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Potential marker proteins for ozone-induced yield reduction in rice

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Abstract: Three proteins - a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1 - were evaluated as potential markers of ozone-induced yield responses in six rice (*Oryza sativa* L.) cultivars ('Kirara 397', 'Koshihikari', 'Nipponbare', 'Takanari', 'Kasalath', 'Suphanburi 90') under ozone stress in laboratory-scale tests. The levels of all three proteins decreased after ozone exposure in cultivars identified as ozone-sensitive while they increased or remained constant after ozone exposure in tolerant cultivars, although ATP synthase tended to decrease. Furthermore, the protein level and grain yield in each cultivar exposed to ozone were significantly positively correlated for all three proteins. Thus, CPN-60 and enolase 1 are potential markers for chronic ozone stress in rice.

1. Introduction

Ozone is a major gaseous pollutant in the troposphere and ozone concentrations have in recent years increased rapidly in developing Asian countries. Indeed, the emission of anthropogenic nitrogen oxides (ozone precursors) in Asia under a no-further-control scenario was predicted to increase by 350% between 1990 and 2020 (Aunan *et al*., 2000).

An elevated ozone concentration will reduce the growth and yield of crop plants including rice, the most important food crop in Asia (Kobayashi *et al*., 1995; Yonekura *et al*., 2005). Many researchers have described the mechanisms responsible for visible injury on plant leaves by acute ozone exposure (reviewed by Kangasjarvi *et al*., 2005). The primary mechanism is oxidative damage caused by an increase in levels of reactive oxygen species (ROS). However, the cause for yield reductions under chronic ozone stress remains unclear. In a previous report we described how ozone sensitivity in evaluated rice cultivars, in terms of visible injury (chlorotic or necrotic lesions), did not coincide with that indicated by the grain yield reduction (Sawada and Kohno, 2009). In addition, conventional evaluation of chronic ozone effects relies on measurements such as growth and yield reductions, which require large-scale studies (e.g. in a field or greenhouse) and long time periods (e.g. about six months). A rapid and small-scale method for early evaluation of chronic ozone effects, such as the use of molecular markers, would make it faster, easier, and less expensive to select ozone-tolerant cultivars.

Kubo *et al*. (2011) reported that during ozone stress, sakuranetin, a flavonone in the phytoalexin family, appears to serve as a molecular marker of the stress response. Sakuranetin contents in rice leaves exposed simultaneously to ozone and high temperature increased only in the three cultivars whose grain yield was unaffected by ozone stress. However, their experiment was performed under both elevated ozone and elevated temperature, making it difficult to determine the separate effect of each factor. Moreover, the ozone concentration was 150 nl l^{-1} (ppb), much higher than ambient ozone levels. Therefore, more practical markers are needed.

Proteomic studies are useful to reveal protein markers associated with various stress tolerance (reviewed by Kosova *et al*., 2011). In a previous study, we conducted differential proteome analysis using three rice cultivars that showed different levels of ozone sensitivity (indicated by the reduction in grain yield) when exposed to elevated ozone during the cultivation season in open-top chambers (Sawada *et al*., 2012). In these cultivars, we observed significant changes in the size of spot that contained three proteins: a 60-kDa chaperonin (CPN-60), chloroplastic ATP synthase, and enolase 1. The change in size of this spot was proportional to their ozone sensitivity, measured as the reduction in grain yield. These results suggest that these proteins are closely involved in the mechanisms that underlie the yield reduction

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that occurs under elevated ozone levels, and therefore they have potential as molecular markers that can predict the ozone-induced yield loss. To clarify the usefulness of these proteins for use in laboratory-scale tests, we investigated the levels of these candidate proteins in the seedlings of six rice cultivars under short-term ozone exposure in growth chambers, and tested for a significant correlation between the protein levels and relative grain yield.

2. Materials and Methods

Chronic ozone exposure

Six rice (*Oryza sativa* L.) cultivars were used in this study: 'Kirara 397', 'Koshihikari', 'Nipponbare' (*japonica* cultivars), 'Takanari' (a hybrid *indica* cultivar), 'Kasalath', 'Suphanburi 90' (*indica* cultivars). Seedlings (n = 40) of each cultivar were grown in seedling boxes for three weeks in a glasshouse under ambient atmospheric conditions, then transplanted into pots (at four plants per pot with a 0.05-m^2 surface area and a 0.015-m^3 volume) in open-top chambers (OTCs; 3.6 × 3.6 m) at an experimental field of the Akagi Testing Center of the CRIEPI (Maebashi, Japan) in the late spring of 2007, 2008, and 2009. Fertilizer was supplied at a rate of N-P_2O_5-K_2O=15-15-15 g m^{-2}. The OTC fumigation system has been described previously (Frei *et al*., 2011). Ozone was added in the chambers using a mass-flow controller combined with a PID controlling system to maintain the designated concentrations. Three ozone-level treatments were established, from transplanting of rice plants into the pots to harvest, for three years with a regular diurnal pattern: charcoal-filtered air (CF), ambient ozone (Ozone ×1), and twice ambient ozone (Ozone ×2). Concentrations of ozone were continuously monitored in each chamber at 3-min intervals using a UV absorption ozone analyzer (ML9810, Monitor Labs, Englewood, CO, USA). Mean ozone concentration, air temperature and relative humidity in the different treatments are summarized in Table 1.

Measurement of the yield

The rice cultivars were harvested between September and November in 2007, 2008, and 2009. Harvesting of each cultivar was conducted when about 80% of the grains had turned yellow. After harvesting, grains were separated from the panicles and categorized into two groups (filled and unfilled grains) using an automatic seed-sorting machine (FV-459A, Fujiwara Seisakusho KK, Tokyo, Japan). The filled grains (rough rice) were weighed to determine the grain yield.

Short-term ozone exposure

Rice seedlings were grown in indoor growth chambers at 28/23°C (day/night), photosynthetic photon-flux density of 400 μmol m^{-2} s^{-1}, with a 12-h photoperiod, and a relative humidity of 60±5%. After two weeks, the 'Kirara 397', 'Koshihikari', and 'Takanari' seedlings were exposed to three levels of ozone (12 h/day) for three days in three individual replicates: CF, ambient ozone (40 ppb), and twice ambient ozone (80 ppb). Similarly, 'Nipponbare', 'Kasalath', and 'Suphanburi 90' seedlings were exposed to CF and 40 ppb of ozone. Ozone was generated with a silent electrical discharge in dry oxygen. The concentration of ozone in the chambers was monitored continuously during exposure with a UV absorption ozone detector (Model 1150, Dylec Inc., Tokyo, Japan). At the end of the exposure, we removed the third leaves, immediately froze them in liquid nitrogen, and stored them in -80°C until the immunoblot analysis was performed.

Immunoblot analysis

Leaves (100 mg) were homogenized in sodium dodecyl sulfate (SDS) buffer (10% (w/v) glycerol, 5% (v/v) β-mercaptoethanol, 2.3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8). Equal amounts of protein samples were separated using 15% SDS-polyacrylamide gel electrophoresis (PAGE). After the SDS-PAGE, the protein samples were transferred onto a polyvinylidene fluoride membrane or they were stained by Coomassie brilliant blue (CBB).

Table 1 - Ozone concentrations and environmental conditions in the open-top chambers during the cropping seasons of rice

		Ozone concentration (ppb)			Temperature (°C)	Relative humidity (%)
		12 h mean	24 h mean	Mean daily Maximum	24 h mean	24 h mean
2007	CF	3.1	2.1	4.1	–	–
	Ozone x1	37.6	31.1	61.3	–	–
	Ozone x2	68.6	56.3	101.7	–	–
2008	CF	4.7	3.9	6.5	21.1	83.4
	Ozone x1	40.4	27.5	57.6	21.3	83.5
	Ozone x2	82.7	57.0	118.3	21.3	81.7
2009	CF	5.1	5.0	9.7	20.6	78.7
	Ozone x1	35.1	27.9	56.9	20.7	78.9
	Ozone x2	73.5	54.7	110.2	20.9	77.4

Measurements of environmental conditions and ozone concentrations were recorded at 3 and 10-minute interval throughout the experiment, respectively. Average values of the two replicate chambers per treatment are shown. 12 h means were calculated for the period from 6:00 to 17:59 hours. The temperature and relative humidity were not measured in 2007.

The blotted membrane was blocked for 1 h in TBS-T (20 mM Tris-HCl, pH 7.6, 150 mM NaCl and 0.1% v/v Tween-20) containing 5% (w/v) nonfat milk (Skim milk; Difco, Sparks, MD, USA). The membrane was subsequently incubated with the monoclonal antibody anti-heat-shock protein 60 (Acris Antibodies GmbH, Herford, Germany), with the polyclonal antibodies anti-ATP synthase β-subunit (AntiProt, Pullach i. Isartal, Germany), and anti-enolase (Aviva system biology, San Diego, CA, USA) at 1:5000 dilutions for 1 h at room temperature. As secondary antibodies, we used anti-mouse or anti-rabbit IgG with conjugated HRP (Bio-Rad Laboratories Inc., Hercules, CA, USA). After incubation for 1 h with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, we detected the immunoblot signals using the ECL plus western blotting detection kit (GE Healthcare, Piscataway, NJ, USA) following the manufacturer's protocols and the results were visualized using an LAS-3000 luminescent image analyzer (Fujifilm, Tokyo, Japan). The relative intensities of the bands were calculated using PD-Quest software (version 8.0.1, Bio-Rad).

3. Results and Discussion

After chronic ozone exposure during three years of growing seasons (from 2007 to 2009) each cultivar showed a similar yield response to ozone in all years of the experiment. The grain yields of 'Kirara 397', 'Takanari' and 'Kasalath' decreased significantly by 15 to 36%, 10 to 21%, and 12 to 19%, respectively, under twice the ambient ozone level (about 80 ppb treatment, daily 12-h mean concentration), although the grain yields did not differ significantly from CF under ambient ozone level (about 40 ppb treatment), except for 'Kirara 397' and 'Takanari' in 2007 (Fig. 1, P <0.05). The grain yields of 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' did not decrease significantly with ozone stress. On this basis, we defined 'Kirara 397', 'Takanari', and 'Kasalath' as ozone-sensitive cultivars, and 'Koshihikari', 'Nipponbare', and 'Suphanburi 90' as ozone-tolerant cultivars.

To confirm whether CPN-60, ATP synthase, and enolase 1 can be used as markers for ozone-induced rice yield loss in laboratory-scale tests, we analyzed the levels of these proteins (Fig. 2A). Levels of CPN-60 decreased significantly after three days of exposure to 40 (the ambient concentration) and 80 ppb (twice the ambient concentration) of ozone in 'Kirara 397' and 80 ppb of ozone in 'Takanari' (Fig. 2B, P<0.05). Levels of ATP synthase and enolase 1 tended to decrease after ozone exposure, although not significantly (except for 'Takanari' exposed to 80 ppb of ozone), in both 'Kirara 397' and 'Takanari'. These cultivars also showed lower grain yield under ozone exposure (Fig. 1). In contrast, levels of CPN-60 and enolase 1 in 'Koshihikari' exposed to 40 ppb of ozone increased significantly and remained the same compared with the levels in CF (P <0.05). Moreover, enolase 1 production also remained constant in 'Koshihikari' at 80 ppb ozone exposure. The level of ATP synthase tended to decrease after ozone exposure in 'Koshihikari'. Because the levels of CPN-60 and enolase 1 decreased and increased at 40 ppb ozone exposure in ozone-sensitive and ozone-tolerant cultivars, respectively, 'Kasalath', 'Nipponbare', and 'Suphanburi 90' seedlings were exposed to CF and

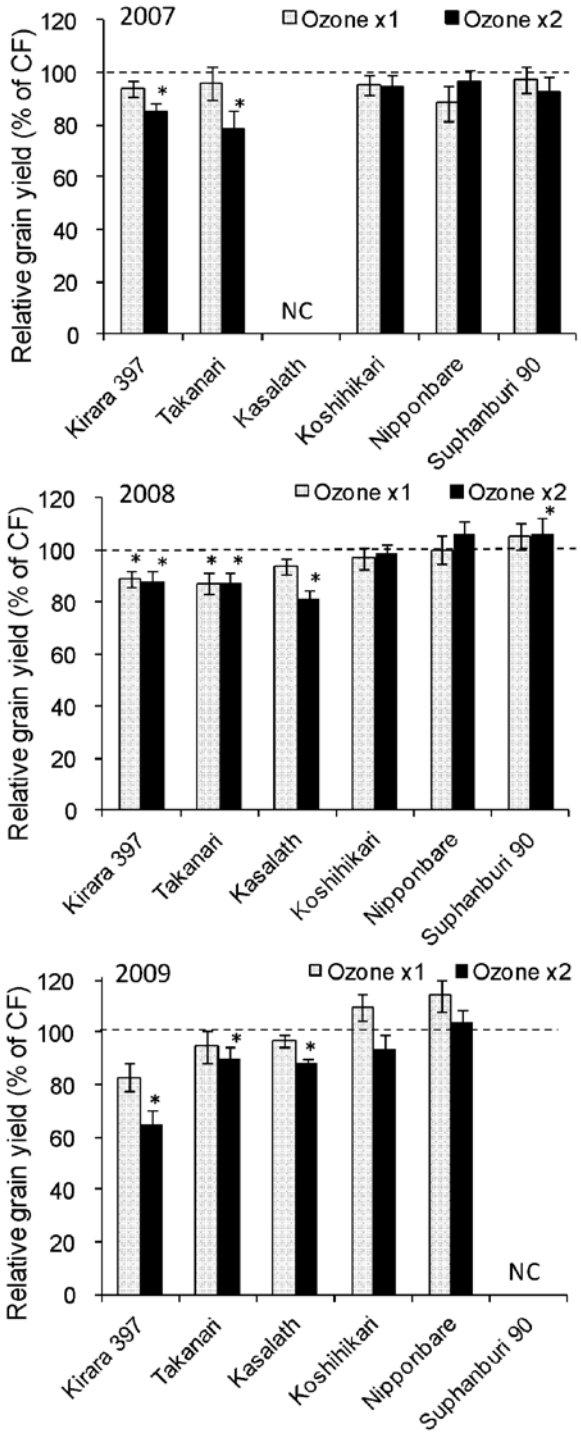

Fig. 1 - Effects of chronic ozone exposure on the grain yields of six rice cultivars in 2007, 2008, and 2009. Values are mean ± SE (n= 40). Asterisk indicates a significant difference compared with CF according to Dunnett's test (P<0.05). 'Kasalath' and 'Suphanburi 90' were not cultivated in 2007 and 2009, respectively, and yields are shown as "NC".

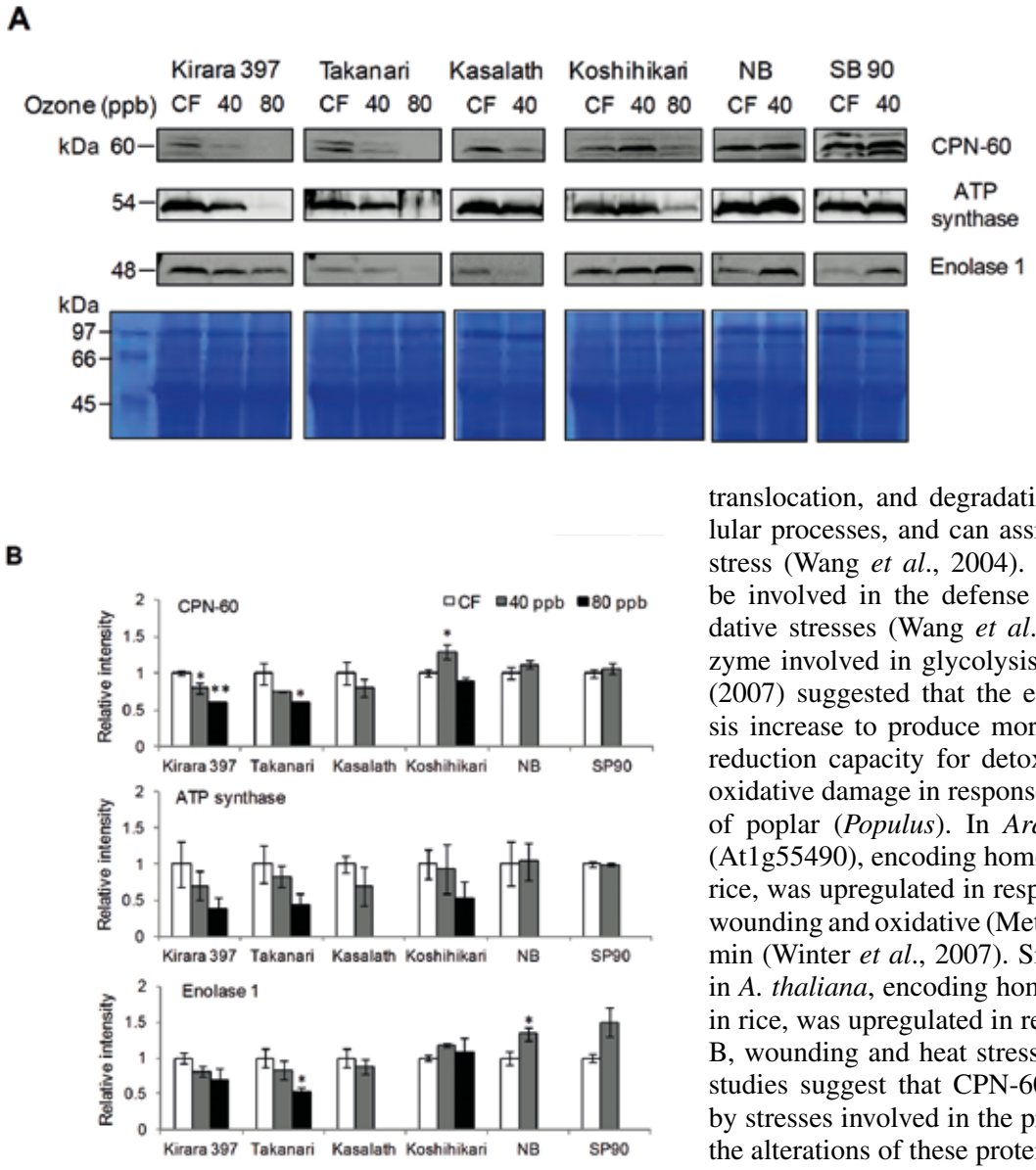

Fig. 2 - (A) Immunoblot analysis of CPN-60, ATP synthase, and enolase 1 in leaves of rice seedlings exposed to charcoal filtered air (CF), or to 40 or 80 ppb of ozone. Immunodetection was performed with antibodies specific to these proteins. (bottom panel) CBB-stained SDS-PAGE showing the quality and loading quantity of the protein samples. (B) Relative intensity for each protein estimated from the immunoblot analysis in panel (A). Values are mean ± SE (n = 3). Asterisk indicates a significant difference compared with CF according to Dunnett's test or *t*-test ($P<0.05$). NB, Nipponbare; SP90, Suphanburi 90.

40 ppb of ozone. The levels of all three proteins increased significantly or were maintained under 40 ppb of ozone in 'Nipponbare' and 'Suphanburi 90', but tended to decrease in 'Kasalath'. Therefore, the levels of CPN-60 and enolase 1 differed between the ozone-sensitive and ozone-tolerant cultivars: they decreased and increased, respectively at least at 40 ppb.

CPN-60 is a molecular chaperone. Many molecular chaperones were originally identified as heat-shock proteins (HSPs), which function in protein folding, assembly, translocation, and degradation during many normal cellular processes, and can assist in protein refolding under stress (Wang *et al*., 2004). CPN-60 (HSP60) appears to be involved in the defense response that mitigates oxidative stresses (Wang *et al*., 2011). Enolase 1 is an enzyme involved in glycolysis in the cytosol. Bohler *et al*. (2007) suggested that the enzymes involved in glycolysis increase to produce more energy and to increase the reduction capacity for detoxification of ROS and repair oxidative damage in response to ozone stress in the leaves of poplar (*Populus*). In *Arabidopsis thaliana*, *CPN60B* (At1g55490), encoding homologous protein to CPN-60 in rice, was upregulated in response to drought, UV-B, heat, wounding and oxidative (Methyl viologen) stress within 30 min (Winter *et al*., 2007). Similarly, *ENO2* (At2G36530) in *A. thaliana*, encoding homologous protein to enolase 1 in rice, was upregulated in response to cold, drought, UV-B, wounding and heat stress (Winter *et al*., 2007). These studies suggest that CPN-60 and enolase 1 are induced by stresses involved in the production of ROS. Therefore, the alterations of these protein levels with ozone exposure might result in ozone-derived ROS rather than ozone itself. However, there has been no report describing whether CPN-60 and enolase 1 influence grain production in crops under environmental stress, although these proteins might not be specific markers to ozone. Further studies will be needed to clarify the relationship between the reduction in grain yield and decreased production of these proteins by ozone-sensitive cultivars.

In order to compare the relative levels of each protein upon short-term ozone exposure with the relative grain yields under chronic ozone exposure we performed a linear regression analysis (Fig. 3). We found significant positive correlations between the levels of CPN-60, ATP synthase, and enolase 1 and the relative grain yield (i.e. yield decreased as the protein concentrations decrease). Therefore, the three proteins may serve as potential markers for chronic ozone stress in rice, although further experiments will be required for ATP synthase that also tended to decrease in 'Koshihikari' at 40 ppb ozone exposure (Fig. 2B). The level of CPN-60 had the highest goodness of fit ($R^2 = 0.786$) with the grain yield. This suggests that the potential ozone-induced yield reduction can be evalu-

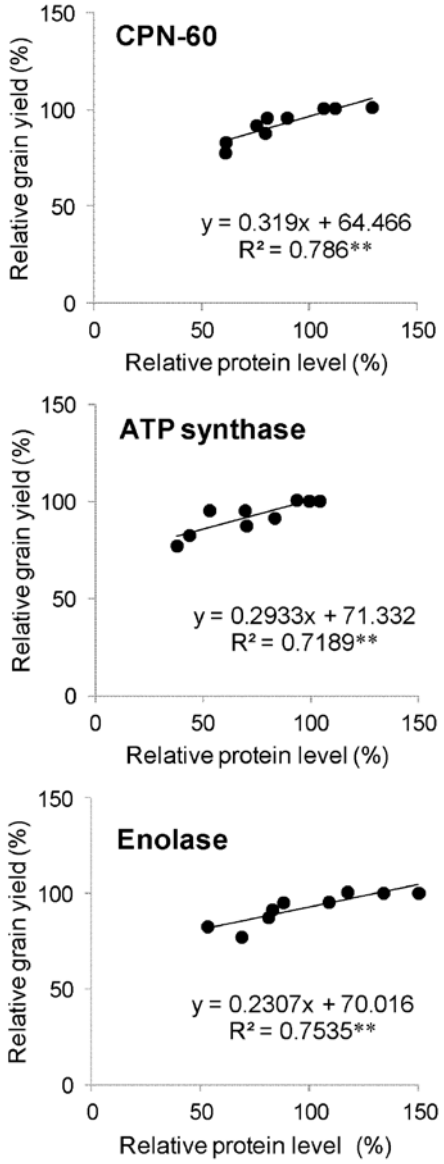

Fig. 3 - Regression analysis for the relative levels of CPN-60, ATP synthase, and enolase 1 in rice seedlings exposed to 40 and 80 ppb ozone, plotted as a function of relative grain yield. The grain yields are relative to the values for six cultivars grown in open-top chambers. Significance levels: **, $P< 0.01$; *, $p< 0.05$.

ated using the level of CPN-60 at the seedling stage in laboratory-scale tests. Moreover, the protein markers that we identified in this study may be useful in crop breeding to quickly select ozone-tolerant rice varieties. Vincent *et al*. (2007) indicated that the inhibition of shoot growth was best correlated with the level of CPN-60 in two wine grape cultivars exposed to salinity and water deficit stress, suggesting that the protein marker is also applicable to other plant or crops.

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