (B). PAP-I (5 ng) and PAP-II (10 ng) were loaded on the left as control proteins. Control represents BSA-inoculated plant. Protein molecular mass markers were represented on the left as kDa.

such as viral infection and damage by insect pests. Young nutrient-deficient and drought-stressed plants expressed less PAP-I than healthy ones (Fig. 9A). The expression of PAP-II was induced by drought stress and damage by insect pests (Fig. 9B). However, the nutrient deficiency may not be a critical factor for the induction of PAP-II expression. Figure 10 showed that the viral infection could induce and/or stimulate the PAP-II expression. Control plant rubbed with BSA somewhat expressed PAP-II, implying that undamaged leaves express PAP-II when any other parts of the plant are wounded.

DISCUSSION

Researches on the application of PAP has been intensively carried out in the past two decades. PAP becomes novel therapeutic agents in relation to potential application in human viral diseases and cancer (Irvin, 1983; Irvin *et al.*, 1992), and potent and putative protective proteins in plants as a defense against viruses (Chen *et al.*, 1993; Lodge *et al.*, 1993). However, molecular information on the PAP expression is still unknown. This work may provide a certain degree of information with respect to physiological roles and actions of PAP *in vivo*.

Poyet *et al.* (1994) reported that PAP-I has 76% similarity to PAP-S in amino acid sequences, whereas PAP-II was only 33% homologous to PAP-I and PAP-S. However, the active site for RIP was conserved among all three PAP proteins. This comparison may lead to a prediction that anti-PAP-II IgG can not cross-react with PAP-I and PAP-S, while anti-PAP-I IgG strongly cross-react with PAP-S. The results shown in Fig. 1 support this prediction, except that anti-PAP-II IgG weakly cross-reacted with PAP-I. Our results will be an important information that in addition to their size differences the different cross-reactivities between anti-PAP-IgGs could provide a clue to distinguish three types of PAPs.

PAP-II was expressed in leaves and stems, while PAP-I was expressed in leaves, stems and roots, except in fruits and seeds. Lin *et al.* (1991) reported that PAP-I is encoded by a multigene family. Therefore, PAP-I expressed in roots (Fig. 2) could be the product of a different gene from one in the leaf. This postulation could be supported by the result shown in Fig. 7 in that PAP-I could not be detected in the cotyledons grown under the continuous darkness. The expression of gene(s) encoding shoot-spec-

ific PAP-I appears to be induced by light. We detected the seed form of PAP (PAP-S) only in seeds. Several types of PAPs, including root PAP-I isoform, present in a plant seem to be related to the distinct physiological role of each protein.

As shown in Figs. 3, 4, 5 and 6, PAP-I was constitutively expressed, while PAP-II expression exhibited seasonal variations. There was only one report that PAP-II is synthesized progressively with the aging of the plant (Houston *et al.*, 1983), but they did not show any data on that in the paper. In our study, PAP-II began to be expressed in May when the pokeweed plant starts shooting. After that, the levels of expression is gradually increased. Even though people have purified PAP-II from the late summer leaves and named it a summer form of PAP (Irvin *et al.*, 1980; Irvin, 1983; Poyet *et al.*, 1994), our results suggest that the name may be not appropriate. Instead they could obtain more PAP-II on the basis of total proteins from the late summer leaves than from spring leaves.

Since PAP-II level was graually increased as time goes by summer from spring, we expected that LD condition and high-light intensity could preferentially stimulate the PAP-II expression. However, the results are exactly opposite. This discrepancy may be resulted from the presence of Rubisco (Ribulose-1,5-bisphosphate carboxylase/oxygenase), about 50% of total soluble proteins in plant leaves. It is probable that a large amount of Rubisco synthesized under LD and high-light conditions may decrease the relative amounts of PAPs in total proteins used in western blot analysis. Another possibility is that PAP-I and PAP-II could be expressed better under low-light intensity than under high-light condition. In general, the pokeweed plants grown in shade look much healthier than grown in open field; they seemed to be more resistant to pathogens. Thus, the plants in shade seem to produce more PAP and thereby they become more resistant to pathogens.

As shown in Figs. 9 and 10, the expression of PAP-II was induced and/or stimulated by biotic stresses and drought stress. The pokeweed plants inoculated with 5 different RNA viruses were protected against viral transmission. These results suggest that under the stress conditions either PAP-II supplements PAP-I action or PAP-II itself has own function.

Based on the our results and fact that three PAPs have same antiviral effects, we would propose a hypothesis: PAP-II appears to have an additive effect in terms of protection of the plant against pathogens during summer time when the plant actively grows and is attacked by various pathogens. However, the exact physiological roles of PAPs *in vivo* still remain to be investigated.

ACKNOWLEDGEMENTS

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