Peroxidase activity in relation to ethylene-induced rice (*Oryza sativa* L.) coleoptile elongation

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(Received 4 April 1, 1996; Accepted June 19, 1996)

Abstract. Changes in the activities of soluble and cell wall-bound peroxidase (EC 1.11.1.7, donor: hydrogen-peroxide oxidoreductase) in relation to the regulation of coleoptile elongation were determined using rice (*Oryza sativa* L. cv. Taichung Native 1) seedlings grown in air or in air containing ethylene. Guaiacol and syringaldazine were used separately as the H donors for peroxidase assays. In air, both guaiacol and syringaldazine cell wall-bound peroxidase activity declined during coleoptile elongation but increased again to the initial level when elongation ceased. Soluble syringaldazine peroxidase activity in air-grown coleoptiles also showed an initial decline followed by a subsequent rise, but soluble guaiacol peroxidase activity remained essentially constant. Ethylene (10 μL/L) enhanced coleoptile elongation but decreased both guaiacol and syringaldazine peroxidase activities, especially the wall-bound forms. 2,5-Norbornadiene (NBD) at 3000 μL/L, an inhibitor of ethylene action, blocked both the ethylene-mediated coleoptile elongation and the decline of peroxidase activity. Ethylene at 50 μL/L reversed the effect of NBD. These results suggest that the activity of peroxidase, especially the cell wall-bound form, is inversely related to the elongation of rice coleoptiles.

Keywords: *Oryza sativa* L., Coleoptile elongation; Ethylene; Peroxidase; 2,5-Norbornadiene.

Abbreviation: NBD, 2,5-Norbornadiene.

Introduction

Rice is one of the plants that can germinate and grow in anoxia or submergence conditions (Alpi and Beevers, 1983; Ishizawa and Esashi, 1984; Raskin and Kende, 1983; Taylor, 1942; Turner et al., 1981). However, the growth of rice seedlings under oxygen-restricted environments is limited to coleoptile elongation (Atwell et al., 1982; Turner et al., 1981). This rapid coleoptile elongation is considered to be a survival mechanism for rice seedlings to allow them to grow out of flood water and obtain oxygen.

Ethylene enhances rice coleoptile elongation (Ishizawa and Esashi, 1984; Ku et al., 1970; Lee and Chu, 1992; Raskin and Kende, 1983; Salter and Kende, 1985) with several physiological factors involved. One is osmoregulation by a stimulated sucrose transport from endosperm to coleoptiles (Ishizawa and Esashi, 1988). Another factor is enhanced cell wall loosening by alteration of wall components. For example, the decline of the level of (1,3), (1,4)-β-D-glucans has been shown to be closely associated with the ethylene-induced cell wall loosening in rice coleoptiles (Hoson et al., 1990).

Peroxidase (EC 1.11.1.7, donor: hydrogen-peroxide oxidoreductase), which is a glycoprotein, has been recognized as mediating the binding of ferulic acid to cell walls by the formation of diferuloyl cross-links to matrix po-

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lysaccharides, and its activity subsequently reduces wall extensibility (Fry, 1979). Peroxidases are also responsible for the assembly of lignins and proteins in cell wall (Fry, 1986; Gaspar et al., 1985; van Huystee, 1987; Iyama et al., 1994; Taiz, 1984; Whitmore, 1978). Higher peroxidase activities are closely associated with reduced growth of plants including peanut hypocotyls (Zheng and van Huystee, 1992), mung bean hypocotyls (Goldberg et al., 1987) and dwarf sorghum (Scherzt et al., 1971). Results from two genotypes of tall fescue (*Festuca arundinacea* Schreb.) with different lengths of leaf elongation zones show that the cessation of elongation is accompanied by an increase of cell wall-bound peroxidase activity (MacAdam et al., 1992).

The activity of peroxidase, especially the cell wall-bound form, is related to ethylene-regulated plant growth. Petioles of *Ranunculus sceleratus* L. elongate faster and have lower peroxidase activities in response to ethylene (Horton, 1993). In contrast, the ethylene-induced inhibition of pea epicotyl elongation is accompanied by a rise of peroxidase activity (Ridge and Osborne, 1970). In the case of the inversion-induced growth inhibition of *Pharbitis* shoots, ethylene levels increase during inversion, and the subsequent increase of wall-bound peroxidase activity is correlated with wall stiffening (Prasad and Cline, 1987). There is also a negative correlation between coleoptile elongation and cell wall-bound peroxidase levels in anoxia-treated rice seedlings (Lee and Lin, 1995).
However the levels of peroxidase activity during ethylene-enhanced elongation of rice coleoptiles has not been previously reported.

**Materials and Methods**

### Plant Cultivation

Rice (*Oryza sativa* L.) seeds of Indica type (cv. Taiichung Native 1) were sterilized with 5% sodium hypochlorite for 10 min. After being washed with distilled H₂O several times, seeds were incubated in Petri dishes at 30°C in darkness as reported previously (Lee and Chu, 1992). For aerobic treatment, ten etiolated seedlings with 2 mm shoots were placed in a Petri dish (8 cm width, 1 cm depth) containing 8 mL distilled H₂O such that the seedlings were not submerged. For ethylene treatment, ten seedlings were placed in a 25 mL vial, and ethylene at 10 μL/L was passed through at a flow rate of 10 mL/min.

Since NBD is a volatile liquid that evaporates quickly in air, we used a closed system for the determinations of effects of norbornadiene (NBD, final concentration 3000 μL/L) on ethylene (final concentration 50 μL/L)-regulated elongation and wall-bound peroxidase activity of rice coleoptiles. Ten etiolated seedlings with 2 mm shoots were placed in a 25 mL Erlenmeyer flask containing distilled H₂O so that the seedlings were not submerged. A plastic pipette (200 μL) was sealed at its tip and set in the flask with open end up. The flask was finally closed with a serum cap. The experiments were started by injecting ethylene gas via the cap into the flask, or injecting liquid NBD via the cap into the sealed tip, or injecting both. Since rice coleoptiles elongated rapidly in the closed system, sampling was performed one day after treatment.

### Determination of Peroxidase Activity

Air-grown or ethylene-treated coleoptiles (about 0.5 g fresh weight) were fixed in liquid nitrogen and then stored in a -70°C freezer for enzyme extraction. Plant samples were homogenized in liquid nitrogen and extracted with 10 mM phosphate buffer (pH 6.4). The ratio of tissue fresh weight (g) and extraction buffer volume (mL) was 1:3. After centrifugation under 1000 g at 4°C for 10 min, the supernatant was used for soluble peroxidase activity determination. The pellet was resuspended in the same volume of extraction buffer. The resuspended pellet was centrifuged at 1000 g at 4°C for 10 min. Washing was repeated at least four times until no peroxidase activity was found in the supernatant. The pellet was then extracted with 3 volumes of 1 M NaCl in buffer for 2 h at 30°C with continuous shaking followed by centrifugation at 1000 g at 4°C for 10 min. After that, the supernatant was used to assay cell wall-bound peroxidase activity. These extracted samples were freshly assayed within 1 h to minimize the loss of enzyme activity. There were three replicated samples in each treatment.

Guaiacol peroxidase activity was analyzed in 10 mM phosphate buffer (pH 6.4) containing 8 mM guaiacol and 2.75 mM H₂O₂ by the methods of Chance and Maehly (1955). The increase of absorbance at 470 nm was recorded within 30 sec after adding 2.75 mM H₂O₂. The determination of syringaldazine peroxidase activity was modified from the method of Pandofigini et al. (1992). Samples were incubated in 100 mM sodium-phosphate buffer (pH 6.0) containing 1.5 mM H₂O₂ and 0.04 mM syringaldazine (dissolved in methanol-dioxane 1:1 v/v). The increase of absorbance at 530 nm was recorded within 1 min. Specific activity of peroxidase was expressed on the basis of mg soluble protein content. Peroxidase activity represents the average of three sample replicates.

### Protein Content Determination

Buffer or NaCl-soluble protein content were determined according to Bradford (1976) with bovine serum albumin as a standard.

### Chemicals

Chemicals were purchased from Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). NBD was obtained from Aldrich (Milwaukee, WI, USA).

### Results

#### Effects of Ethylene on Coleoptile Length

Coleoptiles elongated rapidly during the first 2 days in air then remained almost unchanged (Figure 1). Ethylene at 10 μL/L enhanced the coleoptile elongation up to 28 mm by 4 days after treatment. When coleoptiles were transferred to air after 2 days of ethylene treatment, the ethylene-enhanced elongation ceased immediately (data not shown). NBD at 3000 μL/L slightly blocked coleoptile elongation, but this inhibitory effect could be partially reversed by increasing the ethylene level to 50 μL/L (Figure 5).

![Figure 1. Length of air-grown or ethylene-treated rice (*Oryza sativa* L. cv. Taiichung Native 1) coleoptiles. Ethylene concentration is 10 μL/L. Bars indicate the standard errors (mean ± SE, n=30; 10 replicates in each of 3 treatments).](image-url)
Guaiacol Peroxidase Activity

Soluble peroxidase activity in air-grown coleoptiles slightly decreased during the growth period, while cell wall-bound peroxidase activity decreased during the first 2 days but rose to the initial level after 4 days (Figure 2). The activity of cell wall-bound peroxidase in ethylene (10 μL/L)-treated coleoptiles showed a similar pattern, but at much lower overall levels than those found in the air controls (Figure 2). The transfer of ethylene-treated seedlings to air, 2 days after ethylene treatment resulted in a rapid increase of cell wall-bound peroxidase activity (data not shown).

Figure 2. Guaiacol peroxidase activity in air-grown or ethylene-treated rice (Oryza sativa L. cv. Taichung Native 1) coleoptiles. Ethylene concentration is 10 μL/L. Bars indicate the standard errors (mean ± SE, n=30; 10 replicates in each of 3 treatments).

Syringaldazine Peroxidase Activity

Both soluble and cell wall-bound peroxidase activity decreased in the beginning of growth period and then increased gradually to levels equal to or higher than the initial ones at day 4 as shown in control samples of Figure 3. The initial decline of peroxidase activity was more marked for the wall-bound enzyme than the soluble enzyme. Ethylene (10 μL/L) caused a marked initial decrease of peroxidase activity (Figure 3). When transferred to air 2 days after ethylene treatment, soluble and cell wall-bound peroxidase activity both increased rapidly to the levels found in the air controls within 12 h (data not shown).

Figure 3. Syringaldazine peroxidase activity in air-grown or ethylene-treated rice (Oryza sativa L. cv. Taichung Native 1) coleoptiles. Ethylene concentration is 10 μL/L. Bars indicate the standard errors (mean ± SE, n=30; 10 replicates in each of 3 treatments).

Coleoptile Length and Peroxidase Activity in Response to Different Concentrations of Ethylene

Changes of coleoptile length and cell wall-bound peroxidase activity in response to various ethylene concentrations are shown in Figure 4. Ethylene, at 0.2 μL/L or higher, enhanced coleoptile elongation, with the maximal effect being observed at 10 μL/L. The activity of both guaiacol and syringaldazine wall-bound peroxidases decreased in response to ethylene levels higher than 0.2 μL/L, with the lowest activity of both observed when seedlings were treated with 10 μL/L. Ethylene also had some effect on the syringaldazine peroxidase activity in the soluble fraction (Figure 3).

Effects of Norbornadiene on Ethylene-Mediated Decrease of Peroxidase Activity

Rice seedlings were treated with ethylene in the presence of NBD in order to further understand the effect of ethylene on the control of peroxidase activity. Figure 5 shows that the promotion of coleoptile elongation by ethylene was completely inhibited by NBD at 3000 μL/L. The activity of ethylene in causing a decrease in both guaiacol and syringaldazine wall-bound peroxidase activities was blocked by 3000 μL/L NBD. However, the effect of NBD at this level could be partially reversed by raising the ethylene level to 50 μL/L (Figure 5).
Discussion

Our results show that wall-bound peroxidase activity is inversely related to the elongation of rice coleoptiles. In air, both guaiacol and syringaldazine wall-bound peroxidase activity decreased as coleoptiles elongated rapidly early in the growth period followed by an increase when elongation ceased at later stage. In ethylene-treated seedlings the increase of coleoptile length correlates with the decrease of both guaiacol and syringaldazine wall-bound peroxidases activity. The inhibition of both ethylene-mediated coleoptile elongation and the decrease of wall-bound peroxidase activity by NBD, confirms the effects of ethylene in both events. The negative correlation between the wall-bound peroxidase activity and coleoptile length were observed when ethylene concentrations were varied. A similar negative role of the cell wall-bound peroxidase in ethylene-regulated plant growth has been observed in anoxia-grown rice coleoptiles (Lee and Lin, 1995), pea epicotyl cells (Ridge and Osborne, 1970) and the inversion-induced growth inhibition of Pharbitis shoots (Prasad and Cline, 1987). In contrast, ethylene treatment could induce an increase of peroxidase activity in several plants such as sweet potato (Bueschner et al., 1975; Gahagan et al., 1968), cucumber (Abeles et al., 1988; Retig and Rudich, 1972; Stermer and Hammerschmidt, 1985), and tobacco (van Loon and Antoniw, 1982). However, these observations were associated with defense systems or with senescence-related chlorophyll degradation.

The reported decrease of endogenous ABA levels in ethylene-treated coleoptile indicates a negative role of internal ABA on ethylene-enhanced coleoptile elongation (Lee et al., 1994). The exogenous application of ABA blocked coleoptile elongation in ethylene-treated rice seedlings (Lee and Lin, 1995). Further, the ethylene-mediated decrease of wall-bound peroxidase activity was blocked by ABA. It has been reported that ABA induces peroxidase gene expression in tomato petioles (Sherf et al., 1993), potato and tomato callus culture (Roberts and Kolattukudy, 1989), and duckweed Spirodela polyrrhiza (Chaloupkova and Smart, 1994). We conclude that ethylene and ABA interact in the regulation of peroxidase activity in rice coleoptiles.

It is known that the extracellular peroxidases, including free and cell wall-bound forms, play important roles in cell wall lignification and in the cross linking between extensin molecules, and between feruloylated polysaccharides (Castillo, 1986; Fry, 1986; van Huystee, 1987; Taiz, 1984). The conversion of ferulic acid to diferulic acid on polysaccharides by peroxidase is closely associated with cell wall rigidity (Fry, 1986). Ferulic acid and diferulic...
acid, which occur in rice endosperms (Shibuya, 1984) and coleoptiles (Tan et al., 1991), associate with cell wall polysaccharides. The accumulation of ferulic and diferulic acid in rice coleoptiles decreases in submergence (Kutscher et al., 1993; Tan et al., 1991). However, an equal ratio of ferulic and diferulic acid in both air-grown and submerged coleoptiles suggest that the increase of feruloylation of hemicelluloses instead of the peroxidation of ferulic acid to diferulic acid is a cause of cell wall stiffening in air-grown coleoptiles (Kutscher et al., 1993; Tan et al., 1991). A similar result was also reported for oat coleoptiles (Kamisaka et al., 1990) and the light-induced growth inhibition of rice coleoptiles (Tan et al., 1992). Therefore, the ethylene-mediated decrease of wall-bound peroxidase activities may be involved in the decrease of peroxidation of ferulic acid to diferulic acid in rice coleoptile wall.

The ethylene-mediated decrease of wall-bound peroxidase activities may be associated with the lowering of isodityrosine formation or lignification of cell wall of rice coleoptiles. Cell wall-bound peroxidase is known to catalyze the insolubilization of hydroxyproline-rich glycoprotein in the wall via the cross-linkage of isodityrosine (Cooper and Varner, 1983; Fry, 1986; Iyama et al., 1994; Waffenschmidt et al., 1993) and the level of insoluble hydroxyproline-rich glycoprotein is suggested to be inversely related to cell wall extensibility (Cleland and Karlsnes, 1967; Sadava and Chrispeels, 1973). In the case of *Pharbitis nil*, the ethylene-mediated cross-linkage of hydroxyproline-rich glycoprotein and lignin is considered to have an important role in the elongation cessation of inverted shoots (Prasad and Cline, 1987). It is also known that ethylene mediates peroxidase-induced lignification (Whitmore, 1978), which makes cell walls more rigid (Wardrop, 1964) and limits cell growth (Marigo and Boudet, 1980). Syringaldazine peroxidase, a lignifying peroxidase (Goldberg et al., 1983), also shows a negative correlation to coleoptile elongation in ethylene-treated rice seedlings, indicating that the peroxidase-mediated lignification may also be involved in ethylene-regulated coleoptile elongation. At present, we are determining the lignin and hydroxyproline-rich glycoprotein contents in order to know whether the cross-linkage between extensin and lignin is involved in the peroxidase-related control of rice coleoptile elongation.

Acknowledgments. The financial support from the National Science Council, Executive Yuen and the Institute of Botany, Academia Sinica, Republic of China is appreciated. Tse-Min Lee thanks these two institutes for providing financial support for postdoctoral research.

Literature Cited


