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Isozymes of papaya polyphenol oxidase

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Abstract. Ring spot virus resistant and tolerant cultivars had lower polyphenol oxidase activities than virus susceptible cultivars in apparently healthy papaya leaves. All the papaya leaves had the same polyphenol oxidase with estimated MW of 53 K. The specific activity of polyphenol oxidase in various papaya tissue or growth states was according to the following order: root > leaf > callus > somatic embryo > stem > suspension cells. Gradient polyacrylamide gel eletrophoresis revealed that there were different isozyme patterns in different cultivars and different tissues of the same cultivar. The isozyme patterns were changed by the incubation of crude enzyme extract or cells at 4°C but remained constant if the samples were frozen.

Key words: *Carica papaya*; L-DOPA; Polyphenol oxidase; Ring spot virus.

Introduction

Polyphenol oxidase (PPO) (E.C. 1.14.18.1) is widely distributed in the plant kingdom. It is well known that the enzyme plays an important role in the browning reaction in fruits and vegetables (Mayer and Harel, 1959). It has been suggested that the enzyme might be associated with many important physiological functions of plants such as defense, growth and differentiation (Brueske and Dropkin, 1973; Maratte, 1973; Retig, 1974; Hyodo and Uritani, 1966; Rylan *et al.*, 1982; Gordon and Paleg, 1961).

So far, there have been very few reports on the polyphenol oxidase of papaya. Pal and Selvaraj (1987) found that the PPO activity significantly changed during the ripening of papaya (*Carica papaya* L.). However, there have been no in depth studies on its properties. In the present work, isozymes of PPO in papaya were found, and their activities and patterns in different tissues, cultivars, and growth stages were compared. These findings provide some insight into the role of PPO in papaya.

Materials and Methods

Materials

The callus and somatic embryos used in this experiment were induced from interspecific hybrids of *Carica papaya* and *Carica cauliflora*. Two maternal *C. papaya* plants, 16-6 and 17-1, were used for pollen fertilization. Callus, somatic embryos, and regenerated plants induced from 16-6 embryo culture were designated as 16-6 callus, 16-6 somatic embryo and 16-6 F1, respectively. The culture medium (A1) used for callus and somatic embryos was 1/2 MS (Murashige and Skoog), supplemented with casein hydrolysate 1 g/L, BA (6-benzyl-adenine) 10 ppm, IAA (indole-3-acetic acid) 2 ppm, ABA (abscisic acid) 1 ppm and agar 8 g/L. Suspension cultures were established in liquid 1/2 MS medium suplemented with 1 g/L casein hydrolysate, BA 10 ppm and 2,4-D (2,4-dichlorophenoxyacetic acid) 3 ppm. The cultures were incubated under 25°C 12 hrs/day light and subcultured every 2-3 weeks.

Two different genotypes of junior green house plantlets were used in this work: *Carica papaya* Tainung 2 and 16-6 F1. Tainung 2 is suceptible to ring spot virus (RSV) while 16-6 F1 is resistant to RSV. The

seed of Tainung 2 and the regenerated 16-6 F1 were planted in artificial soil and cultivated in a green house at 25°C for three months. The leaf, stem and root were used for the experiment.

Several types of mature field plant leaves were obtained from the field of the Fongsan Horticulture Experimental Station, Fongsan, Kaoshiung, Taiwan. These included cultivars Tainung 2 (susceptible to RSV), 16-6 F1 (resistant to RSV), *Carica cauliflora* (wild type, resistant to RSV), Tainung 5 and Florida (tolerant to RSV).

L-3,4-dihydroxyphenylalanine (L-DOPA), acrylamide, bisacrylamide, ammonium persulfate and tetramethylenediamine (TEMED) were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). A high molecular weight calibration kit was purchased from the Pharmacia Co. (Uppsala, Sweden). All other chemicals and biochemicals were of reagent grade.

Enzyme Assay

Freshly prepared 0.9 ml of 3.8 mM L-DOPA in 20 mM phosphate buffer (pH 7.0) was mixed with 0.1 ml of PPO solution and incubated at 25°C. The absorbance change at 475 nm was measured by a Beckman DU-50 spectrophotometer. The rate of reaction was calculated from the initial linear portion of the curve. One unit causes a $\triangle$OD (absorbance change) at 475 nm of 0.001/min under the specified conditions.

Measurement of Protein

The protein content was determined by the Bio-Rad protein dye binding method following the instructions of the manufacturer using bovine serum albumin as a standard. The samples were assayed in triplicate.

Enzyme Extraction

Callus, somatic embryos or cells from suspension culture were suspended in 5 volume of potassium phosphate buffer (20 mM, pH 7.0) and homogenized on ice by Polytron homogenizer. The ground slurry was filtered through 4 layers of cheesecloth. After centrifugation at 7,000x g for 30 min in a refrigerated centrifuge (4°C), the supernatant was used to determine enzyme activity.

Root, stem and leaf samples were first cut into small pieces and then extracted using the aforementioned procedure.

Isozyme Analysis

Polyphenol oxidase isozymes from various papaya samples were analyzed by 5-12% linear gradient gel eletrophoresis with a Mighty Small II 7 cm vertical slab unit (Hoefer Scientific Instrument) according to Hames (1981) and stained for enzyme activity by incubating the gel in DOPA solution (3 mg/ml in 20 mM potassium phosphate buffer, pH 7.0). Standard proteins (high molecular weight calibration kit from Pharmacia) including thyroglobulin (669 K), ferritin (440 K), catalase (232 K), lactate dehydrogenase (140 K) and albumin (67 K) were run in the same slab gel and separately stained by coomassie blue. The molecular weight can be estimated from the plot of log MW vs. log %T (T is the acrylamide gel concentration).

Results and Discussion

To elucidate the possible connection between PPO activities and the defense mechanism of the papaya, PPO activities of mature papaya leaves from various cultivars were compared. As shown in Table 1, the

Table 1. *Comparison of polyphenol oxidase activity from the leaves of various cultivars of papaya*

Sample	Specific activity (units/mg protein)
Tainung No. 2	105±7.5
Tainung No. 5	55±5.0
16-6	12±1.5
Florida	8±1.2

PPO activities (specific activity) of healthy mature leaves from different cultivars were according to the following order: Tainung No. 2 (virus susceptible) > Tainung 5 (virus tolerant) > 16-6 (virus resistant) > Florida (virus tolerant). It appeared that virus resistant and tolerant cultivars had lower PPO activities than virus susceptible cultivars in healthy leaves. Thus, PPO might be related to the papaya defense mechanism against virus infection since it causes oxidative polymerization of toxic phenolics, making them have less effect on virus. The difference in PPO activity was not due to the differential expression of isozyme genes since only one major band with estimated M. W. of 53 K (designated as band 7) appeared in the zymogram of all the aforementioned leaf samples. (Fig. 1)

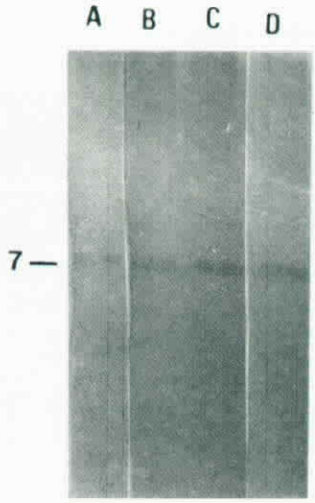

Fig. 1. Zymogram of leaf PPO from various cultivars of papaya. Only one major band (corresponding to band number 7 in Table 3) appeared in the zymogram of all leaf samples tested. A: 16-6 F1; B: Tainung 2; C: Tainung 5; D: Florida.

Table 2. *Comparison of polyphenol oxidase activity from various tissues of 16-6 F1 papaya*

Sample	Specific activity (units/mg protein)
Somatic embryos	118±10
Leaf	184±12
Suspension cell	20±1.8
Root	191±21
Callus	126±11
Stem	104±10

PPO activities from suspension culture cells (stationary phase), callus, somatic embryo, root, stem and leaf were quite different. As shown in Table 2, the specific activity followed the order: root > leaf > callus > somatic embryo > stem > suspension cell. The high specific activity of PPO in the root and leaf samples suggested that PPO played important role in these tissues. It has been suggested that PPO is involved in auxin biosynthesis (Gordon and Paleg, 1961) and inactivation (Briggs and Ray, 1956). Therefore, it could be related to the growth and differentiation of papaya. This needs to be further investigated.

Further analysis of PPO by enzyme activity staining following electrophoresis on gradient polyacrylamide gel electrophoresis revealed that there were different isozyme patterns in different cultivars and different tissues (Table 3). For 16-6 F1, there were six bands designated as bands 1 (MW 669 K), 2 (MW 320 K), 3 (MW 230 K), 4 (MW 176 K), 6 (MW 75 K) and 7 (MW 53 K) in both root and stem. There were three bands (1, 6 and 7) in the suspension cells and embryos, two bands (1 and 6) in the callus and one band (7) in the leaf, respectively. For Tainung 2 cultivar, there were four bands (1, 3, 6 and 7) in the root, two bands (3 and 7) in the stem and one band (7) in the leaf, respectively.

From Fig. 1 and Table 3, it is obvious that the leaf sample from all papaya cultivars and all the tissues (except callus) of 16-6 and Tainung 2 cultivars contained band 7. Therefore, this isozyme is highly conserved in papaya evolution and should have an important physiological function. Bands 2, 3 and 4 did not

Table 3. *Isozyme variations of papaya polyphenol oxidase in different cultivars and tissues*

Band No.	MW[1] (KD)	16-6 F1						Tainung 2		
		Root	Stem	Suspension cells	Embryo	Callus	Leaf	Root	Stem	Leaf
1	669	+[2]	+	+	+	+	−	+	−	−
2	320	+	+	−	−	−	−	−	−	−
3	230	+	+	−	−	−	−	+	+	−
4	176	+	+	−	−	−	−	−	−	−
5	120	−	−	−	−	−	−	−	−	−
6	75	+	+	+	+	+	−	+	−	−
7	53	+	+	+	+	−	+	+	+	+

[1] M. W of each isozyme was estimated by comparing the mobility with standard proteins in linear gradient gel electrophoresis as described in "Method".

[2] +: Presence of isozyme band; −: Absence of isozyme band.

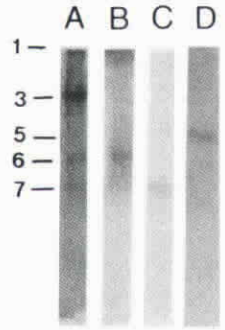

Fig. 2. Effect of 4°C incubation on the multiple forms of PPO in various samples of papaya. Tainung 2 root enzyme extract: Fresh extract (A); extract incubated at 4°C for 3 months (B); 17-1 F1 somatic embryo enzyme extract: Fresh extract (C); Extract incubated at 4°C for 3 months (D).

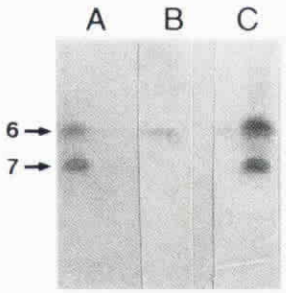

Fig. 3. Effect of storage temperature on the multiple forms of PPO in 16-6 F1 suspension cultured cells. A: cells stored at 0°C for 7 months with medium; B: cells stored at 4°C for 11 months with medium; C: cells stored at -37°C for 7 months with medium.

exist in tissue cultured cells, callus and somatic embryo but appeared in the stem and root of 16-6 cultivar. Apparently, these are the isozymes which have an important function in the differentiated tissues and can be used as markers of differentiation.

The isozyme patterns were changed by the incubation of crude enzyme extract or cells at 4°C. Fig. 2 showed that no isozyme patterns of Tainung 2 and 17-1 (somatic embryo) changed after being stored at 4°C for 3 months. Fresh extract of Tainung 2 roots showed four bands (1, 3, 6, and 7, lane A), but only two bands (1 and 6, lane B) could be seen after incubation. As for 17-1 somatic embryos, fresh extract showed two bands (5 and 7, and C in Fig. 2) but only one band, 5 (lane D), remained after incubation. The results revealed that low MW PPO tends to change into a high MW form during 4°C incubation, possibly due to aggregation induced by cold temperature. The whole cells stored at either 0°C or - 37°C had the same isozyme pattern (bands 6 and 7) as fresh cells while the cells stored at 4°C had only one band, 6 (Fig. 3). Since the cells at 4°C were still in a metabolically active state, the change in isozyme pattern may have been induced by cold-stress.

The different isozyme patterns could be due to aggregation, differences in amino acid composition, variable percentage of covalently linked carbohydrates, partial proteolysis and other posttranslational modifications (Lodish, 1981; Paulson, 1989; Wold, 1981; Chock *et al.*, 1980). These isozymes could have quite different properties (Shaw and Chu, 1989; Chu and Shaw, 1989; Shaw *et al.*, 1989; Harel *et al.*, 1965) and play different roles in growth, differentiation and other physiological functions such as the defense mechanism. Further studies on the structure and function of purified PPO isozymes are necessary to answer these questions. Whatever the reasons for the change in isozyme patterns during sample storage (Figs. 2 and 3), these observations did convey an important message to the researchers using isozymes as genetic markers, i.e., environmental change and storage conditions could greatly change isozyme patterns. Therefore, extreme precautions must be taken in the interpretation of data and the handling of samples.

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木瓜多酚氧化酶之同功異構酶

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木瓜輪點病之抗病與耐病品種木瓜葉片之多酚氧化酶較感病品種者爲低。所有葉片之多酚氧化酶均僅具一種分子量爲53 K之酶。不同組織或生長狀態之多酚氧化酶則具有不同之比活性，其依序爲：根＞葉＞癒傷組織＞體胚＞莖＞懸浮細胞。電泳圖譜顯示不同品種與不同組織之同功異構酶圖譜亦大不相同。同功異構酶圖譜會因粗酵素液或細胞貯藏在4°C下而改變，然而若將樣品予以冷凍則其圖譜可以維持不變。