# Immuno-Localization of Peroxidase in Pumpkin (*Cucurbita ficifolia* Bouché) Seedlings Exposed to High-Dose Gamma Ray

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**Peroxidases (PODs) were localized in pumpkin tissues by using an immuno-gold labeling technique in combination with transmission electron microcopy (TEM). Polyclonal antibodies raised in rabbits against horseradish peroxidase were utilized. The localization patterns of gold particles for peroxidases from tissues of both control and gamma-irradiated plants were typically present in the plasma membrane, cytosol, and cell walls. However, particle densities were remarkably increased on the cell corner middle lamellae of parenchyma cells, especially in the petioles. Based on these results, we propose that growth inhibition is associated with cell-wall stiffening, as related to the formation of cross-links among cell wall polymers. In addition, the densities of POD following gamma irradiation vary according to cell and tissue types in pumpkin.**

*Keywords:* gamma irradiation, immuno-gold labeling, peroxidase, pumpkin, seedling growth

Following exposure to high-doses of gamma rays, plants exhibit a wide range of symptoms, including reduced or inhibited germination and seedling growth (Kim et al., 2004, 2005; Wi et al., 2005a, 2006). These gamma rays can damage a number of vital metabolic constituents and important biomolecules (Kim et al., 2004, 2005), and can also cause irreversible injury to cell membranes (Wi et al., 2005a). However, despite extensive knowledge of the biochemical and ultrastructural effects of gamma irradiation, no studies have been reported concerning how cell walls are affected following such treatment.

Plants are frequently exposed to various types of physical, chemical, and biological stresses, which may induce defensive mechanisms (Larcher, 1995; Somssich and Hahlbrock, 1998). Among these are biochemical and structural responses that lead to the strengthening of cells (Iiyama et al., 1994; Ingham et al., 1998). Peroxidase (POD, EC 1.11.1.7) is widely found in cell walls and cytoplasm (Kim et al., 2002; Wi et al., 2005b). This important enzyme is involved in a variety of cellular functions, including IAA oxidation, lignification, suberization, cell elongation and growth, and the regulation of cell wall biosynthesis and plasticity (Espelie et al., 1986; Lagrimini et al., 1987; Chanda and Singh, 1997; Kim et al., 2002; Wi et al., 2005b). The growth rates of axial organs have long been correlated with differences in POD activities and the formation of phenolic cross-linkages between cell wall components; mediation by cell wall-associated POD enzymes possibly accounts for reduced plant cell expansion (Goldberg et al., 1987; MacAdam et al., 1992; Chen and Kao, 1995; Hohl et al., 1995; Lee et al., 1995; Bacon et al., 1997; Lin and Kao, 1999).

Histochemical and cytochemical investigations have contributed to our understanding at the ultrastructural level. Localization of POD uses various procedures, including cytochemical staining and immuno-fluorescence assays with polyclonal antibodies. Although these histochemical methods are helpful at the tissue level, they do not have enough resolution at the cellular level. In contrast, immuno-cytochemistry utilizes the specificity of the antigen-antibody reaction to visualize the presence and location of target antigens via electron microscopy at the cellular level (Kim et al., 2002; Wi et al., 2005b).

In this study, we applied immuno-gold labeling to establish the location of induced POD, and to examine spatially the differences in distribution patterns for this enzyme after gamma irradiation. To aid in this visualization, we used a polyclonal antibody against horseradish POD in combination with transmission electron microscopy.

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## MATERIALS AND MTHODS

### Plant Materials

Seedlings of pumpkin (*Cucurbita ficifolia* Bouché) were cultivated in a greenhouse on the experimental farm at the Advanced Radiation Technology Institute, Jeongeup, Korea. Uniformly sized seedlings were held for 9 d in polyethylene bags containing a bed-soil mixture before being exposed to 1 kGy generated by a gamma irradiator ($^{60}$Co, ca. 164,000 Ci capacity; AECL, Canada) at the Korea Atomic Energy Research Institute, Korea. This dose was confirmed with a thermoluminescence dosimeter (Alaine, Germany). Samples were collected from leaves, petioles, cotyledons, and hypocotyls 19 d after the radiation treatment. Symptoms that indicated exposure to high-dose gamma rays (1 kGy) included leaf curling and the yellowing of cotyledons (data not shown), both of which are responses to an imbalance in the levels of plant growth regulators.

### Immuno-Cytochemistry of Peroxidase

To evaluate their immuno-cytochemistry, tissue samples were cut into small pieces with a razor blade, then immediately soaked for 2 h at 4°C in a mixture of 0.1% (v/v) glutaraldehyde and 4% (v/v) paraformaldehyde prepared in 50 mM sodium cacodylate buffer (pH 7.4). They were then washed three times with the same buffer, displaced in an ethanol series, and embedded in LR-white resin (London Resin, UK).

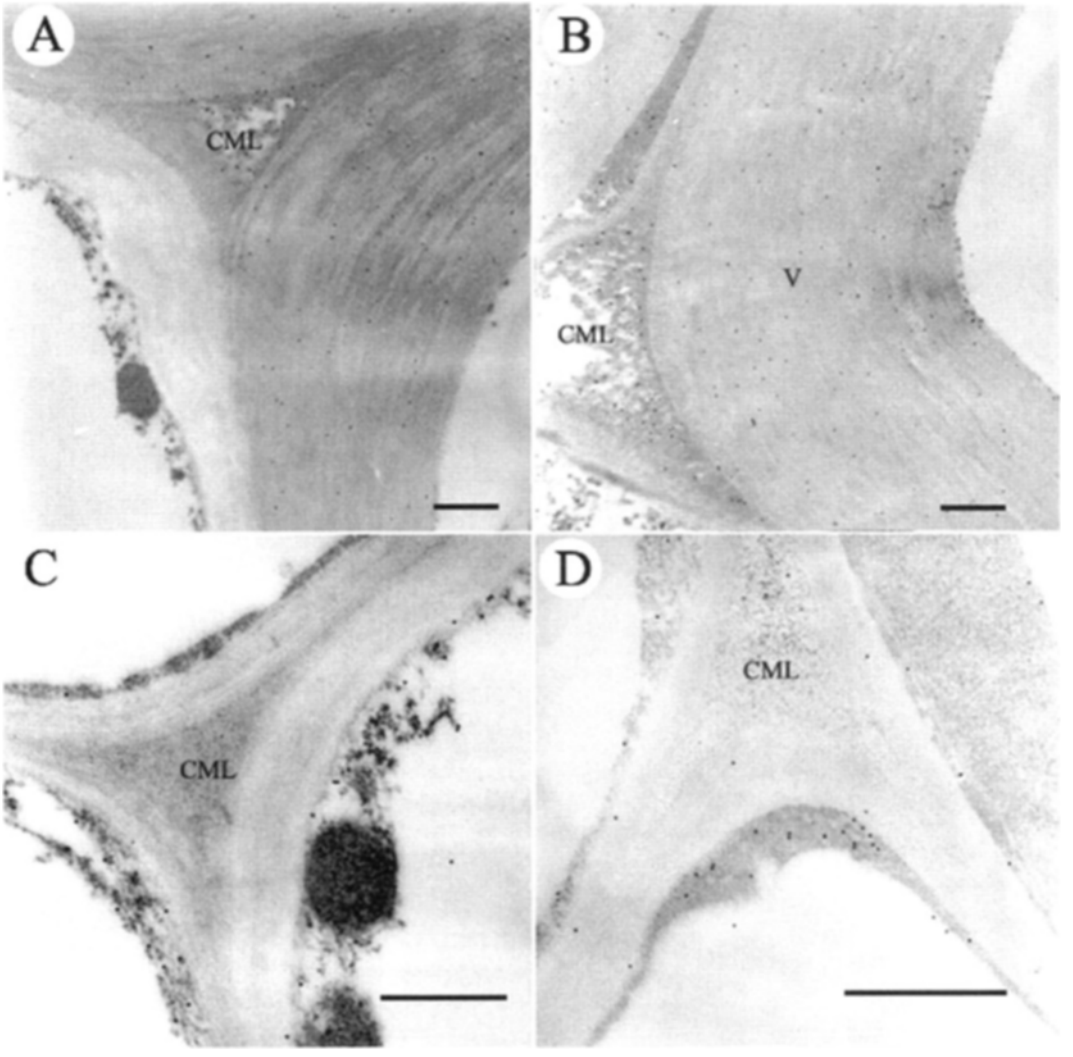


**Figure 1.** Immuno-localization of peroxidase in vessels (**A** and **B**) and parenchyma cells (**C** and **D**) of leaves from control plant (**A** and **C**) and plant irradiated with 1 kGy (**B** and **D**). CML, cell corner middle lamellae; V, vessel wall. Bar = 0.5 μm.

Conventional indirect immuno-gold labeling was employed for the localization of POD (Kim et al., 2002). Ultra-thin sections (70 to 90 nm) were mounted on uncoated nickel grids (300 mesh). After incubating for 30 min with 0.1 M glycine in phosphate-buffered saline (PBS, pH 7.4), the sections were incubated for 40 min with 5% (v/v) normal goat serum in PBS to avoid nonspecific binding of the antibody. They were then incubated with the antiserum against horseradish POD type III (approximate molecular weight 44,000; Sigma, USA), which was produced by Research Genetics (USA). Following dilution in PBS with 0.05% (v/v) Tween 20, the sections were labeled for 2 h with goat-anti-rabbit antiserum conjugated to colloidal gold (10 nm particle size), then examined via transmission electron microscopy (TEM) (J-1010; Jeol, Japan) after counterstaining with 4% (w/v) uranylacetate. Control samples also were treated as described above, with the exception that rabbit pre-immune serum was used instead of the primary antibody. To better evaluate the localization patterns of POD, the density of gold labels (number per $\mu m^{-2}$) was calculated in the area of the scanned microphotographs by image analyzer (Image-Pro Plus; Media Cybernetics, USA).

## RESULTS AND DISCUSSION

Immuno-gold labeling of POD was conducted for vessels and parenchyma cells from control and irradiated plants (Fig. 1-4). Gold particles appeared as

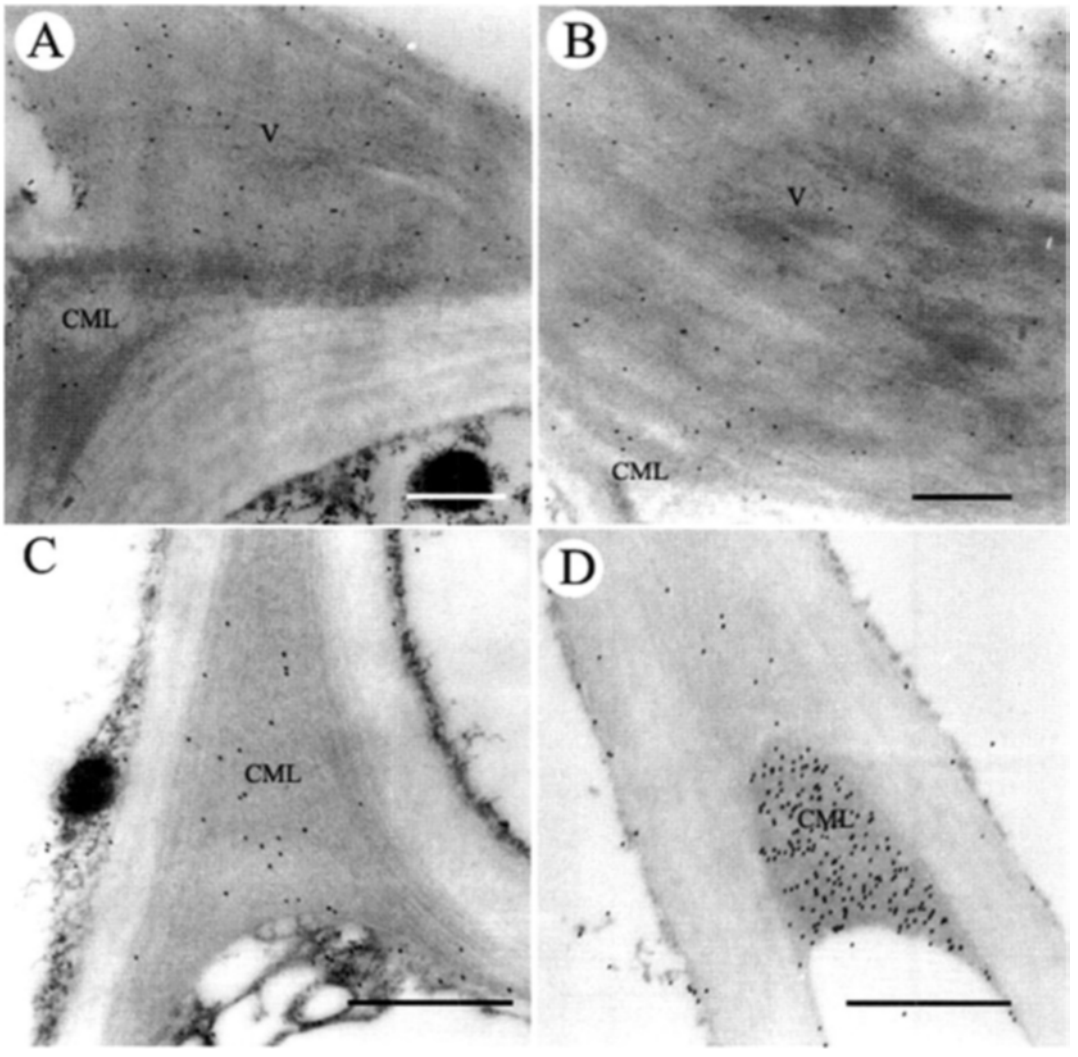


**Figure 2.** Immuno-localization of peroxidase in vessels (**A** and **B**) and parenchyma cells (**C** and **D**) of petioles from control plant (**A** and **C**) and plant irradiated with 1 kGy (**B** and **D**). CML, cell corner middle lamellae; V, vessel wall. Bar = 0.5 μm.

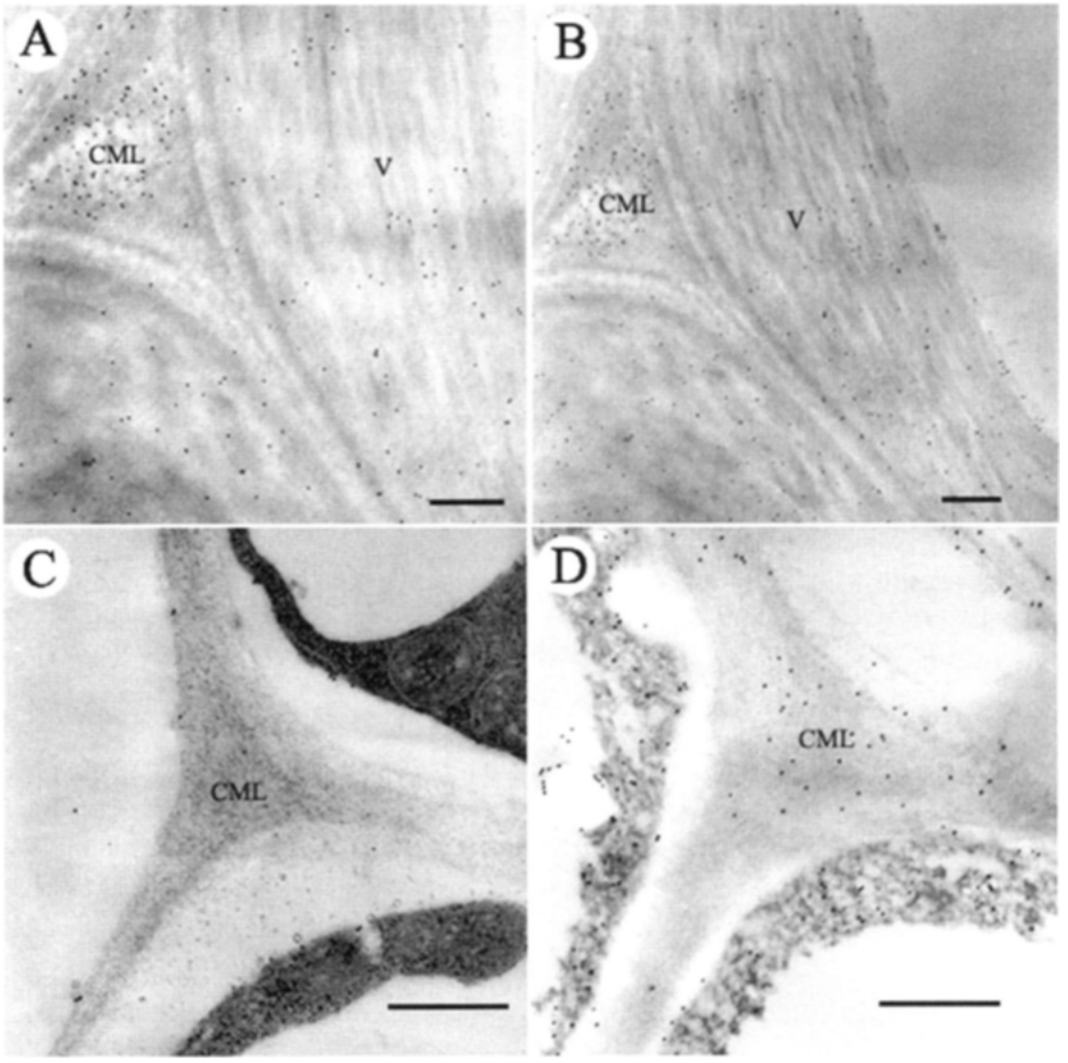


**Figure 3**. Immuno-localization of peroxidase in vessels (**A** and **B**) and parenchyma cells (**C** and **D**) of cotyledons from control plant (**A** and **C**) and plant irradiated with 1 kGy (**B** and **D**). CML, cell corner middle lamellae; V, vessel wall. Bar = 0.5 μm.

black marks. For both plant sources, POD labels were observed in the plasma membrane, cytosol, cell wall, and middle labella. These particles were not detected when sections were incubated in the non-immune serum (data not shown).

As markers of POD in our control and irradiated samples, gold particles in the leaves were distributed in the cytosol and cell walls for both vessels and parenchyma cells (Fig. 1). In the vessel walls, their distribution was uniform in the cell walls and middle lamellae in both plats (Fig. 1A, 1B). In contrast, the dispersal of gold particles in control-plant parenchyma cells was limited to the plasma membrane and cytosol (Fig. 1C), and was labeled only in small numbers on the cell walls of irradiated samples (Fig. 1D). Patterns were similar between control and irradiated leaves for vessels and parenchyma cells. In the parenchyma cells of irradiated petioles, the particles were mainly distributed on the cell corner middle lamella (Fig. 2D); this pattern was almost the same for the irradiated vessels (Fig. 2A, 2B). In contrast, the control-plant samples showed only sparse labeling in the cell corner middle lamella (Fig. 2C). For the cotyledons (Fig. 3) and hypocotyls (Fig. 4), the particle distribution patterns in vessels and parenchyma cells were similar to those observed with the petioles, with localization being found mostly on the cell corner middle lamellae in parenchyma cells (Fig. 3D, 4D).

To better evaluate the localization pattern of POD, quantitative analysis of the gold particles was carried out (Table 1). In the irradiated samples, particle density was greatly increased in the cell corner middle

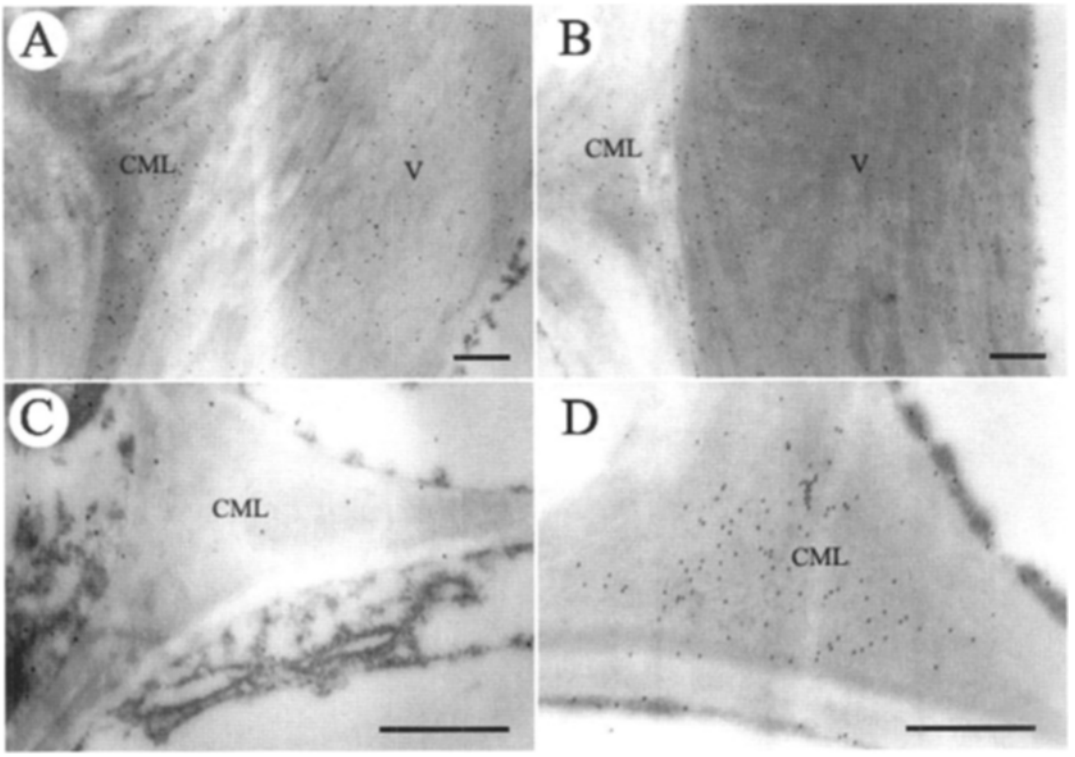


**Figure 4.** Immuno-localization of peroxidase in vessels (**A** and **B**) and parenchyma cells (**C** and **D**) of hypocotyls from control plant (**A** and **C**) and plant irradiated with 1 kGy (**B** and **D**). CML, cell corner middle lamellae; V, vessel wall. Bar = 0.5 μm.

**Table 1.** Quantitative evaluation of the distribution of gold particles as a marker of peroxidase activity (gold particles per $\mu m^2$). Data represent means of five fields ± standard errors (n = 5).

| | Vessel | | | | Parenchyma cell | | | |
|---|---|---|---|---|---|---|---|---|
| | Cell wall | | Middle lamella | | Cell wall | | Middle lamella | |
| | control | 1 kGy | control | 1 kGy | control | 1 kGy | control | 1 kGy |
| Leaf | 9.4 ± 1.3 | 9.1 ± 1.8 | 19.6 ± 4.2 | 39.1 ± 3.9 | 2.1 ± 0.5 | 6.1 ± 0.7 | 1.2 ± 0.1 | 8.2 ± 2.3 |
| Petiole | 11.9 ± 1.4 | 12.7 ± 0.9 | 14.4 ± 3.9 | 12.1 ± 0.7 | 2.8 ± 0.8 | 8.8 ± 0.9 | 21.4 ± 3.6 | 233.3 ± 19.2 |
| Cotyledon | 10.9 ± 2.1 | 15.2 ± 2.3 | 38.3 ± 4.7 | 36.8 ± 3.9 | 4.3 ± 1.1 | 19.0 ± 2.9 | 6.1 ± 1.9 | 31.7 ± 4.6 |
| Hypocotyl | 16.2 ± 1.2 | 16.6 ± 1.8 | 31.1 ± 2.9 | 27.9 ± 3.9 | 8.6 ± 1.3 | 9.5 ± 1.7 | 5.4 ± 0.9 | 77.5 ± 4.9 |

lamellae of the parenchyma cells, whereas no marked difference in distribution was observed in the vessel walls. Labeling intensities in that region were 5- to 15-fold higher than those found in the controls. The greatest increase in POD intensity (at 19 d after treatment) was observed on cell corner middle lamellae from irradiated petioles. This rise in density suggests that gamma irradiation induces POD generation on the cell walls and cell corner middle lamellae of parenchyma cells.

Various tissue types sampled from irradiated pumpkin seedlings showed increased POD densities in their cell walls and cell corner middle lamellae of the parenchyma cells (Table 1). However, POD labeling in the vessels was homogeneously distributed in the walls and cell corner middle lamellae, with differences being only negligible between control and irradiated plants. Peroxidase has extremely broad substrate specificity and exists in a multitude of isoenzyme forms. Type III POD plays a role in lignin biosynthesis (Whetten et al., 1998), and also binds to pectic molecules in the cell walls (Wi et al., 2005b). Because POD may be bound to cell wall polymers (Whetten et al., 1998), an essential role has been proposed in the stiffening of cell walls through the formation of biphenyl bridges between cell wall polymers

and, thus, a reduction in cell wall extensibility (Fry, 1986). Such a reduction in leaf growth rates and the cessation of cell expansion under stress conditions may be associated with changes in cell wall-bound POD activity (Lin and Kao, 2001). Thus, our observations of intensive labeling in the middle lamellae further support the suggestion that POD inhibits seedling growth.

In conclusion, we have discovered here that 1) the localization pattern of POD varies according to cell and tissue type in pumpkin, 2) POD is more inductive via gamma irradiation in parenchyma cells than in the vessels, and 3) the density of gold particles, especially in the petioles and hypocotyls, is mainly increased by gamma rays in the cell corner middle lamellae.

## ACKNOWLEDGEMENT

This project was supported by a grant from the Nuclear R&D Program of the Ministry of Science and Technology, Korea.

Received November 15, 2005; Accepted January 25, 2006.

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