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Sequential Induction of Ethylene, Lipoxygenase, and Ascorbate Peroxidase in Senescing Soybean Callus

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Abstract: Bursts of ethylene production occurred in twice at an early exponential (EEP) and prestationary (PSP) phases, respectively, during growth of callus tissue isolated from the root of soybean seedlings. The second burst of ethylene production at PSP was smaller in magnitude than the earlier one at EEP, but was followed by increases in both guaiacol peroxidase (GuPOX) and ascorbate peroxidase (AsPOX). The increase in AsPOX activity was also preceded by an increase in lipoxygenase (LOX) activity. Treatment of the tissue with the ethylene antagonist 2,5-norbonadiene (NBD) resulted in substantial reduction in LOX and AsPOX activities during this period. GuPOX activity was reduced only slightly, if any, by NBD. Role of ethylene in the sequential induction of LOX and AsPOX in senescing callus tissue is discussed.
Key words: ascorbate peroxidase, ethylene, lipoxygenase, senescence, soybean callus

Ethylene is a gaseous hormone which promotes senescence in plants (Abele *et al.*, 1992). Activities of several enzymes associated with induction and/or progress of senescence are controled by the hormone. Lipoxygenase (LOX) catalyzes the incorporation of molecular oxygen into fatty acids to form fatty acid hydroperoxides (Vick and Zimmerman, 1987), is involved in senescence of plant tissue (Sylvestre *et al.*, 1989), and its activity is increased by ethylene treatment (Ievinsh, 1992). Some peroxidases (POXs) catalyzing the oxidation of a substrate coupled with the reduction of hydrogen peroxide can also be induced during ethylene-mediated senescence (Abeles *et al.*, 1988).

Soybean callus tissue provides an excellent model system for studies on senescence. Upon subculture the callus tissue undergoes growth cycle with a typical sigmoid pattern reaching a stationary phase in about 20 days. The tissue exhibits a visible sign of senescence by turning brown in color even before reaching the stationary phase.

In the present work, sequential induction of ethylene, LOX and POXs was investigated in callus from soybean roots undergoing senescence.

Materials and Methods

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Material

Soybean (*Glycine max* L. PALDAL) seeds were obtained from the Crop Experiment Station in Suwon, Korea. Root segments from 3 day-old etiolated seedlings were used for callus induction. The callus (1.5 g) was subcultured every 18 days, and used as a material for experiments. The B5 medium containing 2,4-D (2 mg/l), kinetin (0.1 mg/l), and 0.8 % agar (w/v) was used for callus induction and subculture.

Ethylene measurements

Ethylene production from the callus tissue was determined with a gas sample withdrawn from a sealed flask by gas chromatography (Shimazu GC-RIA, 3 m stainless column; air, 0.4 kg/cm^2; carrier N_2, 50 ml/min; H_2, 0.6 kg/cm^2) equipped with an active alumina column (80/100 mesh).

Treatment with 2,5-norbornadien (NBD)

A sterilized eppendorf tube (1.5 ml) containing NBD was placed in a sealed callus culture flask (100 ml) under aseptic conditions. The tube was suspended in the flask using a sterilized thread. The final concentration of NBD in the flask was 1,000 ppm.

Assay of peroxidases (POXs)

Frozen callus was homogenized with 25 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM ascorbate. The homogenate was cen-

trifuged for 30 min at 13,000×*g*. The supernatant was concentrated by microcentrifuge filter (Millipore, NMWL; 5,000). Soluble proteins (3 μg) were used for determination of POX activity. Guaiacol peroxidase (GuPOX) activity was assayed employing the method described by Wakamatsu and Takahama (1993), where increases in absorbance value at 470 nm per min were determined. The method of Katsumi *et al.* (1994) was used for assay of ascorbate peroxidase (AsPOX) activity, where decreases in absorbance value at 290 nm per min were determined. Specific activity of POX (unit/μg protein) was determined by enzyme kinetics software from Pharmacia.

Detection of ascorbate peroxidase activity in native gels

Mittler and Zilinskas' method (1993) was modified to detect AsPOX activity in native gels. Soluble proteins (2~3 μg) were subjected to discontinuous PAGE (5% stacking gel and 10% separating gel) under nondenaturing and nonreducing conditions. The carrier buffer contained 2 mM ascorbate and the separation was carried out at 4℃. The separating gel was equilibrated with 50 mM sodium phosphate buffer (pH 7.0) and 2 mM ascorbate for 30 min. The equilibrium buffer was changed every 10 min. The gel was incubated with 50 mM sodium phosphate buffer (pH 7.0) containing 4 mM ascorbate and 2 mM H_2O_2 for 20 min. The gel was subsequently washed with sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM nitroblue tetrazolium (NBT) with gentle agitation. The reaction was allowed to continue for 10 min and stopped by a wash with deionized water.

Detection of lipoxygenase (LOX) isozymes

Funk's method (1985) was used to detect LOX isozyme activity. Isoelectric focusing was carried out with a Mini IEF Cell (Bio-Rad). Frozen callus was homogenized with 50 mM HEPES buffer (pH 7.5). The homogenate was centrifuged and the supernatant was concentrated as described in the method for POX assay. Prefocusing was conducted for 15 min at 100 V and for 15 min at 200 V. Focusing was conducted for 1 h at 450 V. The activity of LOX isozymes in the gel was stained with 100 ml of 50 mM HEPES buffer (pH 7.5) containing 120 μl linoleic acid and 30 mg *o*-dianisidine. Segments of focused gel containing IEF marker was stained with 0.2% Coomassie brilliant blue R-250 in 28% EtOH and 14% acetic acid. Destaining was carried out with 28% EtOH in 14% acetic acid. The activity of LOX on the agarose gel was determined by an image analyzer from Pharmacia (Uppsala, Sweden).

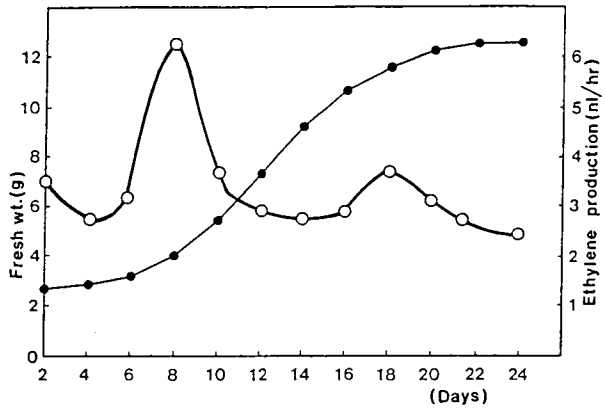

Fig. 1. The kinetics of growth (closed circle) and ethylene production (open circle) in soybean callus. Culture fasks (100 ml) were sealed with a silicon plug for 24 h before withdrawing a 1 ml gas sample to measure ethylene.

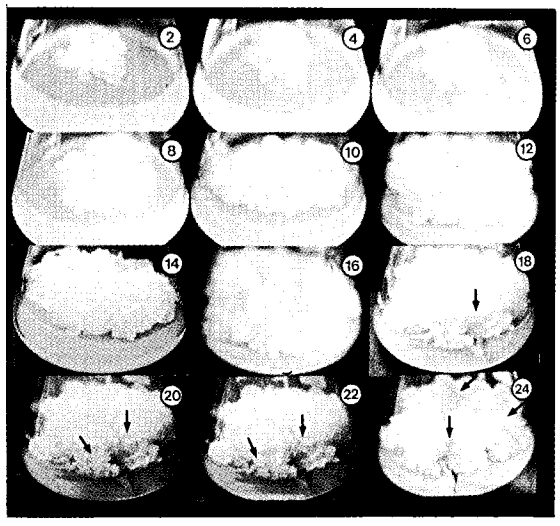

Fig. 2. Photographs of soybean callus at different growth phases. The numbers indicate days after subculture. The arrows indicate browning sites on the callus.

Results

Growth of, and ethylene production from callus tissue during a 24 days culture period are shown in Fig. 1. A burst of ethylene production occurred early in the exponential phase peaking at day 8. It is believed that the ethylene peak may be associated with the onset of active exponential growth of the tissue. This ethylene peak was followed by a second, smaller peak of ethylene production at day 18 just prior to the stationary phase. The second ethylene peak coincides with appearance of browning on the tissue (Fig. 2). The color persisted throughout the stationary phase where the tissue underwent senescence leading eventually to the cellular death. It can therefore be assumed that the second ethylene peak might be related to the onset

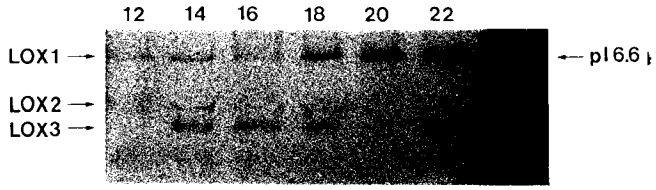

Fig. 3. The activity of lipoxygenase (LOX) isozymes from the soybean callus. The numbers mean days after subculture.

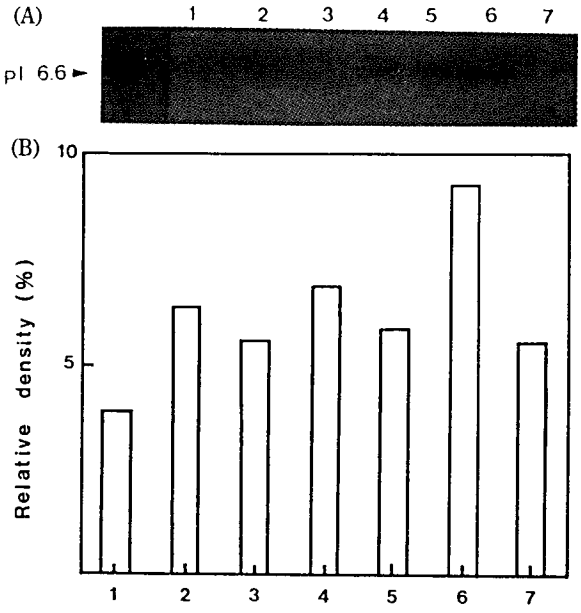

Fig. 4. Effect of 2,5-norbornadine (NBD) on the activity of lipoxygenase1 (LOX1). (A) Activity of LOX1 on agarose gel. 1: Activity at day 17. 2: Activity at day 18. 3: Activity at day 18, pretreated with 1,000 ppm NBD for 1 day. 4: Activity at day 19. 5: Activity at day 19, pretreated with 1,000 ppm NBD for 2 day. 6: Activity at day 20. 7: Activity at day 20, pretreated with 1,000 ppm NBD for 3 day. Soluble protein (20 μg) from soybean callus was loaded and focused. Agarose gel (1%) containing 100 μl pharmarlyte (pH 3~10) was used for the isoelectric focusing. (B) Analysis of the activity of LOX1 on the agarose gel by an image analyzer.

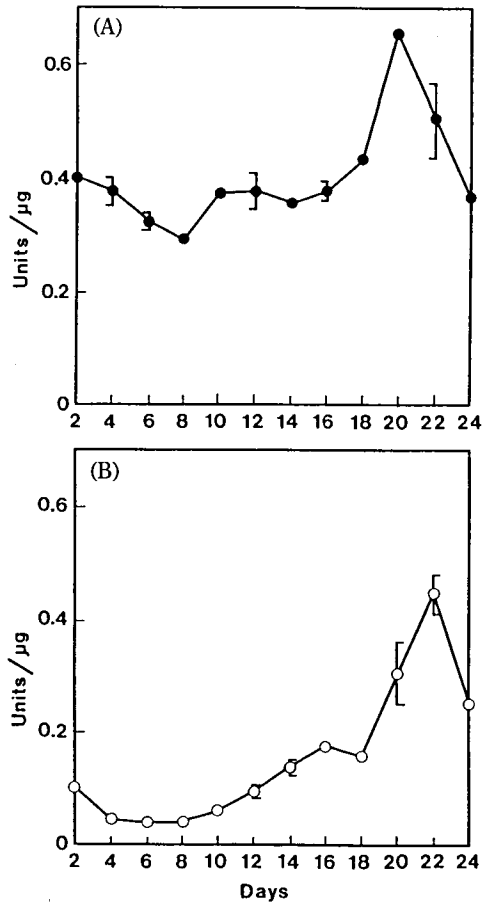

Fig. 5. The peroxidase activity in soybean callus. (A) Guaiacol peroxidase (GuPOX). (B) Ascorbate peroxidase (AsPOX).

of senescence.

At least three isozymes of lipoxygenase (LOX1, LOX 2, and LOX3) having different pI values can be separated as shown in Fig. 3. Of these three forms, LOX1 (pI 6.6) appeared to be a major form with the highest activity. It can also be clearly seen that the LOX1 activity started to emerge at day 18 and persisted thereafter. Data in Fig. 4 indicate that the LOX1 activity was reduced significantly by treatment of the callus tissue with the ethylene antagonist NBD, suggesting that the increased enzyme activity may have resulted from elevated levels of endogenous ethylene production from the tissue.

Changes in peroxidase activity were monitored throughout the entire culture period. Data illustrated in Fig. 5 indicate that both guaiacol peroxidase (GuPOX) and ascorbate peroxidase (AsPOX) activities increased steadily from about day 8 reaching a peak at day 20 for the former and day 22 for the latter. Both activities subsided following the peaks. It is noticed that the peak enzyme activity occurred after a lag of 2 days following the onset of elevated LOX1 activity for GuPOX, and 4 days for AsPOX, respectively.

Treatment of the callus tissue with NBD resulted in a substantial reduction in AsPOX activity, whereas GuPOX activity was little affected, if any, by the same treatment (Fig. 6). These results indicate that endogenously produced ethylene may be causally related to the peak of enzyme activity for AsPOX, but probably not for GuPOX.

AsPOX could also be separated on a native gel into two isozymes, slow moving AsPOXS and fast moving AsPOXF, during the stationary phase as shown in Fig. 7. Activities of both forms appeared maximal at day 22, and already decreased substantially at day 24, in accordance with the data of Fig. 5B. Both activities of AsPOX isozymes were also found to be clearly suppressed after the tissue was grown in the presence of NBD.

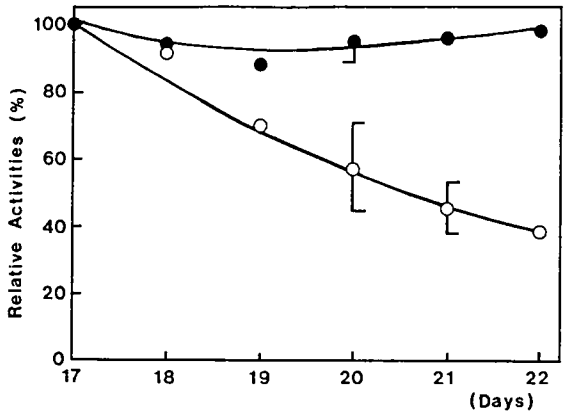

Fig. 6. Suppression of AsPOX activity by NBD. Values are presented as percent of the control (without NBD) for GuPOX (closed circles) and AsPOX (open circles). NBD was applied at day 17.

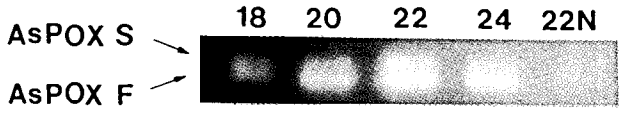

Fig. 7. The activity of AsPOX isozymes (S and F) from soybean callus. The numbers denote days after subculture. N means the treatment with NBD.

Discussion

Senescence represents cellular processes with a series of programmed events leading ultimately to the death of a cell. A number of enzymes are known to be under the direct or indirect control of the senescence-inducing hormone ethylene. The two enzymes investigated in the present work, lipoxygenase (LOX) and peroxidase (POX), are both known to be induced by ethylene (Abeles *et al.*, 1992). Based on the time course data obtained in the present work (Figs. 1, 3, and 5), however, action of ethylene to induce lipoxygenase seems more likely to be direct compared with induction of perxidase, although both are suppressed by the ethylene antagonist NBD.

Lipid peroxidation by lipoxygenase yields hydrogen peroxide as a by-product (Kumar and Knowles, 1993). Because of its toxicity, cells must get rid of excess hydrogen peroxide, the reaction being catalyzed by POX (Mittler and Zilinskas, 1991). Activities of both guaiacol peroxidase (GuPOX) and ascorbate peroxidase (AsPOX) sharply increase during the stationary phase, but only AsPOX activity could be reduced by NBD. It is thus implied that increased activity of AsPOX was attributable to ethylene produced, whereas that of GuPOX was not. AsPOX activity was slightly stimulated in 2 days by treatment of the callus with the ethylene-releasing agent 2-chloroethyl phosphonic acid (CEPA), but the magnitude of the stimulation was very small, and it quickly subsided even in the presence of CEPA (data not shown). In view of the long lag period for AsPOX induction following the ethylene peak and also the lack of a significant effect of CEPA, ethylene is unlikely to have a direct action on the enzyme induction observed in the stationary phase. One plausible speculation would be that accumulation of hydrogen peroxide as a result of induction of lipoxygenase by ethylene induced AsPOX by a mechanism of substrate induction. This possibility will be checked in future studies.

In the present work we identified AsPOX as being distinct from the major family of plant peroxidase GuPOX (Santos *et al.*, 1996). AsPOX from soybean callus is so labile that the enzyme activity became undetectable in 3 h following extraction, whereas GuPOX activity was found stable for many hours (data not shown). AsPOX in plants is known to exist in at least four distinct types based on subcellular localization i.e., the cytosol, thylakoid membranes, stroma, and glyoxisomal membranes (Mittler and Zilinskas, 1991; Miyake *et al.*, 1993; Bunkelmann and Trelease, 1996). While AsPOXs localized in thylakoid and stroma were major AsPOXs in matue leaves generating hydrogen peroxide during photon-utilization processes of photosynthesis (Amako *et al.*, 1994), glyoxysomal AsPOX activity was detected mainly in growing oilseedings during β-oxidation of fatty acids stored in the cotyledons (Bunkelmann and Trelease, 1996). In this work, we have used heterotrophic callus using sucrose as an energy source under dark conditions. Therfore, our AsPOX is believed to be mostly from the cytosol. Based on the ability of AsPOX to prevent the ascorbate-dependent reduction of nitroblue tetrazolium in the presence of hydrogen peroxide (Mittler and Zilinskas, 1993), we were able to separate two types of AsPOX isozymes from soybean callus. The profile of variation of AsPOX isozyme activity on the native gel was in excellent agreement with that determined by the photometric method. It was recently reported that cytosolic AsPOX from *Arabidopsis* might be encoded by a small multigene family (Santos *et al.*, 1996).

Two kinds of POXs which were detected during ethylene-induced senescence degraded chlorophyll of cucumber cotyledons *in vitro*, and it was interpreted that ethylene-induced POXs are involved in the stimulation of chlorophyll catabolism in senescing tissues (Abeles *et al.*, 1988). It remains to be seen whether peroxidases, depending on their subcellular localization or compartment, have on one hand senescence-promoting action in the chloroplast and on the other hand senescence-delaying action in the cytosol through hydrogen peroxide-scavenging effects.

Acknowledgement

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