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Short Communication

Cell wall peroxidase against ferulic acid, lignin, and NaCl-reduced root growth of rice seedlings

Chuan Chi Lin, Ching Huei Kao*

Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China

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Summary

The changes in cell wall peroxidase activity against ferulic acid (FPOD) and lignin level in roots of NaCl-stressed rice seedlings and their correlation with root growth were investigated. Increasing concentrations of NaCl from 50 to 150 mmol L^{-1} progressively decreases root growth. The reduction of root growth by NaCl is closely correlated with the increase in FPOD activity extracted from the cell wall. In contrast, lignin level was reduced by NaCl. Since proline and ammonium accumulations are associated with root growth inhibition caused by NaCl, we determined the effect of proline or NH_4Cl on root growth and FPOD in roots. Exogenous application of NH_4Cl or proline markedly inhibited root growth and increased FPOD activity in rice seedlings in the absence of NaCl. An increase in FPOD activity in roots preceded inhibition of root growth caused either by NaCl, NH_4Cl, or proline. Our results suggest that cell-wall stiffening catalyzed by FPOD may participate in the regulation of root growth reduction of rice seedlings caused by NaCl.

Key words: ammonium – lignin – NaCl – *Oryza sativa* – peroxidase – proline

Abbreviations: DW dry weight. – FPOD peroxidase against ferulic acid. – FW fresh weight

Introduction

NaCl is known to inhibit plant growth. However, the mechanism underlying this inhibition is not yet clear (Greenway and Munns 1980, Munns and Termaat 1986, Rengel 1992). It has been shown that inhibition of leaf or root growth of cereals is not caused by decreased turgor (Cramer 1992, Munns 1993, Neumann et al. 1994). In contrast, NaCl-induced inhibition in growth has often been related to measured decreases in the plastic extensibility of the growing cell walls in root and leaf expansion zones (Cramer 1992, Neumann 1993, Prichard et al. 1993, Chazen and Neumann 1994, Neumann et al. 1994, Chazen et al. 1995). Neumann (1993, 1997) suggests that rapid, metabolically regulated changes in the physical properties of growing cells caused by osmotic or other effects, appear to be a factor regulating maize leaf growth response to root salinization. Neumann et al. (1994) also demonstrated that root growth inhibition caused by salinity was associated with cell wall stiffening. A key role of cell-wall peroxidases in the stiffening of the cell wall, and consequently, in the growth

* E-mail corresponding author: kaoch@ccms.ntu.edu.tw

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reduction of cell elongation, has been postulated (Fry 1986). Recently, we have reported that ionically bound peroxidase activity was associated with growth inhibition of rice seedling root caused by NaCl (Lin and Kao 1999). This ionically bound fraction of peroxidases, removable from homogenized tissues with high ionic strength buffers, has been equated with the cell wall fraction. However, Mader et al. (1986) argued that at least some ionically bound peroxidase activity might be an artifact of homogenization. Therefore, to test the hypothesis that an increase in cell wall peroxidase activity is associated with growth inhibition of seedling roots of rice, data from peroxidase activity associated with cell walls is required. Guaiacol is usually used as a substrate to assay peroxidase activity. However, guaiacol is not the natural substrate for the peroxidase in the cell-wall stiffening process. Ferulic acid has been identified as being ester linked to arabinoxylans in monocotyledonous plants (Kato and Nevins 1985, Hartley and Ford 1989). A key role in the cell-wall stiffening of dimerization of ferulic acid catalyzed by cell-wall POD has been reported (Sanchez et al. 1996). Thus, ferulic acid appears to be the suitable substrate to establish the relationship between cell-wall peroxidase activity and NaCl-inhibited root growth of rice seedlings. The present investigation was therefore designed to study the changes in peroxidase activity against ferulic acid (FPOD) associated with cell walls during growth reduction of rice seedling roots caused by NaCl.

It is known that peroxidases also influence plant growth through lignin synthesis (Siegel 1953). Lignification of the stems of the halophyte *Suaeda maritima* has been shown to be negatively correlated with salinity (Hagege et al. 1988). In *Phaseolus* sp., salinity caused an earlier and stronger lignification of root vascular tissues (Cachorro et al. 1992). However, it has been reported that salinity had no effect either on lignin content in tomato roots or reduced lignin level in the internodes of *Atriplex prostrata* grown under salt conditions (Peyrano et al. 1997, Wang et al. 1997). Therefore, effect of NaCl on lignin level in roots of rice seedlings was also studied in the present investigation.

Materials and Methods

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5 % sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in a Petri dish (20 cm) containing distilled water at 37 °C under dark condition. After a 1-day incubation, uniformly germinated seeds were selected and transferred to Petri dishes (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 mL of distilled water or test solutions. Each Petri dish contained 20 germinated seeds. Each treatment was replicated 4 times. The germinated seeds were allowed to grow at 27 °C in darkness. To avoid the loss by evaporation and taken up by the seeds, a further 3 mL of distilled water or test solution was added to each Petri dish on day 3 of the growth. Fresh weight (FW) and dry weight (DW) of roots were measured at the times indicated.

Cell walls were prepared by homogenizing roots in ice cold phosphate buffer (50 mmol L^{-1}, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1,000 g_n, and washed at least four times with 50 mmol L^{-1} phosphate buffer (Lee and Lin 1995). The pellet was collected and used as a cell wall fraction.

POD ionically bound to the cell wall was extracted with 1 mol L^{-1} NaCl. Cell walls prepared as described above were incubated in 1 mol L^{-1} NaCl for 2 h with shaking at 30 °C, and centrifuged at 1,000 g_n. The supernatant was used for FPOD assay. Ferulic acid POD (FPOD) was assayed according to Sanchez et al. (1996). The oxidation of ferulic acid was measured spectrophotometrically following the absorbance decrease at 310 nm in a reaction mixture containing 1.35 mL Na-phosphate buffer (0.2 mol L^{-1}, pH 5.8), 0.5 mL ferulic acid (240 µmol L^{-1}), 0.5 mL H_2O_2 (3 mmol L^{-1}) and 0.15 mL enzyme extract. One unit of FPOD was defined as a decrease of 1 A_{310} per min.

The lignin level in roots was measured by the Sasaki et al. (1996) method, a method originally described by Morrison (1972). Roots were homogenized with a pestle in a mortar in 95 % ethanol. The homogenate was centrifuged at 1,000 g_n for 5 min. The pellet was washed three times with 95 % ethanol and twice with a mixture of ethanol and hexane (1 : 2, v/v). The material was allowed to air dry and its lignin level measured. The dried sample was washed one time with 2 mL acetyl bromide in acetic acid (1 : 3, v/v). Then 1 mL acetyl bromide in acetic acid (1 : 3, v/v) was added to the pellet and incubated at 70 °C for 30 min. After cooling of the mixture to room temperature, 0.9 mL of 2 mol L^{-1} NaOH and 0.1 mL 7.5 mol L^{-1} hydroxylamine hydrochloride were added, and the volume was made up to 10 mL with acetic acid. After centrifugation at 1,000 g_n for 5 min, the absorbance of the supernatant was measured at 280 nm (A_{280}).

For all measurements, each treatment was repeated four times. All experiments described here were repeated at least three times. Similar results and identical trends were obtained each time. The data reported here is from a single experiment.

Results and Discussion

Root growth was followed by measuring FW and DW of roots. Figure 1 shows the effect of NaCl on root growth of rice seedlings. Increasing concentrations of NaCl from 50 to 150 mmol L^{-1} progressively decreased root growth. The reduction of root growth with increasing NaCl concentrations is correlated with an increase in FPOD activity extracted from cell walls (Fig. 1). Sanchez et al. (1996) demonstrated a negative relationship of FPOD and growth capacity in pine hypocotyl, suggesting a role for peroxidase in the cell-wall stiffening. Furthermore, the ability of peroxidases to catalyze wall cross-linking through ferulic acid esterified to polysaccharides has already been shown (Whitemore 1976). It seems that cell-wall stiffening catalyzed by FPOD may participate in the regulation of root growth reduction of rice seedlings under NaCl conditions.

It is known that ammonium strongly inhibits the growth of many plants (Haynes and Goh 1978). Exogenous application of NH_4Cl was also found to reduce root growth of rice seedlings (Lin and Kao 1996 a). It has also been shown that NaCl was effective in stimulating the accumulation of ammonium in roots of rice seedlings, and that accumulation of ammonium in roots preceded inhibition of root growth and increase in ion-

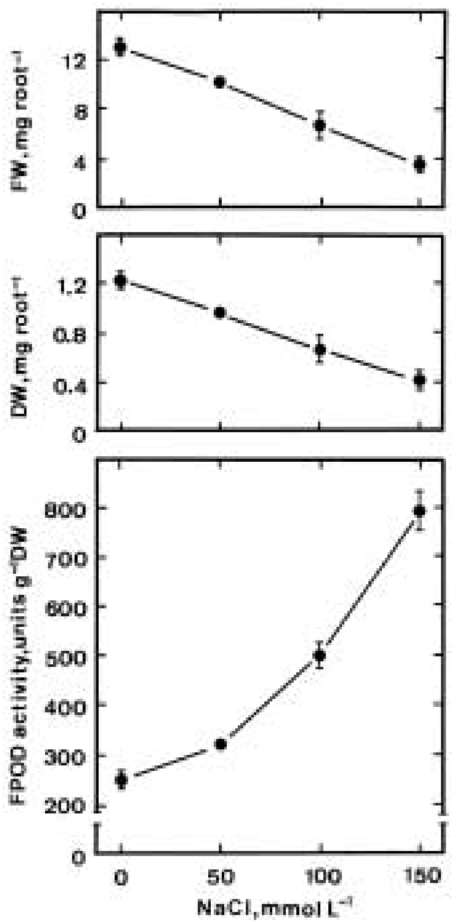

Figure 1. Effect of NaCl on root growth and FPOD activities in roots of rice seedlings. FW, DW, and FPOD activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

ically bound peroxidase activity caused by NaCl (Lin and Kao 1996 a, Lin and Kao 1999). If the increase in FPOD activity is important in regulating growth reduction of rice seedling roots caused by NaCl, then exogenous application of NH_4Cl would be expected to increase FPOD activity in roots of rice seedlings. Figure 2 shows that growth of and FPOD activity in roots of seedlings are decreased and increased, respectively, with the increasing of NH_4Cl concentrations.

We have previously shown that proline accumulation is correlated with root growth inhibition of rice seedlings induced by NaCl (Lin and Kao 1996 b), and that exogenous application of proline in the absence of NaCl resulted in a reduction of root growth (Chen and Kao 1995, Lin and Kao 1996 b). Exogenous application was also found to increase ionically bound peroxidase activity in roots of rice seedlings (Chen and Kao 1995, Lin and Kao 1996 b). In the present investigation, we demonstrated that the reduction of root growth with increasing proline concentrations is correlated with an increase in FPOD activity extracted from cell walls (Fig. 3).

The observations that rice seedlings treated with NH_4Cl or proline, which resulted in an increase in FPOD activity in roots, reduced root growth in the same way that NaCl did, further supports our suggestion that cell-wall stiffening catalyzed by FPOD may participate in the regulation of root growth reduction of rice seedlings.

To test the causal relationship between FPOD activity and root growth reduction caused by NaCl, NH_4Cl or proline, 2-day-old seedlings were transferred to distilled water, NaCl, NH_4Cl, and proline, respectively, for 4, 8, 12, and 16 h. Changes in FPOD and root growth were then monitored. As indicated in Table 1, an increase in FPOD activity was observed at 8 h after treatment, whereas root growth reduction was observed at 12 h after treatment, indicating that an increase in FPOD activity in roots preceded inhibition of root growth caused by NaCl, NH_4Cl, or proline. In previous studies, we observed that proline or ammonium accumulation occurred at 4 h after NaCl treatment (Lin and Kao 1996 a, b). Clearly, the links between NaCl treatment, proline, ammonium, FPOD, and root growth are well established.

Peroxidases were found to be related to lignin synthesis (van Huystee 1987, Sato et al. 1993, Polle et al. 1994). Lignification is part of cell wall differentiation and irreversibly inhibits cell elongation (Sauter and Kende 1992). It has been

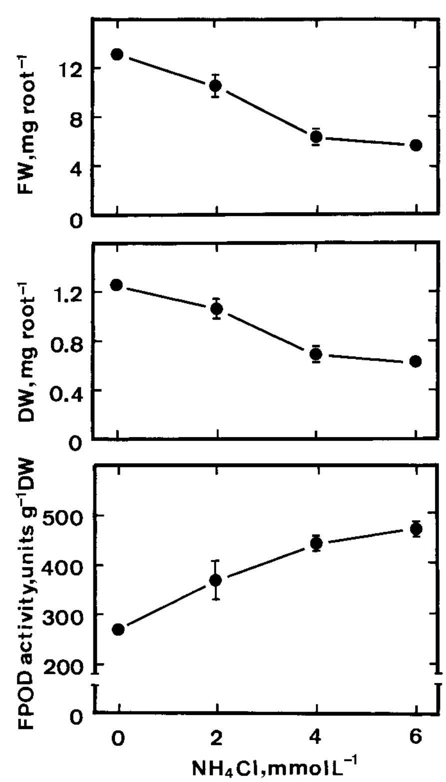

Figure 2. Effect of NH_4Cl on root growth and FPOD activities in roots of rice seedlings. FW, DW, and FPOD activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

Table 1. Changes in root growth and FPOD activities in roots of NaCl-, proline-, or NH_4Cl-treated rice seedlings. Rice seeds were germinated in distilled water for 2 days, and then were transferred to distilled water, NaCl (150 mmol/L) and NH_4Cl (4 mmol/L), respectively.

		Time, h				
		0	4	8	12	16
FW (mg root^{-1})	H_2O	6.45 ± 0.21	6.86 ± 0.17	7.32 ± 0.19	8.48 ± 0.17	9.82 ± 0.31
	NaCl		6.74 ± 0.15	6.92 ± 0.32	7.21 ± 0.15	7.94 ± 0.24
DW (mg root^{-1})	H_2O	0.61 ± 0.02	0.65 ± 0.02	0.70 ± 0.02	0.81 ± 0.02	0.93 ± 0.03
	NaCl		0.64 ± 0.01	0.66 ± 0.03	0.68 ± 0.02	0.75 ± 0.02
FPOD (units g^{-1} DW)	H_2O	212 ± 19	247 ± 26	238 ± 15	266 ± 24	268 ± 15
	NaCl		237 ± 28	305 ± 28	374 ± 19	444 ± 28
FW (mg root^{-1})	H_2O	7.13 ± 0.29	7.75 ± 0.18	8.65 ± 0.13	9.25 ± 0.19	10.25 ± 0.06
	Proline		8.06 ± 0.26	8.78 ± 0.15	8.72 ± 0.14	9.12 ± 0.21
DW (mg root^{-1})	H_2O	0.68 ± 0.03	0.74 ± 0.02	0.82 ± 0.01	0.88 ± 0.02	0.97 ± 0.01
	Proline		0.77 ± 0.03	0.83 ± 0.02	0.83 ± 0.01	0.87 ± 0.02
FPOD (units g^{-1} DW)	H_2O	249 ± 13	275 ± 13	277 ± 16	255 ± 7	268 ± 119
	Proline		284 ± 25	317 ± 8	324 ± 10	335 ± 9
FW (mg root^{-1})	H_2O	7.48 ± 0.37	7.74 ± 0.31	8.49 ± 0.25	9.18 ± 0.12	10.12 ± 0.30
	NH_4Cl		7.93 ± 0.19	8.43 ± 0.03	8.72 ± 0.08	8.84 ± 0.18
DW (mg root^{-1})	H_2O	0.71 ± 0.04	0.74 ± 0.03	0.81 ± 0.03	0.87 ± 0.01	0.96 ± 0.03
	NH_4Cl		0.75 ± 0.02	0.80 ± 0.01	0.83 ± 0.01	0.84 ± 0.02
FPOD (units g^{-1} DW)	H_2O	246 ± 20	267 ± 6	281 ± 15	278 ± 14	294 ± 25
	NH_4Cl		286 ± 21	335 ± 6	358 ± 7	412 ± 47

shown that salinity caused lignification of plants (Hagege et al. 1988, Cachorro et al. 1992). Thus, it is of great intreast to know whether NaCl has an effect on lignin level in roots of rice seedlings. Contrary to our expectation, increasing concentration of NaCl progressively decreased lignin level in roots of rice seedlings (Fig. 4). It has been shown that syringaldazine, a hydrogen donor, has a particularly high affinity for peroxidases associated with lignification (Goldberg et al. 1983). Recently, we used syringaldazine as the substrate to establish whether ionically bound peroxidase activity is related to the reduction of root growth caused by NaCl. It was shown that the increase in ionically bound peroxidase

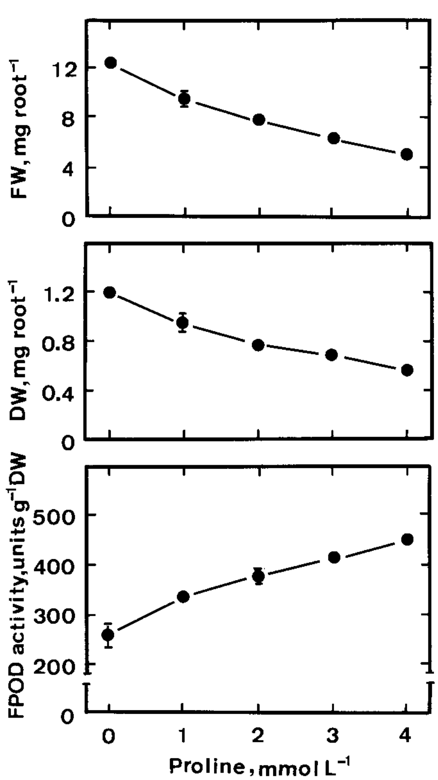

Figure 3. Effect of proline on root growth and FPOD activities in roots of rice seedlings. FW, DW, and FPOD activities were determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

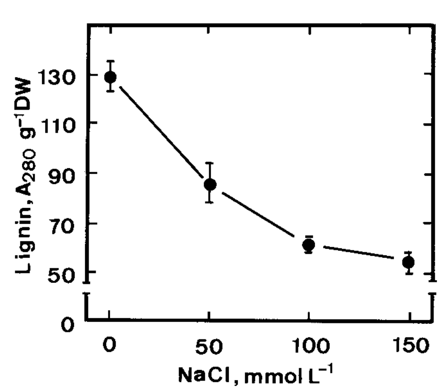

Figure 4. Effect of NaCl on the levels of lignin in roots of rice seedlings. Lignin was determined after 5 days of treatment. Vertical bars represent standard errors (n=4).

against syringaldazine was only observed at a concentration of 150 mmol L^{-1} (Lin and Kao 1999). It is clear that lignification plays no role in regulating root growth reduction of rice seedlings caused by NaCl.

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