

H₂O₂ metabolism during senescence of rice leaves: changes in enzyme activities in light and darkness

Chin Jung Chang & Ching Huei Kao*

Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China
(* author for correspondence: fax: 886-2-23620879)

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Abstract

The possible role of H₂O₂ metabolism on light-regulated senescence of detached rice leaves was investigated. Light retards senescence but at the same time accumulates more H₂O₂. Light treatment resulted in an increase in malondialdehyde level in detached rice leaves but no membrane leakage was observed in light-treated detached leaves. It seems that there was no direct relationship between lipid peroxidation and deterioration in membrane integrity. The results obtained suggest that retardation of senescence by light is closely related to high activities of superoxide dismutase and ascorbate peroxidase.

Abbreviations: APOD = ascorbate peroxidase; CAT = catalase; GR = glutathione reductase; MDA = malondialdehyde; POD = peroxidase; SOD = superoxide dismutase

1. Introduction

Hydrogen peroxide (H₂O₂) is a constituent of oxidative metabolism. It is a product of peroxisomal and chloroplastic oxidative reactions [6]. H₂O₂ itself is an active oxygen species. H₂O₂ can also react with superoxide radicals to form more powerful oxygen free radicals and hydroxyl radical in the presence of trace amounts of Fe or Cu [4, 26]. The hydroxyl radicals initiate self-propagating reactions leading to peroxidation of membrane lipids and destruction of proteins [2, 4, 11]. Free radicals and lipid peroxidation are widely considered to be major contributors to leaf senescence [7, 8, 16, 24]. Cellular damage caused by free radicals and lipid peroxidation might be reduced or prevented by protective metabolism involving antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APOD), glutathione reductase (GR), catalase (CAT), and peroxidase (POD). SOD catalyzes the dismutation of two molecules of superoxide into oxygen and H₂O₂. APOD reduces H₂O₂ to water, with ascorbate as electron donor [1]. GR plays a part in the control

of endogenous H₂O₂ through an oxido-reduction cycle involving glutathione and ascorbate [10, 25]. CAT and POD are implicated in removal of H₂O₂. The present investigation was conducted to compare the changes in the activities of enzymes related to H₂O₂ metabolism of rice during senescence in light and darkness.

2. Materials and methods

Rice (*Oryza sativa* L. cv. Taichung Native 1) was cultured as previously described [14]. The apical 3-cm segments excised from the third leaves of 12-day-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 mL of test solutions. Each treatment was replicated 4 times. Incubation was carried out at 27 °C in the light (40 μmol m⁻²s⁻¹) or in darkness.

For protein determination, leaf segments were homogenized in 50 mM sodium phosphate buffer (pH 6.8). The extracts were centrifuged at 17,600 *g* for 20 min, and the supernatants were used for deter-

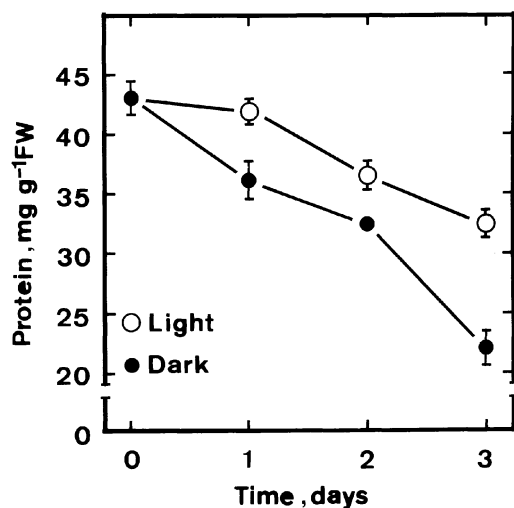


Figure 1. Changes in level of protein in detached rice leaves treated with light or darkness. Vertical bars represent standard errors ($n = 4$).

mination of protein by the method of Bradford [5]. Protein levels were expressed as mg g^{-1} fresh weight. Malondialdehyde (MDA) was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer [12]. MDA level is routinely used as an index of lipid peroxidation and was expressed as nmol g^{-1} fresh weight. Conductivity of the medium containing leaf segments was determined directly using a conductivity meter (Suntex conductivity meter, Taipei, Taiwan). The conductivity of the medium containing no leaf segments was used for correction.

The H_2O_2 level was colorimetrically measured as described by Jana and Choudhuri [13]. H_2O_2 was extracted by homogenizing 50 mg leaf tissue with 3 mL of phosphate buffer (50 mM, pH 6.5). The homogenate was centrifuged at 6,000 g for 25 min. To determine H_2O_2 levels, 3 mL of extracted solution was mixed with 1 mL of 0.1% titanium sulphate in 20% (v/v) H_2SO_4 and the mixture was then centrifuged at 6,000 g for 15 min. The intensity of the yellow colour of the supernatant was measured at 410 nm. H_2O_2 level was calculated using the extinction coefficient $0.28 \mu\text{mol}^{-1} \text{cm}^{-1}$.

For extraction of enzymes, leaf tissues were homogenized with 0.1 M sodium phosphate buffer (pH 6.8) in a chilled pestle and mortar. The homogenate was centrifuged at 12,000 g for 20 min and the resulting supernatant was used for determination of enzyme activity. The whole extraction procedure was carried out at 4 °C. CAT activity was assayed by measuring

Table 1. Effect of light and dark pretreatments on levels of protein in detached rice leaves treated with paraquat

Pretreatment	Treatment	Protein level, mg g^{-1} FW
Light	H_2O	36.2 ± 0.3
	Paraquat	30.0 ± 0.4
Dark	H_2O	30.4 ± 0.1
	Paraquat	15.3 ± 0.6

Ten detached rice leaves were pretreated with either light or darkness for 2 days and then transferred to either water or paraquat (10 μM) for 12 h in the light. Each treatment (10 detached rice leaves) was replicated 4 times.

the initial rate of disappearance of H_2O_2 [15]. The decrease in H_2O_2 was followed as the decline in optical density at 240 nm, and activity was calculated using the extinction coefficient ($40 \text{ mM}^{-1} \text{ cm}^{-1}$ at 240 nm) for H_2O_2 [15]. POD activity was measured using a modification of the procedure of MacAdam et al. [17]. Activity was calculated using the extinction coefficient ($22.6 \text{ mM}^{-1} \text{ cm}^{-1}$ at 470 nm) for tetraguaical. SOD was determined according to Paoletti et al. [20]. APOD was determined according to Nakano and Asada [19]. The decrease in ascorbate concentration was followed as a decline in optical density at 290 nm and activity was calculated using the extinction coefficient ($2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ at 290 nm) for ascorbate. GR was determined by the method of Foster and Hess [9]. Enzyme activity was expressed on the basis of gram fresh weight.

3. Results

The senescence of detached rice leaves was followed by measuring the decrease of protein. Figure 1 shows the changes in protein level in detached rice leaves under light and dark conditions. The noticeable effect of light on the retardation of protein loss in detached rice leaves was evident at one day after incubation.

Figure 2 demonstrates that light treatment resulted in a marked increase in MDA level. In contrast to the effect of light on lipid peroxidation, light treatment resulted in no increase in membrane leakage of leaf tissue (Figure 2). An increase in membrane leakage of leaf tissue held in darkness was observed (Figure 2). Figure 3 shows that H_2O_2 level increased significantly in detached rice leaves in either light or darkness. However, there was greater accumulation of H_2O_2 in the

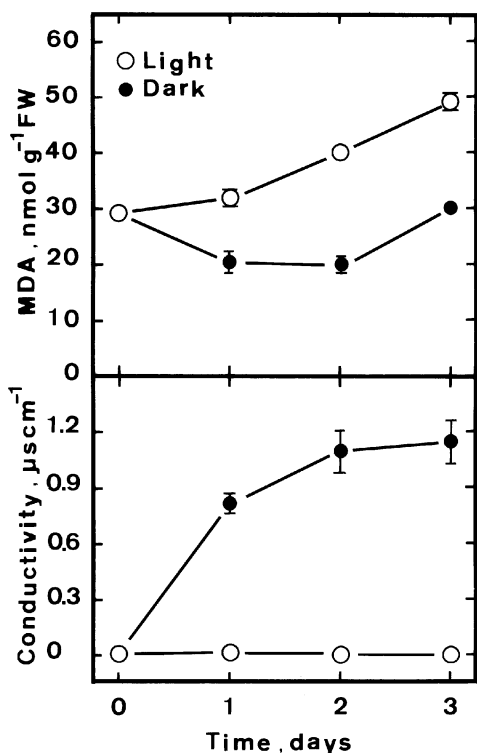


Figure 2. Changes in conductivity in the medium and level of MDA in detached rice leaves treated with light or darkness. Vertical bars represent standard errors (n = 4).

light than in the dark. Also shown in Figure 3 is the decline in SOD and CAT activities in detached rice leaves under light and dark conditions. The decline of SOD activity was found to be greater in the dark than in the light (Figure 3). However, the decline of catalase activity was greater in the light than in the dark. POD and APOD activities increased significantly in detached rice leaves in both light and darkness (Figure 3). However, the effect was more pronounced in the light at a later stage of incubation than in the dark. There was no difference in GR activity in detached rice leaves between light and dark treatments. Table 1 shows that rice leaves pretreated with light resulted in a reduction of toxicity of paraquat, judged by the changes in total protein levels.

4. Discussion

Light is generally known to retard leaf senescence [27]. It has been reported that H₂O₂ promotes senescence in detached rice leaves and suggested that the accumula-

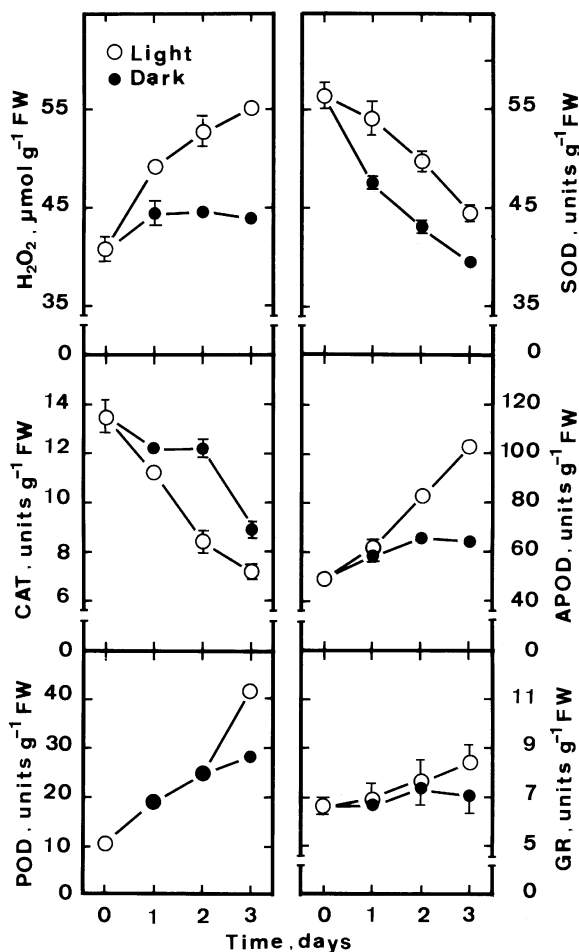


Figure 3. Changes in level of H₂O₂ and activities of CAT, POD, SOD, APOD and GR in detached rice leaves treated with light or darkness. Vertical bars represent standard errors (n = 4).

tion of H₂O₂ may be responsible for inducing rice leaf senescence [21]. The present investigation, however, shows that light retards senescence but at the same time causes more H₂O₂ to accumulate when compared with dark controls (Figures 1 and 3). Activity of catalase, the enzyme responsible for eliminating H₂O₂ in leaves, was greater in darkness than in the light (Figure 3). This may result in a higher H₂O₂ level in light-treated rice leaves. However, the possibility that H₂O₂ in light-treated leaves is less utilized in metal-catalyzed Haber-Weiss reaction to form hydroxyl radicals [4] can not be excluded.

The results we obtained with detached rice leaves incubated in the light showed that there was no direct relationship between lipid peroxidation and deterioration in membrane integrity, since the MDA level

markedly increased in light-treated leaves, whereas conductivity in the medium remained unchanged (Figure 2). These results suggest that in the light lipid reserves may undergo peroxidation serving as a detoxification mechanism and thus protecting the membranes and leading to retardation of senescence. Antioxidative enzymes play important roles in stress tolerance and have been implicated in the adaptation and ultimate survival of plants during periods of environmental stress. Light is one of the important environmental factors which is likely to influence the levels of antioxidative enzymes [4], because the potential for the production of activated oxygen species is greatly enhanced by high-light stress in plants [2]. Activities of SOD and APOD have been found to be much lower in wheat seedlings grown under low-light condition than in those grown under high-light condition [18]. In the present investigation, we used low-light ($40 \mu\text{mol m}^{-2}\text{s}^{-1}$) for light treatment. We were able to show that SOD and APOD activities were higher in detached leaves held in light than in those held in darkness (Figure 3). This would explain why light retards senescence of detached rice leaves. It has been demonstrated that transgenic plants overexpressing SOD and APOD had increased resistance to paraquat-mediated oxidative stress [3, 22, 23]. Thus, it would be interesting to know whether light-treated detached rice leaves are more resistant to paraquat than dark-treated leaves. To test this, detached rice leaves were pretreated with either light or darkness for 2 days and then transferred to either water or paraquat ($10 \mu\text{M}$) for 12 h in the light. It is indeed that pretreatment reduced the toxicity of paraquat (Table 1).

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References

- Asada K (1992) Ascorbate peroxidase – a hydrogen peroxide scavenging enzyme in plants. *Physiol Plant* 85: 235–241
- Asada K and Takahashi M (1987) Production and scavenging of active oxygen in photosynthesis. In: Kyle DC and Arntzen CJ (eds) *Photoinhibition*, pp 227–287. Amsterdam: Elsevier
- Bowler C, Slooten L, Vandenbranden S, De Rycke R, Booterman J, Sysbesma C, Van Montagu M and Inze D (1991) Mangane superoxide dismutase can reduce cellular damage mediated by oxygen radicals in transgenic plants. *EMBO J* 10: 1723–1732
- Bowler C, Van Montagu M and Inze D (1992) Superoxide dismutase and stress tolerance. *Annu Rev Plant Physiol Plant Mol Biol* 43: 83–116
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254
- Del Rio L, Sandalio LM, Palma JM, Bueno P and Corpus FJ (1992) Metabolism of oxygen radicals in peroxisomes and cellular implications. *Free Rad Biol Med* 13: 557–580
- Dhindsa RS, Plumb-Dhindsa P and Reid DM (1982) Leaf senescence and lipid peroxidation: effects of some phytohormones, and scavengers of free radicals and singlet oxygen. *Physiol Plant* 56: 453–457
- Dhindsa RS, Plumb-Dhindsa P and Thorpe TA (1991) Leaf senescence: correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. *J Exp Bot* 32: 93–101
- Foster JG and Hess JL (1980) Responses of superoxide dismutase and glutathione reductase activities in cotton leaf tissue exposed to an atmosphere enriched in oxygen. *Plant Physiol* 66: 482–487
- Foyer CH and Halliwell B (1976) The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. *Planta* 133: 21–25
- Halliwell B (1987) Oxidative damage, lipid peroxidation and antioxidant protection in chloroplasts. *Chem Phys Lipids* 44: 327–340
- Heath RL and Packer L (1968) Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125: 189–198
- Jana S and Choudhuri MA (1981) Glycolate metabolism of three submerged aquatic angiosperms during aging. *Aquat Bot* 12: 345–354
- Kao CH (1980) Senescence of rice leaves IV. Influence of benzyladenine on chlorophyll degradation. *Plant Cell Physiol* 21: 1255–1262
- Kato M and Shimizu S (1987) Chlorophyll metabolism in higher plants. VII. Chlorophyll degradation in senescing tobacco leaves: phenolic-dependent peroxidative degradation. *Can J Bot* 65: 729–735
- Kunnert KJ and Ederer M (1985) Leaf aging and lipid peroxidation: the role of antioxidant vitamin C and E. *Physiol Plant* 65: 85–88
- MacAdam JW, Nelson CJ and Sharp RE (1992) Peroxidase activity in the leaf elongation zone of tall fescue. *Plant Physiol* 99: 872–878
- Mishra NP, Fatma T and Singhal GS (1995) Development of antioxidative defense system of wheat seedlings in response to high light. *Physiol Plant* 95: 77–82
- Nakano Y and Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22: 867–880
- Paoletti F, Aldinucci D, Mocali A and Capparini A (1986) A sensitive spectrophotometric method for the determination of superoxide dismutase activity in tissue extracts. *Anal Biochem* 154: 536–541
- Parida RK, Kar M and Mishra D (1978) Enhancement of senescence in excised rice leaves by hydrogen peroxide. *Can J Bot* 56: 2937–2941
- Perl A, Perl-Treves R, Galili S, Aviv D, Shalgi E, Malkin S and Galan E (1993) Enhanced oxidative-stress defense in trans-

- genic potato expressing tomato Cu, Zn superoxide dismutase. *Theor Appl Genet* 85: 568–576
23. Sen Gupta A, Webb RP, Holaday AS and Allen RD (1993) Overexpression of superoxide dismutase protects plants from oxidative stress. Induction of ascorbate peroxidase in superoxide dismutase-overexpressing plants. *Plant Physiol* 103: 1067–1073
 24. Sharma YK and Davis KR (1994) Ozone-induced expression of stressed-related genes in *Arabidopsis thaliana*. *Plant Physiol* 105: 1089–1096
 25. Smith IK, Vierheller TL and Thorne C (1989) Properties and functions of glutathione reductase in plants. *Physiol Plant* 77: 449–456
 26. Thompson JE, Legge RL and Barber RF (1987) The role of free radicals in senescence and wounding. *New Phytol* 105: 317–444
 27. Thimann KV (1980) The senescence of leaves. In: Thimann KV (ed) *Senescence in Plants*, pp 85–115. Boca Raton: CRC Press