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Effects of irradiance and copper on the activity of ascorbate oxidase in detached rice leaves

L.-M. CHEN and C.H. KAO

Department of Agronomy, National Taiwan University, Taipei 106, Taiwan, Republic of China

Abstract

The effects of copper on the activity of ascorbic acid oxidase (AAO) in detached rice leaves under both light and dark conditions and in etiolated rice seedlings were investigated. $CuSO_4$ increased AAO activity in detached rice leaves in both light and darkness, however, the induction in darkness was higher than in the light. In the absence of $CuSO_4$, irradiance (40 μmol m^{-2} s^{-1}) resulted in a higher activity of AAO in detached rice leaves than dark treatment. Both $CuSO_4$ and $CuCl_2$ increased AAO activity in detached rice leaves, indicating that AAO is activated by Cu. Sulfate salts of Mg, Mn, Zn and Fe were ineffective in activating AAO in detached leaves. $CuSO_4$ was also observed to increase AAO activity in the roots but not in shoots of etiolated rice seedlings.

Additional key words: Cu metalloenzyme, *Oryza sativa*.

Introduction

Ascorbic acid oxidase (AAO, EC 1.10.3.3) is a Cu metalloenzyme. It catalyzes the oxidation of ascorbic acid to dehydroascorbic acid. The definitive roles of AAO is not clear, although it has been suggested that the enzyme may participate in a redox system involving ascorbic acid (Weis 1975) and in growth promotion (Lin and Varner 1991, Takahama and Oniki 1994).

It has been shown that AAO is induced by adding copper into cell cultures from pumpkin (Esaka *et al.* 1988a, b, 1989, 1990, 1992), cucumber (Cho *et al.* 1989, Sekiya *et al.* 1990), or zucchini (Pitari *et al.* 1993). It is not known whether or not Cu activates AAO in other tissues, such as leaves and roots. In this study, detached rice leaves and etiolated rice seedlings were used to examine the effect of Cu on AAO activity.

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Abbreviations: AAO - ascorbic acid oxidase; CHI - cycloheximide.

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Fax: (+886) 2 2362 0879, e-mail: kaoch@cc.ntu.edu.tw

Materials and methods

Fifty rice (*Oryza sativa* L. cv. Taichung Native 1) seedlings were planted on a stainless net floating on half-strength Johnson's modified nutrient solution (pH 4.2) in a 500 cm^3 beaker (Kao 1980). The nutrient solution was replaced every three days. Rice plants were grown for 12 d in a greenhouse under natural light and controlled temperature 30 °C during the day and at 25 °C at night. The apical 3 cm of the third leaf was used for the experiment. A group of 10 segments was floated in a Petri dish containing 10 cm^3 distilled water or $CuSO_4$ solutions. Incubation was carried out at 27 °C in darkness or under irradiance 40 μmol m^{-2} s^{-1}. For the etiolated seedlings, seeds were sterilized with 2.5 % sodium hypochlorite for 15 min and washed extensively with distilled water. These seeds were then germinated in Petri dishes containing distilled water at 37 °C under darkness. After 1 d, uniformly germinated seeds were selected and transferred to a Petri dish containing 2 sheets of *Whatman No. 1* filter paper moistened with 10 cm^3 distilled water or various concentrations of $CuSO_4$ (pH 5.5). Each treatment was replicated 4 times. The germinated seeds were allowed to grow at 27 °C in darkness and additional 3 cm^3 distilled water or treatment solutions was added to each Petri dish on day 3 of the growth. Roots or shoots of seedlings grown in darkness were harvested after 5 d to determine fresh mass (f. m.) and AAO activity.

Plant tissues (40 mg) were homogenized in 4 cm^3 of 50 mM sodium phosphate buffer (pH 6.8) in a chilled mortar and pestle at 4 °C. The homogenates were centrifuged at 17 600 *g* for 20 min, and the supernatants were used to assay AAO activity as described by Esaka *et al.* (1988a). AAO activity was assayed by a spectrophotometer (*U-2000*, *Hitachi*, Tokyo, Japan) by following the decrease in absorbance at wavelength 265 nm (A_{265}) of the reaction mixture containing 1 cm^3 of 100 μM ascorbic acid, 1.8 cm^3 of 50 mM sodium phosphate buffer (pH 6.8) and 0.2 cm^3 of enzyme extract. One unit (U) of enzyme activity was defined as a decrease of 0.01 A_{265} per minute.

All experiments described here were repeated four times. Similar results and identical trends were obtained. The data reported here are from a single experiment.

Results

Under both light and dark, increasing concentrations of $CuSO_4$ from 0.01 to 10 mM progressively increased AAO activity in detached rice leaves (Table 1). After 24-h incubation in darkness, treatment with 10 mM $CuSO_4$ increased AAO activity 3- to 4-fold. However, only about 50 % increase in AAO activity in detached rice leaves treated with 10 mM $CuSO_4$ for 24 h under light was found. These results indicated that $CuSO_4$ was more effective in the dark than under light. In the absence of $CuSO_4$, AAO activity did not change during the 24 h in the dark but increased under irradiance of 40 μmol m^{-2} s^{-1} which resulted in a higher activity of AAO under this irradiance than in the dark (Table 2). Increases in AAO activities, as a consequence of $CuSO_4$ treatment, were detected 4 h after that start of incubation in the dark

(Table 2). When detached rice leaves were exposed to $CuSO_4$, both in the dark and irradiated, AAO activities were found to increase up to 24 h (Table 2).

Table 1. Effects of various $CuSO_4$ concentrations on the AAO activity in detached rice leaves under dark or light. Detached rice leaves were floated on solutions of $CuSO_4$ at different concentrations for 24 h. Means ± SE ($n = 4$).

$CuSO_4$ [mM]	AAO [U g^{-1}(f.m.)] dark	light
0	4.23 ± 0.18	12.17 ± 0.09
0.01	6.58 ± 0.87	14.28 ± 0.12
0.10	9.44 ± 0.40	15.00 ± 1.28
1.00	11.87 ± 0.12	15.38 ± 0.29
10.00	16.37 ± 1.38	18.58 ± 0.58

Table 2. Time courses of AAO activity in detached rice leaves treated with $CuSO_4$ (10 mM) or distilled water under dark or light. Means ± SE ($n = 4$).

Time [h]	AAO [U g^{-1}(f.m.)] H_2O		$CuSO_4$	
	dark	light	dark	light
0	3.35 ± 0.15	3.35 ± 0.15	3.35 ± 0.15	3.75 ± 0.15
4	2.99 ± 0.57	5.60 ± 0.41	4.76 ± 0.49	7.30 ± 0.54
8	3.40 ± 0.22	6.66 ± 0.37	5.41 ± 0.21	10.80 ± 0.78
12	3.39 ± 0.20	8.28 ± 0.28	9.24 ± 0.39	14.62 ± 0.42
24	4.53 ± 0.86	11.86 ± 0.36	14.68 ± 0.27	16.92 ± 0.77

To investigate whether other divalent metals also increase the activities of AAO in detached rice leaves, we tested Mg, Zn, Fe, and Mn (Table 3). Results indicate that, under both irradiance and dark, Mg, Zn, Fe, and Mn did not cause an increase in

Table 3. Effects of various divalent metals on AAO activities in detached rice leaves. AAO activities were determined after 24 h treatment of metals (10 mM) in the dark or light. Means ± SE ($n = 4$).

Treatment	AAO [U g^{-1}(f.m.)] dark	light
H_2O	4.00 ± 0.26	11.37 ± 0.55
$CuSO_4$	11.16 ± 0.76	15.36 ± 0.86
$CuCl_2$	13.66 ± 0.79	15.59 ± 0.46
$MgSO_4$	2.75 ± 0.43	11.33 ± 0.36
$ZnSO_4$	2.25 ± 0.24	4.85 ± 0.34
$FeSO_4$	1.65 ± 0.43	2.65 ± 0.84
$MnSO_4$	1.75 ± 0.54	9.26 ± 0.45

AAO activities in detached rice leaves. In addition, $CuSO_4$ and $CuCl_2$ were equally effective in increasing AAO activity, indicating that it was induced by Cu rather than by SO_4^{2-} or Cl^-.

To understand whether the effects of Cu^{2+} on AAO activities are specific for green leaf tissue, the effects of $CuSO_4$ on AAO activities in shoots and roots of etiolated rice seedlings were also studied. Increasing concentrations of $CuSO_4$ from 10 to 50 mM progressively decreased root growth, however, no reduction of shoot growth was observed. The differential effect of Cu on root and shoot growth could be explained by the fact that Cu is accumulated mainly in roots and to a minor extent in shoots (Fernandes and Henrigues 1991). As found in light-grown leaves, $CuSO_4$ was effective in increasing AAO activities in roots of etiolated rice seedlings (Table 4). However, $CuSO_4$ had no significant effect on AAO activities in etiolated shoots (Table 4). This is expected if accumulation of less Cu in etiolated shoots was taken into consideration.

Table 4. Effects of $CuSO_4$ concentrations on the fresh mass and AAO activities in etiolated rice seedlings. Seeds were germinated at various $CuSO_4$ concentrations for 5 d in the dark. Means ± SE ($n = 4$)

$CuSO_4$ [μM]	Fresh mass [mg organ^{-1}]		AAO [U g^{-1}(f.m.)]	
	shoot	root	shoot	root
0	25.02 ± 0.42	12.94 ± 0.57	5.15 ± 0.15	1.15 ± 0.25
10	26.21 ± 1.30	9.29 ± 0.40	5.08 ± 0.14	2.50 ± 0.30
20	24.41 ± 1.00	7.51 ± 0.20	5.72 ± 0.24	3.41 ± 0.17
30	25.72 ± 0.50	6.55 ± 0.21	5.33 ± 0.39	7.31 ± 0.39
40	26.53 ± 1.20	5.80 ± 0.14	5.17 ± 0.10	8.65 ± 0.17
50	22.60 ± 1.30	3.93 ± 0.45	5.92 ± 0.36	10.50 ± 0.52

Discussion

Arrigoni *et al.* (1981) reported that many plant tissues lacked AAO activity. In the present study, we demonstrated that AAO is present in green leaves of rice and roots and shoots of etiolated rice seedlings. AAO activity in rice shoot apices during panicle formation has also been reported (Reddy *et al.* 1986).

There have been reports that the activities of several enzymes in plant tissue is induced by Cu^{2+}. Bligny *et al.* (1986) has reported that laccase activity in sycamore cells is increased by adding Cu^{2+}, which is component of laccase. Ikeda *et al.* (1982) have also reported that cytochrome aa_3 content in cultured tobacco cells increases after subculturing in higher Cu^{2+}. Delhaize *et al.* (1986) have reported that the amount of diamine oxidase protein in clover leaves is controlled by the copper concentrations of the leaves. We now report that the increase in AAO activity in detached rice leaves or roots of etiolated rice seedlings treated with $CuSO_4$. Our results are consistent with those of previous work in which AAO in cultured cells of fruit tissues was activated by Cu^{2+} (Esaka *et al.* 1988a,b, 1992, Sekiya *et al.* 1990,

Pitari *et al.* 1993).

Of particular interest is the finding that Cu^{2+} is more effective in activating AAO in detached rice leaves in the dark than in the light. This is mainly due to the fact that AAO activity in detached rice leaves in the absence of Cu in the dark is much lower than that in the light. We also demonstrated that in the absence of $CuSO_4$ irradiance was effective in increasing AAO activity when compared with dark controls. However, Esaka *et al.* (1988a) showed that light had no effect on the AAO activity in pumpkin callus. Phytochrome-mediated induction of AAO in mustard seedlings has been reported (Van Poucke *et al.* 1969, Drumm *et al.* 1972, Acton *et al.* 1974).

Lin and Varner (1991) proposed that AAO may be involved in zucchini cell growth. Esaka *et al.* (1992) showed that AAO was involved in cell growth or cell division of pumpkin fruits. More recently, Takahama and Oniki (1994) reported that one of the causes of the enhancement of elongation growth by indole-3-acetic acid in epicotyls from *Vigna angularis* is related to an increase in AAO activity. However, it seems unlikely that the increase in AAO activity is related to root growth of rice seedling, because Cu, which increases AAO activity, reduced root growth of rice seedlings. It is clear that AAO is not universally associated with cell growth in plants and is most likely confined to certain plant species.

References

Acton, G.J., Drumm, H., Mohr, H.: Control of synthesis *de novo* of ascorbate oxidase in the mustard seedling (*Sinapis alba* L.) by phytochrome. - Planta **121**: 39-50, 1974.

Arrigoni, O., Dipierro, S., Borraccino, G.: Ascorbate free radical reductase, a key enzyme of the ascorbic acid system. - FEBS Lett. **125**: 242-244, 1981.

Bligny, R., Gaillerd, J., Douce, R.: Excretion of laccase by sycamore (*Acer pseudoplatanus*) cells. - Biochem. J. **237**: 583-588, 1986.

Cho, H.J., Aimi, T., Paik, S.Y., Murook, Y.: Secretory production of ascorbate oxidase by cultured cells of cucumber. - J. Ferment. Bioeng. **68**: 193-199, 1989.

Delhaize, E., Dilworth, M.J., Webb, J.: The effect of copper nutrition and developmental state on the biosynthesis of diamine oxidase in clover leaves. - Plant Physiol. **82**: 1126-1131, 1986.

Drumm, H., Brunning, K., Mohr, H.: Phytochrome-mediated induction of ascorbate oxidase in different organs of a dicotyledonous seedling (*Sinapis alba* L.). - Planta **106**: 259-267, 1972.

Esaka, M., Imagi, J., Suzuki, K., Kubota, K.: Formation of ascorbate oxidase in cultured pumpkin cells. - Plant Cell Physiol. **29**: 231-235, 1988a.

Esaka, M., Uchida, M., Fukui, H., Kubota, K., Suzuki,. K.: Marked increase in ascorbate oxidase protein in pumpkin callus by adding copper. - Plant Physiol. **88**: 656-660, 1988b.

Esaka, M., Fukui, H., Suzuki,. K., Kubota, K.: Secretion of ascorbate oxidase by suspension-cultured pumpkin cells. - Phytochemistry **28**: 117-119, 1989.

Esaka, M., Suzuki,. K., Kubota, K.: Stimulation of ascorbate oxidase secretion from cultured pumpkin cells by eosin yellowish and potassium salicylate. - Phytochemistry **29**: 1547-1549, 1990.

Esaka, M., Fujisawa, K., Goto, M., Kisu, Y.: Regulation of ascorbate oxidase expression in pumpkin by auxin and copper. - Plant Physiol. **100**: 231-237, 1992.

Fernandes, J.C., Henriques, F.S.: Biochemical, physiological, and structural effects of excess copper in plants. - Bot. Rev. **57**: 246-273, 1991.

Ikeda, T., Matsumoto, T., Obi, Y.: Influences of copper concentration on cytochrome aa_3 formation and growth in cultured tobacco cells. - Agr. biol. Chem. **46**: 565-566, 1982.

Kao, C.H.: Senescence of rice leaves. IV. Influence of benzyladenine on chlorophyll degradation. - Plant Cell Physiol. **21**: 1255-1262, 1980.

Lin, L.-S., Varner, J.-E.: Expression of ascorbic acid oxidase in zucchini squash (*Cucurbita pepo* L.). - Plant Physiol. **96**: 159-165, 1991.

Pitari, G., Chichiricco, G., Marcozzi, G., Rossi, A., Maccarrono, M., Avigliano, L.: Expression of ascorbate oxidase in cultured zucchini cells. Effect of copper and abscisic acid. - Plant Physiol. Biochem. **31**: 593-598, 1993.

Reddy, K.P., Khan, P.A., Mohanty, G.B., Kumar, K.B.: Ascorbate oxidase activity in rice shoot apices during panical initiation. - Plant Cell Physiol. **27**: 725-728, 1986.

Sekiya, J., Mizuno, K., Kimura, O., Shimose, N.: Ascorbate oxidase in cucumber calli and enhancement of enzyme activity by copper sulfate. - Soil Sci. Plant. Nutr. **36**: 43-51, 1990.

Takahama, U., Oniki, T.: The association of ascorbate and ascorbate oxidase in the apoplast with IAA-enhanced elongation of epicotyls from *Vigna angularis*. - Plant Cell Physiol. **35**: 257-266, 1994

Van Poucke, M., Barthe, F., Mohr, H.: Phytochrome-mediated induction of ascorbic acid oxidase in mustard seedlings. - Naturwissenschaften **56**: 417, 1969.

Weis, W.: Ascorbic acid and electron transport. - Ann. N. Y. Acad. Sci. **258**: 190-200, 1975.