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Effects of Various Ions on the Cellular and Secretory Isoperoxidases in Rice Suspension Culture

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Abstract : The effects of several ions on the specific activity and isozyme patterns of cellular and secretory isoperoxidases were studied in suspension-cultured cells of rice (*Oryza sativa* L.). Peroxidase release into the culture medium occurred in the absence of added calcium. The addition of calcium ion greatly stimulated the secretion of cationic isoperoxidases such as C2 and C3 into the medium; a maximum 11 fold increase of secretions occurred in the presence of 5 mM $CaCl_2$, and the secretion was accomplished within 1 hour after the addition of $CaCl_2$. About a 10 fold increase of the peroxidase secretion into the medium did occur with 0.5% NaCl, whereas cellular isoperoxidase levels were reduced notably. About a 6 fold increase of the specific activity of cellular isoperoxidase was found in 5 mM $NiCl_2$-treated cell, while $NiCl_2$ had no effect on the secretion of peroxidase into the medium. Various concentrations of KCl did not change peroxidase secretion, but 5 mM $ZnCl_2$ reduced peroxidase secretion greatly. The major secretory isoperoxidases stimulated by $CaCl_2$, NaCl and cellulase were composed of cationic isoperoxidases C2 and C3, which were found to be localized in the cell wall of rice by examination of the enzyme in the protoplast. Furthermore, the secretion rates of secretory isoperoxidases were increased rapidly when cellulase was treated in the absence of the osmotic stabilizer of 0.4 M mannitol. These results suggest that the stimulations of secretory isoperoxidase levels seem to be due to the stimulation of secretion into the culture medium of rice.
Key words : cellular isoperoxidase, rice, secretory isoperoxidase

Plant peroxidase (EC 1.11.1.7) is widely distributed in all higher plants (van Huystee and Cairns, 1982) and it has been implicated in a wide range of metabolic processes including lignin biosynthesis, indole-3-acetic acid oxidation and response to pathogen (Fry, 1986).

In Korean radish there are at least eight isoperoxidases, and some of them were isolated and their enzymatic properties were studied at the protein (Yoo and Kim, 1987; Lee and Kim, 1990; Lee and Kim, 1994; Lee *et al.*, 1994), carbohydrate (Kim and Kim, 1996) and gene level (Park and Kim, 1996). In the case of rice (*Oryza sativa* L.), four peroxidase components from green leaves and two forms of peroxidases were isolated and characterized (Hiroyuki *et al.*, 1991). Moreover rice cationic peroxidase, PO-C1, which was induced in incompatible interactions between the vascular pathogen *Xanthomonas oryzae* pv oryzas and rice, was also purified (Scott *et al.*, 1995).

One of the main functions of peroxidases in various sources is known to be related with the defense enzyme complex, ensuring the detoxification of the reactive oxygen species (Bakardjieva *et al.*, 1996). Moreover, the changes of peroxidase activity and isozyme patterns have been reported to be involved in the influence of different environmental factors, including the metal ion effect (Antje *et al.*, 1996), salt effect (Bakardjieva *et al.*, 1996) and air pollution damage (Karege *et al.*, 1982) etc. In this respect, many authors began to find information concerning the metabolic response of plants to different stress factors.

Usually plant cell cultures produce various enzymes and secrete some of them into the medium. However, very little information is available on the effectors on which peroxidase secretion into the medium is dependent. The present study describes the effect of several ions on the secretion of isoperoxidases into the medium from rice (*Oryza sativa* L.) in order to develop an effective production system.

Materials and Methods

Experimental plant

A rice callus line, *Oryza sativa* L., was maintained routinely in AA2 media. The rice cell suspension culture of 20 ml was aseptically transferred to 80 ml of

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AA2 medium in a 250 ml flask and then grown for 14 days.

Peroxidase assays and protein determination

The peroxidase activity with guaiacol as a substrate was assayed by a modified procedure of Kim *et al.* (1980). The assay mixture contains 40 mM phosphate buffer, 15 mM guaiacol, 5 mM H_2O_2 and 50 μl of enzyme preparation in a total volume of 1 ml. The reaction was initiated by the addition of H_2O_2, and the increase in absorbance at 470 nm was measured using a UV/VIS spectrophotometer. Protein was determined by the method of Lowry *et al.* (1951).

Gel electrophoresis

Starch gel electrophoresis was performed as described by Kim *et al.* (1980). Isoperoxidase bands were visualized by placing the gel in a solution of 100 mg of 3-amino-9-ethylcarbazole in 10 ml of N,N-dimethyl-formamide, 50 mM of acetate buffer (pH 5.0), 10 mM $CaCl_2$ and 0.2 ml of 30% H_2O_2. The same quantities of proteins were loaded for all of the gel analysis.

Effect of various ions on the cellular and secretory isoperoxidases

The effects of various ions on the secretion of rice callus peroxidase were examined by comparing the specific activities and isoperoxidase patterns between rice cell and medium cultured with various ions such as NaCl, $CaCl_2$, KCl, $NiCl_2$ and $ZnCl_2$. The concentrations of four test ions except NaCl fell within the range of 0 and 10 mM, and the concentration range of NaCl was between 0 and 2%. The rice cell culture was maintained with various concentrations of indicated ions for 1 week, and then filtered through Whatman paper (No. 1) for the separation of rice cells from medium extracts. The collected cells were resuspended with a minimum volume of fresh AA2 media for the measurement of cellular isoperoxidase levels. The activities of secretory isoperoxidases were measured in the remaining medium extract.

Determination of NaCl treated cell volume

Experiments on the treatment of NaCl were performed by adding NaCl to log phase cells. The growth rate of suspension cells was determined by measurement of packed cell volume. Cells were collected in a 15 ml conical tube by centrifugation for 20 min at 2,500 rpm and the volume of collected cells was measured.

Protoplast isolation and culture

Rice cell suspension culture (10 ml) in AA2 media was incubated with cellulase (20 mg/ml) in the presence of 0.6 M mannitol for 5 hours in darkness and 25°C at a rotary shaker speed of 50 rpm. The protoplast suspension solution was filtered through a 40 mesh cell filtration unit in order to remove the debris. Protoplast susupension was centrifuged at 100 rpm for 3 min and the supernatant was removed. The remaining protoplasts were resuspended with 5 ml of culture medium. Washing by centrifugation was repeated two more times and the protoplasts were finally resuspended with a 2 ml culture medium (Constabel, 1975).

Results

Effects of $CaCl_2$ on the cellular and secretory isoperoxidases

The effects of $CaCl_2$ on the specific activity of total peroxidase and isoperoxidase patterns were compared between cell and medium extracts of rice (Fig. 1). In the cell, there existed three cationic isoperoxidases named C1, C2 and C3, and three anionic isoperoxidases named A1, A2 and A3 when subjected to starch gel electrophoresis at pH 7.0. On the contrary, the medium contains secretory isoperoxidases mainly composed of cationic isoperoxidases such as C2 and C3. Peroxidase release into the culture medium occurred in the absence of added calcium. Addition of calcium ion greatly stimulated the secretion of cationic isoperoxidases such as C2 and C3 into the medium, and a maximum 11 fold increase of secretions occurred in terms of specific activities in the presence of 5 mM $CaCl_2$ (Table 1). The stimulation of the secretion was rapid, being evident

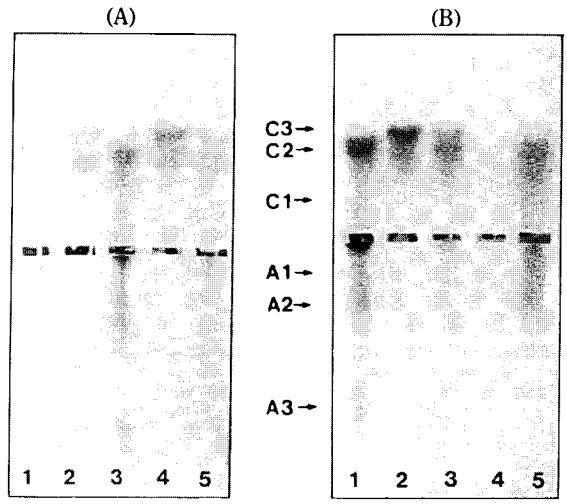

Fig. 1. The effect of $CaCl_2$ on the patterns of cellular and secretory isoperoxidases from rice grown for 1 week. (A) lane 1, 0 mM $CaCl_2$ (medium): lane 2, 1 mM $CaCl_2$ (medium): lane 3, 1 mM $CaCl_2$ (cell): lane 4, 3 mM $CaCl_2$ (medium): lane 5, 3 mM $CaCl_2$ (cell). (B) lane 1, 0 mM $CaCl_2$ (cell): lane 2, 5 mM $CaCl_2$ (medium): lane 3, 5 mM $CaCl_2$ (cell): lane 4, 10 mM $CaCl_2$ (medium): lane 5, 10 mM $CaCl_2$ (cell).

Table 1. Effects of various ions on the specific activities of cellular and secretory isoperoxidases in rice suspension culture

		Specific activity of isoperoxidase ($\triangle A_{470}$/min/mg)	
		Cellular	Secretory
$CaCl_2$ (mM)	0	6	1
	1	1.6	4.4
	3	1	4.6
	5	1.5	11.2
	10	1.6	0.5
NaCl (%)	0	1.9	0.1
	0.5	0.5	1.1
	1	0.5	0.6
	2	0.4	0.6
KCl (mM)	0	0.2	0.1
	5	0.27	0.2
	10	0.2	0.16
$NiCl_2$ (mM)	0	0.2	0.4
	1	0.44	0.4
	5	1.2	0.6
	10	0.8	0.5
$ZnCl_2$ (mM)	0	0.5	0.4
	1	0.3	0.2
	5	0.2	0.07
	10	0.4	0.08

Each value is the mean of triple test.

within 1 hour after the addition of $CaCl_2$ (Fig. 2).

Effects of NaCl and KCl on the cellular and secretory isoperoxidases

The effects of NaCl on the specific activity of peroxidases and isoperoxidase patterns were examined in the cell and medium extracts (Fig. 3). A significant increase of specific activity (10 fold) did occur in the 0.5% NaCl treated medium, which might be due to the stimulation of secretion of secretory isoperoxidases such as C2 and C3 as judged by starch gel electrophoregram and specific activity. In the NaCl-treated cell, the decrease of total peroxidase activity was notable. Therefore the decrease of total peroxidase level in the NaCl-treated cell seems to be correlated with the stimulation of the excretion of cationic isoperoxidases into the medium. Not

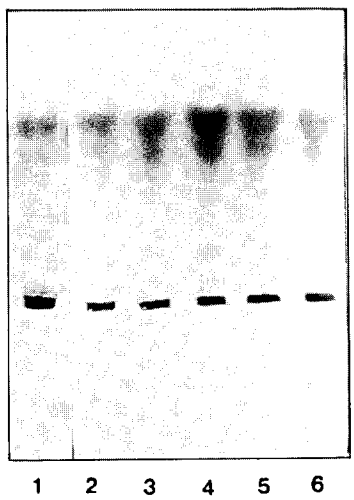

Fig. 2. Effects of 5 mM $CaCl_2$ on the secretion of secretory isoperoxidases at various culture times. Lane 1, 0 hour: lane 2, 0.5 hour: lane 3, 1 hour: lane 4, 6 hours: lane 5, 12 hours: lane 6, 24 hours.

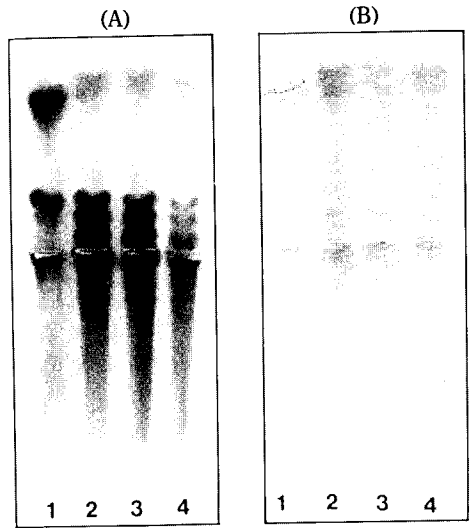

Fig. 3. Effects of NaCl on the patterns of cellular and secretory isoperoxidases from rice grown for 1 week. (A) Rice cell was used. lane 1, 0% NaCl: lane 2, 0.5% NaCl: lane 3, 1% NaCl: lane 4, 2% NaCl. (B) Culture medium was used. lane 1, 0% NaCl: lane 2, 0.5% NaCl: lane 3, 1% NaCl: lane 4, 2% NaCl.

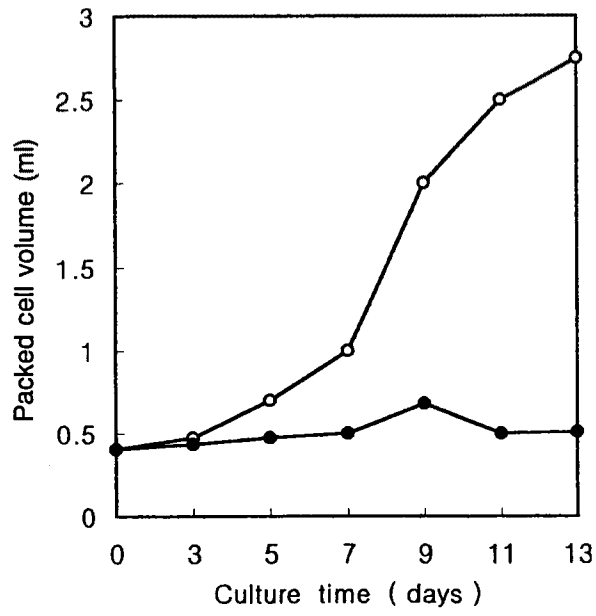

Fig. 4. The growth pattern of rice suspension culture. Suspension culture cells were incubated in the presence or absence of 0.5% NaCl (○—○: control, ●—●: NaCl).

only the increase of peroxidase secretion but also the reduction of cell volume was found with NaCl treatment (Fig. 4). This may be the result of dehydration of the cell under osmotic stress as shown in wheat seedling (Larssson *et al.*, 1989) and carrot callus (Moon *et al.*, 1997).

When various concentrations of KCl were treated, KCl did not change the secreted isoperoxidase levels greatly in terms of specific activities (Table 1) or the starch gel electrophoregram (data not shown).

Effects of cell wall degrading enzyme

Cell wall degrading enzyme such as cellulase (20 mg/ml) was treated in the presence or absence of mannitol as an osmotic buffer to study the effect of the cell wall degrading enzyme on peroxidase secretion. The level of secreted peroxidases in the medium increased more rapidly in the absence of the osmotic stabilizer of 0.4 M mannitol as shown in Fig. 5. The major secretory isoperoxidases by cellulase were composed of C3 and C2 (data not shown).

Effects of $NiCl_2$ on the cellular and secretory isoperoxidases

The effects of $NiCl_2$ on the specific activity of peroxidases and isoperoxidase patterns were examined in the cell and medium extracts (Fig. 6). Notably a significant increase of specific activity was observed in the $NiCl_2$-treated cell, and a maximum 6 fold increase of total cellular peroxidase did occur with 5 mM $NiCl_2$. However, $NiCl_2$ seemed to have little effect on the secretion of secretory peroxidases on the whole.

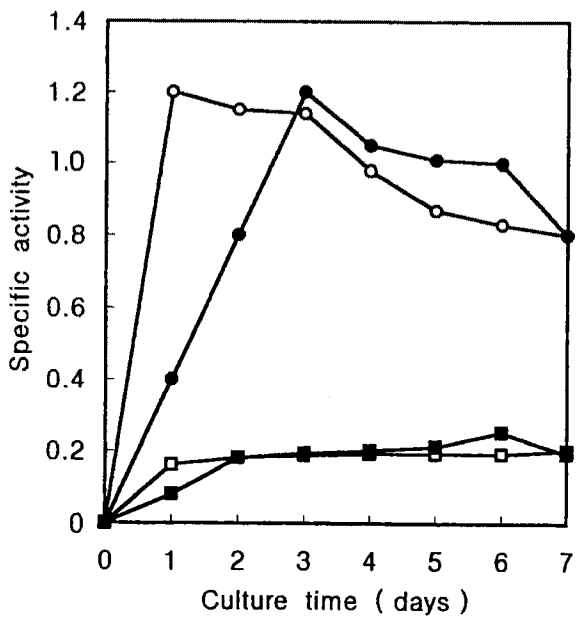

Fig. 5. Effects of cell wall degrading enzyme of cellulase on the secretion of secretory isoperoxidases in the presence or absence of 0.4 M mannitol. a, cellulase without mannitol: b, cellulase with mannitol: c, mannitol only: d, control. The specific activity of peroxidase was designated as $\triangle A_{470}$/min/mg protein (○—○: a, ●—●: b, □—□: c, ■—■: d).

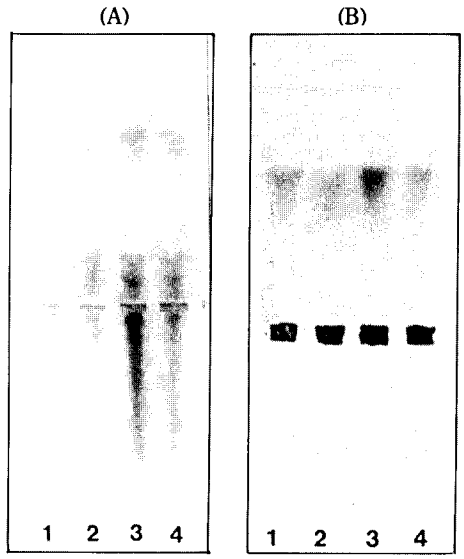

Fig. 6. Effects of $NiCl_2$ on the patterns of cellular and secretory isoperoxidases from rice grown for 1 week. (A) Rice cell was used. lane 1, 0 mM $NiCl_2$: lane 2, 1 mM $NiCl_2$: lane 3, 5 mM $NiCl_2$: lane 4, 10 mM $NiCl_2$. (B) Culture medium was used. lane 1, 0 mM $NiCl_2$: lane 2, 1 mM $NiCl_2$: lane 3, 5 mM $NiCl_2$: lane 4, 10 mM $NiCl_2$.

Effects of $ZnCl_2$ on the cellular and secretory isoperoxidases

The specific activity and isoperoxidase patterns of rice were examined in the cell and medium treated with various concentrations of $ZnCl_2$ (Fig. 7). About a 6 fold decrease of specific activity was found in the medium treated with 5 mM $ZnCl_2$ (Table 1). In particular, a significant decrease of C3 secretion occurred as judged by starch gel electrophoregram (Fig. 7). However, the specific activity of cellular peroxidase was not greatly affected by $ZnCl_2$. In flax seedlings, $ZnCl_2$ increased the activity of all the peroxidase isozymes. However, the activity of the main cationic isozyme decreased in the hypocotyls and cotyledons, showing a different response of the cationic and anionic peroxidases to $ZnCl_2$ under a stress condition (Fieldes and Gerhardt, 1981).

Protoplast preparation

In order to identify cell wall associated isoperoxidases, the isoperoxidase pattern was examined in the protoplast culture. As shown in Fig. 8 the protoplast culture consisted mainly of anionic isoperoxidases. Therefore all of the cationic isoperoxidases seemed to be localized in the cell wall of rice. The secretion of cationic isoperoxidases might be performed easily because of

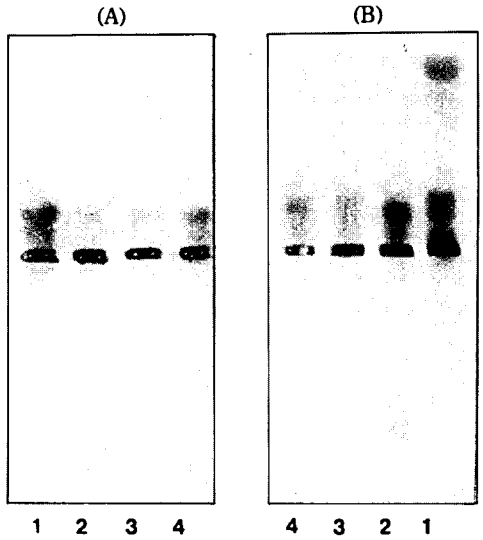

Fig. 7. Effects of $ZnCl_2$ on the patterns of cellular and secretory isoperoxidase from rice frown for 1 week. (A) Rice cell was used. lane 1, 0 mM $ZnCl_2$; lane 2, 1 mM $ZnCl_2$; lane 3, 5 mM $ZnCl_2$; lane 4, 10 mM $ZnCl_2$. (B) Culture medium was used. lane 1, 0 mM $ZnCl_2$; lane 2, 1 mM $ZnCl_2$; lane 3, 5 mM $ZnCl_2$; lane 4, 10 mM $ZnCl_2$.

their cellular location. Similar results of high level secretions of cationic peroxidase were reported in the peanut (*Arachis hypogaea* L.) (van Huystee and Maldonade, 1982). On the other hand, anionic isoperoxidase A3, proved to be localized in the cell wall, was found to be the major secretory enzyme in tobacco callus (Woo and Kim, 1987).

Fig. 8. Comparison of isoperoxidase patterns between cell and protoplast culture from rice. (A) Intact cell of rice was used. (B) Protoplast formed from rice was used.

Discussion

Cell suspension cultures provide a convenient system for investigating salt response at the cellular and secretory level. The salt effect can be readily applied uniformly to a relatively homogenous population of cells. Therefore changes of isoperoxidase patterns in suspension cultures are now being widely used as an indication of tissue responses during development (Thorpe and Gaspar, 1978), stress (Thomas and Delince, 1979) and exogenously applied hormones (Birecka and Galston, 1970).

Rice suspension culture produced cellular and secretory isoperoxidases, and the isoperoxidase levels varied with the treatment of various ions. Secretory isoperoxidase levels increased with Ca^{++} and Na^{+} treatment being the last cations the most effective to stimulate peroxidase secretion. A maximum 11 fold increase of cationic isoperoxidase (C2 and C3) secretion occurred in the presence of 5 mM $CaCl_2$ and the secretion reduced rapidly in the presence of 10 mM $CaCl_2$, suggesting the secretion of peroxidase into the medium was regulated by the concentration of extracellular calcium as reported by Sticher *et al.* (1981). NaCl supplementation resulted in a maximum 10 fold increase of secretory peroxidase levels, although with a reduction of cell growth. Kato *et al.* (1991) reported that NaCl induced peroxidase excretion into the medium rather than activate peroxidase synthesis in horseradish hairy roots. Interestingly, about a 6 fold increase of total cellular peroxidase level was found in the presence of 5 mM $NiCl_2$, suggesting the role in detoxification of increased peroxidase levels under stress conditions, as suggested by Gaspar *et al.* (1991). $NiCl_2$ has been reported to change the cell wall plasticity (Pandolfini and Gabbrielli, 1993) and cell permeability in *Triticum aestivum* L. seedlings. However, $NiCl_2$ showed little stimulating effect on the secretion of rice peroxidase. KCl did not change peroxidase secretion, but 5 mM $ZnCl_2$ reduced peroxidase secretion greatly. The secretory isoperoxidases stimulated by $CaCl_2$, NaCl and cellulase were commonly composed of cationic isoperoxidases C2 and C3, which were found to be localized in the cell wall of rice by protoplast study. Moreover, the increases of secretory isoperoxidase levels were correlated with the decrease of cellular isoperoxidase levels. In addition, the levels of secretory isoperoxidases were rapidly increased in the absence of the osmotic stabilizer of 0.4 M mannitol when cellulase was treated. Therefore, based on the above results, the stimulation of secretory peroxidase levels seems to be due to the stimulation of release into the culture medium rather than the increase of peroxidase synthesis and for other reasons as well.

References

Antje, B., Ahmed, A., Marina, A., Andreas, B. and Karl-Josef, D. (1996) in Plant Peroxidases: Biochemistry and Physiology (Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., eds.) pp. 374-379, University of Agriculture, Vienna and University of Geneva.

Bakardjieva, N. T., Christova, N. V. and Christov, K. (1996) in Plant Peroxidases: Biochemistry and Physiology (Obinger, C., Burner, U., Ebermann, R., Penel, C., Greppin, H., eds.) pp. 345-351, University of Agriculture, Vienna and University of Geneva.

Birecka, H. and Galston, A. W (1970) *J. Exp. Bot.* **21**, 735.

Constabel, F. (1975) in Plant Tissue Culture Methods (Gamborg, O. L., ed.) pp. 11-16, N. R. C., Ontario.

Fieldes, M. A. and Gerhardt, K. E. (1981) *J. Cell. Sci.* **48**, 345.

Fry, S. C (1986) *Annu. Rev. Plant Physiol.* **37**, 165.

Gaspar, T., Penel, C. Hagege, T. and Greppin, H. (1991) in Biochemical, Molecular and Physiological Aspects of Plant Peroxidases (Lobarzewski, J., Greppin, H., Penel, C., Gaspar, Th., eds.) pp. 249-280, University of M. Curie-Sklodowska, Lublin.

Hiroyuki, I., Nobutsugu, H., Akira, O. and Yuko, O. (1991) *Agric. Biol. Chem.* **55**, 2445.

Karege, F., Penel, C. and Greppin, H (1982) *Plant Physiol.* **64**, 353.

Kato, Y., Uozumi, N., Kimura, T., Honda, H. and Kobayashi, T. (1991) *Plant Tissue Culture Letters.* **8**, 158.

Kim, S. H. and Kim, S. S. (1996) *Phytochemistry* **42**, 287.

Kim, S. S., Wender, S. H. and Smith, E. C. (1980) *Phytochemistry* **19**, 165.

Koo, W. and Kim, S. S. (1987) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **20**, 58.

Larsson, M. Larsson, C. M., Whitford, P. N. and Clarkson, D. T. (1981) *J. Exp. Bot.* **40**, 1265.

Lee, M. Y. and Kim, S. S. (1990) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **23**, 440.

Lee, M. Y. and Kim, S. S. (1994) *Phytochemistry* **35**, 287.

Lee, M. Y., Choi, Y. and Kim, S. S. (1994) *Plant Physiol. Biochem.* **32**, 259.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **93**, 265.

Moon, S. Park, S. H. and Cho, B. H. (1997) *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **30**, 21.

Pandolfini, T. and Gabbrielli, R. (1993) in Plant Peroxidases: Biochemistry and Physiology (Welinder, K. G., Rasmussen, S. K., Penel, C., Greppin, H., eds.) pp. 417-421, University of Geneva.

Park, J. H. and Kim, S. S. (1996) *J. Biochem. Mol. Biol.* (formerly *Korean Biochem. J.*) **29**, 137.

Scott, A. Y., Ailan, G., James, A. G., Frank, F. W. and Jan, E. L. (1995) *Plant Physiol.* **107**, 1333.

Thomas, P. and Delincee, H. (1979) *Phytochemistry* **18**, 917.

Thorpe, T. A and Gaspar, T. (1978) *In Vitro* **14**, 522.

Van Huystee, R. B. and Cairns, W. L. (1982) *Phytochemistry* **21**, 1843.

Van Huystee, R. B. and Maldonado, B. (1982) *Physiol. Plant* **54**, 88.

Yoo, W. I. and Kim, S. S. (1988) *Korean Biochem. J.* (presently *J. Biochem. Mol. Biol.*) **21**, 207.