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Tropospheric Ozone Poses a Rising Threat to Yield Stability in Rice: Tolerance Mechanisms and Underlying Genetic Factors

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ABSTRACT

Rice (*Oryza sativa* L.) is the major staple food for most of the developing countries in Asia, whose populations are predicted to steadily increase in this century. However, global change will put increasing pressure on crop production, as it is associated with a range of abiotic stress factors that limit crop yields. One such stress factor is tropospheric ozone (O_3), whose rising background concentrations are a consequence of rising emissions of precursor gases such as nitrogen oxides and volatile organic compounds. Surface O_3 levels that exceed the critical threshold beyond which yield declines are expected are now common in many rice growing areas of South and East Asia and climate models predict further increases for the next three decades. It is therefore imperative to assess the potential to develop new breeding lines with improved tolerance to ozone exposure. This review summarizes the current state of research on the effect of ozone on rice, focusing on recent efforts in the breeding for ozone tolerance. Moreover, novel data are presented that characterize physiological and agronomic features of previously reported quantitative trait loci (QTL) for ozone tolerance, including two QTLs influencing leaf bronzing (*OzT3* and *OzT9*) and one QTL influencing dry weight (*OzT8*). In addition, the first ozone response data of 'IR64', the most widely-grown tropical rice cultivar in South and South-east Asia, is presented, including data on photosynthetic performance and yield loss under season-long ozone exposure.

Keywords: abiotic stress, antioxidant, breeding, global change

CONTENTS

INTRODUCTION

The global problem of rising background tropospheric ozone concentrations and its impact on agriculture

Tropospheric ozone (O_3) is a secondary air pollutant whose current background concentrations are causing adverse effects on vegetation in various regions of the world (Ashmore 2005; Booker *et al.* 2009). It is produced by a series of photochemical reactions involving nitrogen oxides (NO_x) or volatile organic compounds, and the increased emission of these precursors into the atmosphere, starting from the era of the Industrial Revolution until today, has resulted in an increase in global background concentrations (Long and Naidu 2002; Fowler 2008).

Ozone negatively affects vegetation by entering through the stomatal pores and quickly reacting with the apoplast of nearby mesophyll cells, producing reactive oxygen species (ROS) and triggering a series of signaling cascades and plant defense responses, which ultimately result in a range of effects, including visible foliar damage, decreased photosynthetic capacity, and/or accelerated senescence (Omasa *et al.* 2000; Long and Naidu 2002; Kangasjarvi *et al.* 2005). For most agricultural crop species, the long-term effects of ozone include decreased biomass accumulation, altered reproductive phenology, and decreased yield (Ashmore and Marshall 1999; Fiscus *et al.* 2005; Feng and Kobayashi 2009).

Due to the nature by which tropospheric ozone is formed, diurnal and seasonal patterns of O_3 show significant variation depending on local meteorological conditions (Fowler

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2008). Although precursors of O_3 are emitted primarily around urban centers, they can travel significant distances by wind, resulting in significant levels of O_3 even in rural areas. Therefore, tropospheric ozone can present a significant threat to agriculture in many areas of the globe.

Historically, the negative impact of O_3 on plants was first observed in the 1950's on natural vegetation in the western United States. In the latter half of the 20th century, elevated O_3 concentrations were a significant issue of concern in urban and rural regions throughout the United States and Europe, causing visible effects in natural communities and economically significant yield losses in agriculture (Fiscus *et al.* 2005; Booker *et al.* 2009). For that reason, a large volume of literature has been produced on ozone's effects on crops such as soybean and wheat. However, relatively little work has been done thus far looking at its impact on rice (*Oryza sativa* L.) and the development of ozone-tolerant lines, since tropospheric ozone was not considered to be a significant threat in Asian regions in the past, and rice was considered to be relatively tolerant to air pollution compared to other crops (Heagle 1989).

In the coming decades, however, large increases in tropospheric ozone are predicted for parts of East, Southeast, and South Asia, where the majority of rice is grown worldwide (Aunan *et al.* 2000; Wang and Mauzerall 2004; Sitch *et al.* 2007). Since rice is the major staple food for most developing countries in Asia, whose populations expected to increase drastically over the next several decades, maintaining or continuing to improve yield in the midst of global change will be one of the major agricultural challenges in the 21st century.

Impact of ozone on rice and food security in the East and South Asian regions

The negative impact of ambient ozone concentrations on rice plants was first identified and studied in the 1970's in Japan (Nakamura *et al.* 1975; Matsuoka *et al.* 1976). Ambient air pollution in Pakistan and Malaysia, of which ozone makes a significant contribution, was found to have negative impacts on rice yield compared to plants grown in carbon-filtered air (Maggs *et al.* 1995; Ishii *et al.* 2004). In China, ambient levels of ozone as early as in 1999 were already found to cause economically significant yield losses (Feng *et al.* 2003). In Japan, controlled ozone fumigation experiments examining rice biomass partitioning and yield were published on the Japanese varieties 'Koshihikari' and 'Nipponbare' (Kobayashi *et al.* 1995). They found significant decreases in yield as well as individual seed weight under ozone exposure. More recently, the effects of elevated ozone on Chinese rice cultivars under open-air field conditions have been investigated, and significant losses in yield and photosynthetic performance were found, specifically in hybrid lines (Pang *et al.* 2009; Shi *et al.* 2009). Therefore, the case that rice yields in various regions of East, South, and South-east Asia are being negatively impacted by ozone has already been well-established.

Meta-analytic analyses of the existing literature regarding impacts of ozone on rice growth and productivity have found significant decreases in photosynthesis, stomatal conductance, biomass, and yield at current ozone concentrations (Ainsworth 2008; Feng and Kobayashi 2009). By 2020, at the ambient ozone concentrations projected for South and East Asia, ozone-induced yield losses in rice are predicted to reach or possibly exceed 20%.

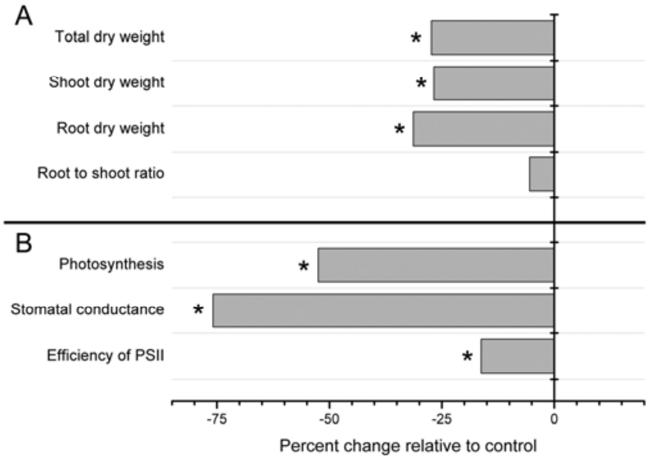

Fig. 1 Effect of ozone on the rice cultivar 'IR64'. 4 week-old seedlings were exposed to 100 ppb O_3 for 7 hr/day for 23 days. (**A**) Dry weight was measured at the end of fumigation, after being dried at 70°C for over 72 hours. (**B**) Photosynthetic gas exchange and chlorophyll fluorescence measurements were taken near midday on Day 21 of fumigation on the second most recently expanded leaves. Asterisks indicate significant treatment effects at $\alpha = 0.05$. N = 4.

Responses of modern tropical rice varieties to ozone

'IR64' is currently the most widely-grown *indica* rice cultivar in the world (Wu *et al.* 2005); however, its sensitivity to ozone has not been tested to date. Therefore, we present here for the first time, the results of a short-term ozone fumigation experiment on 'IR64' plants at the seedling stage to reveal its short-term physiological responses, as well as a season-long ozone exposure to assess impacts on yield.

Month-old 'IR64' seedlings were exposed to moderately high ozone (100 ppb, 7 hrs/day) in open-top chambers for 21 days and photosynthetic gas exchange, chlorophyll fluorescence, and biomass measurements were taken at the end of fumigation. 'IR64' showed very large decreases in photosynthetic activity and biomass under the 3-week ozone exposure (**Fig. 1**). Shoot dry weight and root dry weight decreased by 27 and 31%, respectively, resulting in an overall decrease of total dry weight of 27% (**Fig. 1A**). There was no significant change in the root to shoot ratio. Photosynthetic carbon assimilation rate and stomatal conductance were both greatly lowered under ozone exposure (53 and 76%, respectively; **Fig. 1B**). Chlorophyll fluorescence measurements of the quantum efficiency of Photosystem II also showed a significant decrease of 16% under ozone.

A season-long exposure to ozone was conducted in open-top chambers at a field site northeast of Tokyo, Japan in the summer of 2009, after which the plants were harvested and processed. The average ambient 12-h daily concentration of O_3 at the site over the entire growing season was approximately 38 ppb, and plants were grown under carbon-filtered (mean daily 12-h average of 6.4 ppb), 1.0 × ambient (38.0 ppb), and 2.0 × ambient (71.0 ppb) conditions. 'IR64' showed negative effects at the 2.0 × ambient O_3 treatment but not the 1.0 × ambient O_3 treatment, when compared against the carbon-filtered control (**Table 1**). There was no effect of ozone on panicle number, but panicle weight per

Table 1 Response of 'IR64' to a season-long fumigation of ozone (O_3). Plants were grown in carbon-filtered, 1.0X ambient, and 2.0X ambient O_3, with an average 12-hr. [O_3] of 6.4, 38.0, and 71.0 ppb, respectively. Total exposure period was 97 days. Different letters after each mean indicate treatment differences at $\alpha = 0.05$. N = 4. Unpublished data.

O_3 treatment level	**Daily mean O_3 (12h, ppb)**	**Panicle number**	**Panicle weight (g/plant)**	**Filled grain ratio (%)**	**Kernel weight (mg)**	**Yield (g/plant)**
CF	6.4	6.7 a	20.28 a	85.0 a	29.1 a	11.55 a
1.0 x	38.0	6.5 a	19.90 ab	82.2 ab	28.7 a	11.64 a
2.0 x	71.0	6.9 a	15.94 b	81.2 b	27.8 b	8.68 b

plant decreased by 21%. The proportion of filled grains also decreased from 85% in the carbon-filtered treatment to 81% at 2.0 × ambient O_3. Individual grain weight decreased from 29.1 to 27.8 mg, and overall yield decreased by 25%.

These results confirm that currently grown tropical rice lines show significant sensitivity to ozone in terms of both their short-term physiological and long-term yield response, at O_3 concentrations which are expected in some regions of Asia as soon as 2020 (Wang and Mauzerall 2004).

BREEDING FOR OZONE TOLERANCE

Screening for natural variation in ozone tolerance within crops

Although the necessity to breed ozone tolerant crops to counteract major crop yield losses is recognized, the fact that ambient ozone concentrations are usually spatially and temporally heterogeneous, along with the dose-specific nature of plant responses to ozone, make it quite difficult to implement large-scale and long-term breeding programs in the field (Ainsworth 2008). This may be one of the reasons why only a few breeding programs have been implemented so far, and evaluation of genotypic tolerance is usually based on small-scale chamber experiments where environmental conditions can be controlled. A few such chamber studies have looked into genotypic differences in ozone tolerance within certain agricultural species. Biswas *et al.* (2008a, 2008b, 2009) screened for ozone tolerance (80-100 ppb, 5-21 days) in wheat by determining visible symptoms, photosynthetic parameters, and growth. They found that primitive cultivated wheat varieties were more resistant to ozone than modern wheat varieties, while hybrid varieties and wild wheat varieties were particularly susceptible. Moreover, the genome donors of modern cultivated wheat appeared to contrast in ozone tolerance, *Aegilops taschii* (DD) being the most sensitive, followed by *Triticum monococcum* (AA), while *Triticum turgidium* ssp durum was fairly tolerant. The year in which wheat varieties were released also approximately correlated with ozone tolerance, with the more recently released varieties showing higher sensitivity to ozone. The ambient ozone concentration at the breeding site did not seem to influence ozone tolerance of wheat varieties.

Burkey and Carter (2009) screened for ozone tolerance (80 ppb, 6 days to several weeks) in 30 genotypes of soybeans representing more than 90% of the genetic base of soybeans grown in North America. Ozone sensitivity was determined based on visual symptoms. They found substantial genetic variation in ozone stress tolerance that can be tapped in breeding, and predicted ozone resistance scores for 247 publicly released soybean cultivars based on ancestral resistance.

Ozone tolerance screening in rice based on biomass and visible injury under high ozone concentration has suggested that genotypic variability exists that can be exploited for breeding (Frei *et al.* 2008; Sawada and Kohno 2009). Rice is also the only cultivated crop for which quantitative trait loci (QTL) for ozone tolerance have been reported. Kim *et al.* (2004) screened for genotypic differences in visible symptoms under acute ozone stress (300 ppb, 3 hrs). They mapped QTLs for visible injury using a mapping population derived from the parents Milyang 23 (tolerant) and Gihobyeo (intolerant). QTLs were located on chromosomes 1, 6, 7, and 11 and explained between 4 and 9% of the phenotypic variation. The first QTLs for chronic exposure to ozone (120 ppb, 10 to 15 days) were reported by Frei *et al.* (2008) in a mapping population derived from the parents 'Nipponbare' (intolerant *japonica* type) and 'Kasalath' (tolerant *aus* type). We will now examine the current progress of the chronic ozone QTL mapping in detail and present the newest data from this project.

QTLs for leaf bronzing

Oxidative stress caused by ozone uptake into leaves leads to the formation of necrotic lesions termed leaf bronzing. The quantification of such symptoms by assigning a leaf bronzing score (LBS) is quite straightforward. Thus large populations can be phenotyped efficiently, making LBS an ideal trait for the mapping of quantitative trait loci (QTL) associated with oxidative stress tolerance under ozone exposure. This approach was adopted by Frei *et al.* (2008) in experiments where plants were exposed to ozone (120 ppb, 7 hrs/day) for 10 to 17 days. After screening a diverse set of 23 rice varieties for LBS, a suitable QTL mapping population was selected based on contrasting tolerance of its parent. The *indica* landrace 'Kasalath' exhibited almost no visible symptoms, whereas the *japonica* variety 'Nipponbare' was moderately sensitive.

Subsequent mapping of QTLs using 98 BC_1F_5 backcross inbred lines of 'Nipponbare' and 'Kasalath' revealed four QTLs associated with leaf bronzing (**Table 2**). The effect of these QTLs was tested in chromosome segment substitution lines (SL) carrying introgressions from 'Kasalath' in the genetic background of 'Nipponbare' at the respective QTL positions. This led to the confirmation of two QTLs termed *OzT3* and *OzT9*. These QTLs influenced leaf symptoms in a contrasting manner. At the QTL *OzT3*, located at approximately 2 cM on chromosome 3, 'Kasalath' alleles led to an increase in LBS. Conversely, at the QTL OzT9, located at approximately 18 cM on chromosome 9, 'Kasalath' alleles reduced leaf bronzing. The contrasting phenotype is seen clearly in the corresponding lines SL15 (containing bronzing-sensitive QTL *OzT3*) and SL41 (containing the bronzing-tolerant QTL *OzT9*) (**Fig. 2**). The youngest fully expanded leaves of both SL41 and SL15 did not show any visible symptoms; this is likely due to the low cumulative dose of ozone that the younger leaves received by the end of the ozone fumigation. However, the older leaves of SL15 showed significant leaf bronzing and necrosis starting from the 3rd youngest leaf. Analysis of total leaf ascorbate concentration found similar contrasts in ozone response between 'Nipponbare', SL15, and SL41 (**Fig. 3**).

These two contrasting QTLs were further characterized by transcriptome profiling of the two SLs and their recurrent parent 'Nipponbare' (Frei *et al.* 2010). Gene expression analyses using the Rice Oligo 4x44 Microarray platform (Agilent Technologies) revealed an abundant number of ozone responsive genes (18342 out of 42475 probes showed significant treatment effect). Only genes that showed geno-

Table 2 QTLs detected under ozone exposure for leaf bronzing, stomatal conductance and relative dry matter. Rice plants were exposed to 120 ppb O_3 for 7 hrs/day for 12 days. Marker nearest to the putative QTL is underlined. (Adapted from Frei *et al.* 2008).

Chromosome	Marker interval	QTL	Position (cM)	LOD	R^2	Positive allele
Leaf bronzing score						
3	R1925-R1927	*OzT3*	2	4.2	17.7	'Nipponbare'
4	R1427-C1016	*OzT4*	9	6.1	24.9	'Nipponbare'
5	C246-R521	*OzT5*	89	4.1	17.3	'Nipponbare'
9	C1454-G103	*OzT9*	18	3.3	14.5	'Kasalath'
Stomatal conductance (mmol H_2O m^{-2} s^{-1})						
5	C1268-R1553	*OzSC5*	55	3.2	14.0	'Kasalath'
Relative shoot dry weight (%)						
8	R202 – R2676	*OzT8*	35	4.0	17.7	'Kasalath'

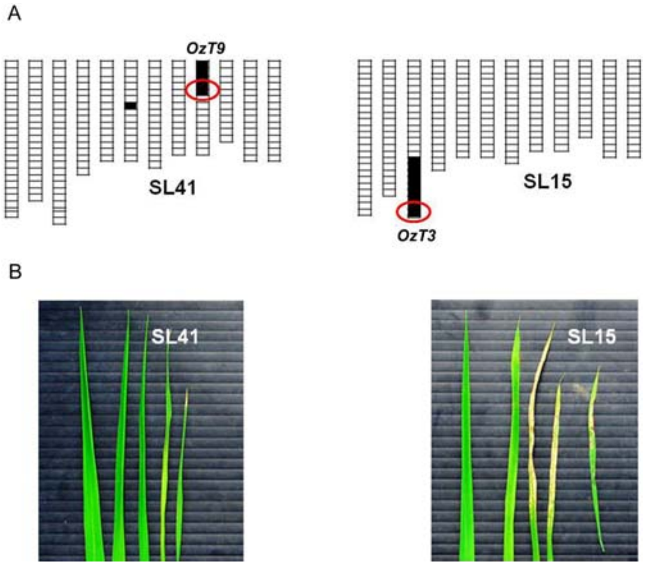

Fig. 2 Substitution lines containing QTLs associated with leaf bronzing under ozone stress. (**A**) Diagram showing the approximate position of 'Kasalath' chromosome inserts (dark bars) within the genome of a leaf bronzing-tolerant substitution line (SL41) and leaf bronzing-sensitive line (SL15). The approximate location of the QTLs identified in Frei *et al.* (2008) is marked within red circles. (**B**) Digital color images of leaves of both lines after ozone exposure. The left-most leaves are the youngest fully expanded, and the right-most leaves are the oldest.

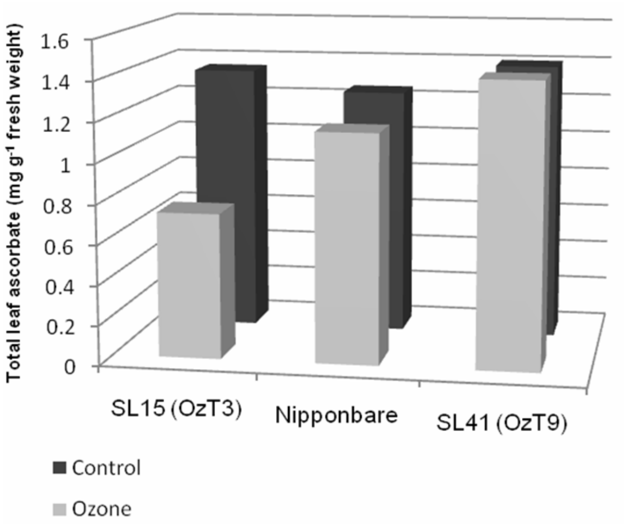

Fig. 3 Total leaf ascorbate concentration in 'Nipponbare', SL15, and SL41. Ascorbate was assayed after 4 days of exposure to ozone (120 ppb, 7 hr/day). Dark bars are control, light bars are ozone treatment. Data adapted from Frei *et al.* (2008).

typic differences in expression were further investigated, focusing on genes belonging to three different functional categories: (i) genes involved in programmed cell death, (ii) genes involved in biosynthesis of antioxidants, and (iii) genes involved in enzymatic ROS detoxification. This approach yielded a number of differentially regulated genes in the categories (i) and (iii), but none in the category (ii). Among these differentially regulated genes, possible tolerance genes were selected based on gene annotation, physical position, and expression pattern.

The most promising candidate gene identified by this approach encoded a putative ascorbate oxidase (AO). It was determined to be a strong candidate gene for various reasons: (i) it was located within the introgression of SL41 at the QTL *OzT9*; (ii) it was highly responsive to ozone and showed more than 30-fold up-regulation; (iii) it showed consistently lower expression in SL41 across experimental conditions, independent time points and independent experiments; (iv) low AO activity had previously been associated with ozone tolerance in spinach (Sanmartin *et al.* 2003); (v) its expression pattern logically explained the whole leaf and apoplastic ascorbate status in contrasting genotypes – SL41 maintained a higher level of reduced ascorbic acid when exposed to ozone and had a higher concentration of apoplastic ascorbic acid.

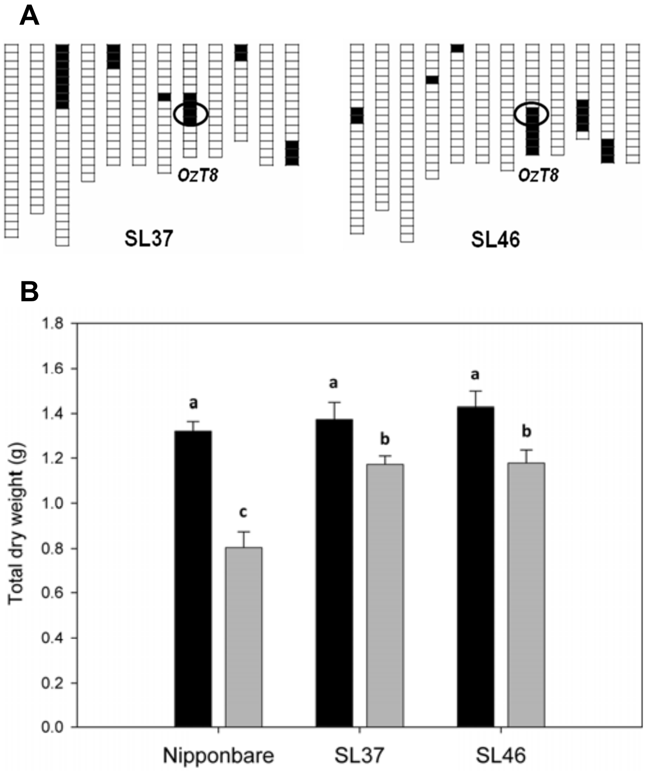

Fig. 4 Comparison of biomass response to ozone stress between 'Nipponbare' and two substitution lines containing the QTL *OzT8*. (**A**) Chromosome maps of SL37 and SL46, using the same notation as in **Fig. 2**. (**B**) Total dry weight of 'Nipponbare', SL37, and SL46 seedlings after exposure to 100 ppb O_3 for 7 hr/day for 23 days. Error bars are standard error of the mean. Different letters above each bar represent differences at the $\alpha = 0.05$ level. N = 4.

Confirmation of a QTL conferring ozone tolerance in dry weight accumulation

Another QTL from Frei *et al.* (2008) which shows potential for breeding is *OzT8*, which is associated with relatively higher dry weight under ozone exposure (**Table 2**). **Fig. 4** shows confirmation of the QTL via comparison of the dry weight of 'Nipponbare' under control and ozone treatments and two substitution lines, SL37 and SL46, which both contain chromosome inserts from 'Kasalath' containing the *OzT8* locus. Exposure to ozone for 3 weeks produced a clear treatment effect in all lines. Although there was no significant difference between any of the genotypes under control conditions, both SL37 and SL46 showed higher dry weight than 'Nipponbare' in the ozone treatment. Recent work has determined that this difference in biomass under ozone stress can be attributed to higher biochemical capacity of photosynthesis (i.e., maximum carboxylation rate of Rubisco and maximum electron transport rate) in the lines containing the *OzT8* locus (Chen *et al.* 2011). Since the presence of *OzT8* results in less biomass loss under ozone stress, there is a possibility that it may also result in effects in yield over the course of the entire growing season.

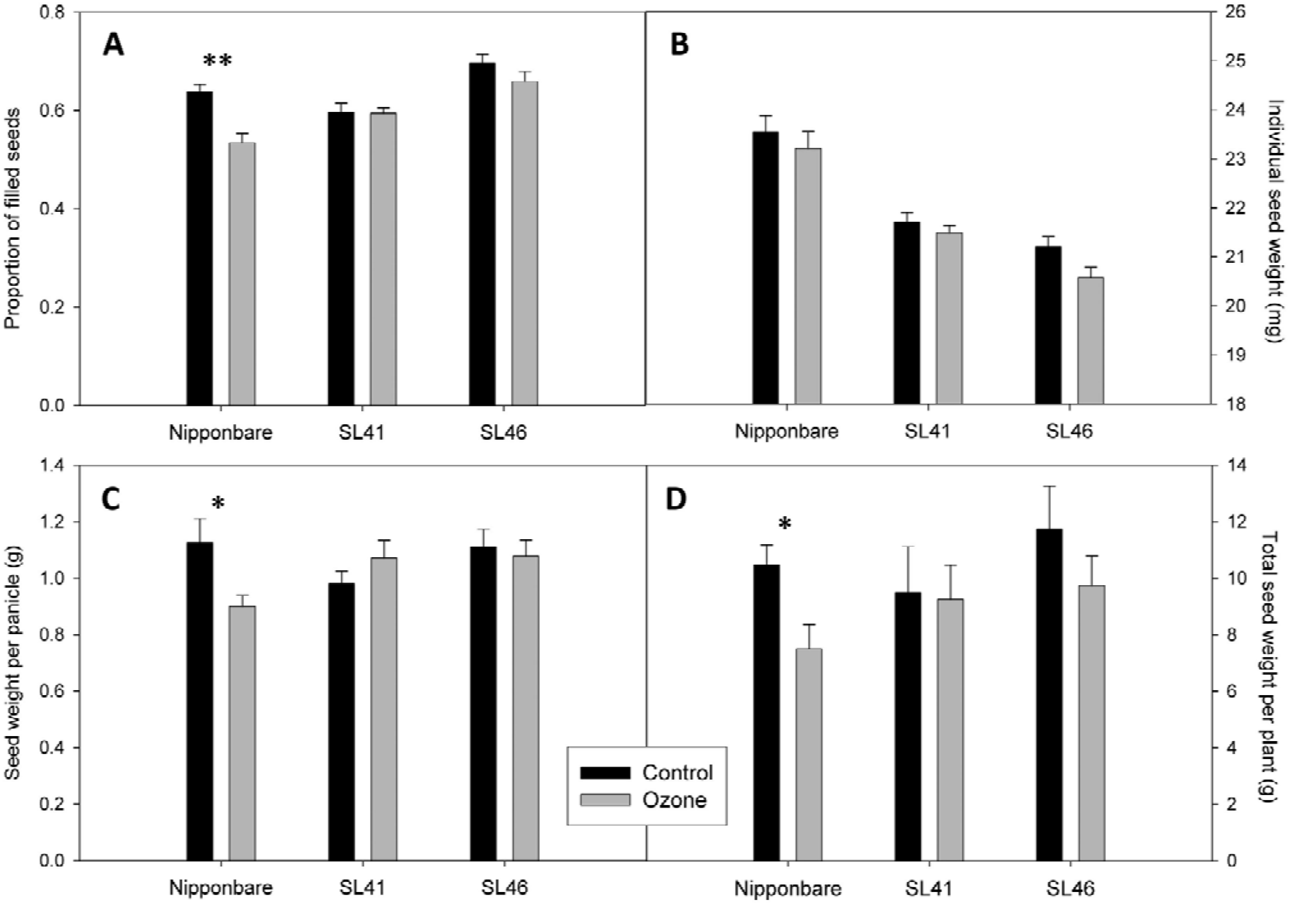

Fig. 5 The effects of ozone on 3 rice lines ('Nipponbare', SL41, and SL46) during reproductive phase. The plants were exposed to 100 ppb O_3 for 7 hr/day for the entire period from heading to grain maturity. (**A**) Proportion of filled spikelets (seed set). (**B**) Average weight of individual seeds. (**C**) Seed weight per panicle. (**D**) Total seed weight per plant. Error bars are standard error of the mean. Asterisks indicate significant treatment difference (*: $p < 0.05$; **: $p < 0.01$). N = 8.

Effect of ozone on rice during the reproductive stage

To test the effects of ozone stress at the reproductive stage in rice and ultimately on yield, we grew 3 lines ('Nipponbare', SL41 containing the tolerance QTL *OzT9*, and SL46 containing *OzT8*) under ambient conditions up until the heading stage. Then, from heading until grain maturity, we exposed the plants to 100 ppb O_3 for 7 h d^{-1} inside growth cabinets. After grain maturity and harvest, yield characteristics were measured.

There was a significant effect of ozone exposure during the reproductive stages on rice fertility and yield (**Fig. 5**). In 'Nipponbare', there was a significant decrease in the percentage of filled seeds, weight of seeds per panicle, and total seed weight per plant (**Fig. 5A, 5C, 5D**). However, SL41 and SL46, the two genotypes containing the ozone tolerance QTLs *OzT9* and *OzT8*, respectively, did not show significant decreases in any of these yield characteristics. Individual seed weight appeared to decrease slightly for all genotypes under ozone, but this was not statistically significant (**Fig. 5B**). These results show that even in rice cultivars which are considered relatively tolerant to ozone, such as 'Nipponbare', further gains in yield under stress conditions can be achieved if QTLs related to tolerance are selected for.

MECHANISM-BASED TARGETS FOR OZONE TOLERANCE BREEDING

Another approach to developing ozone-tolerant crops is to target particular genes related to known mechanisms of ozone response and tolerance.

Antioxidants in ozone defense

Detoxification of reactive oxygen species (ROS) is a major tolerance mechanism that can be targeted in the breeding of ozone resistant cultivars of crops. Plants possess a broad range of antioxidants (reviewed by Blokhina *et al.* 2003), including antioxidant enzymes (such as superoxide dismutase, peroxidases, catalase, etc.) and non-enzymatic antioxidants (such as ascorbic acid, glutathione, tocopherol, etc.). Many of these components of the antioxidant system are responsive to ozone or have been shown to be involved in ozone tolerance. Due to the abundant wealth of literature on this topic, the following sections will focus on studies employing gene suppression or over-expression to establish definite links between certain antioxidant genes and ozone tolerance.

The ascorbic acid metabolism pathway (**Fig. 6**) constitutes the core of plant's ROS detoxification system. It consists of three general sections, all of which have previously been shown to be involved in ozone tolerance:

(i) Biosynthesis of ascorbic acid from its precursor glucose-6-P via various enzymatic conversion steps. Numerous studies have documented that total foliar ascorbic acid level correlates with ozone tolerance in plants (e.g. Lee 1991; Robinson and Britz 2000; Burkey and Eason 2002). Taking advantage of this correlation, Conklin *et al.* (1996) used foliar injury of *Arabidopsis thaliana* mutants exposed to high ozone concentration as a simple phenotypic trait that allowed for rapid selection of mutants that were deficient in ascorbic acid. This approach not only demonstrated the importance of ascorbic acid biosynthesis for ozone tolerance, but even more importantly led to the identification of the genes involved in the ascorbic acid biosynthetic pathway in

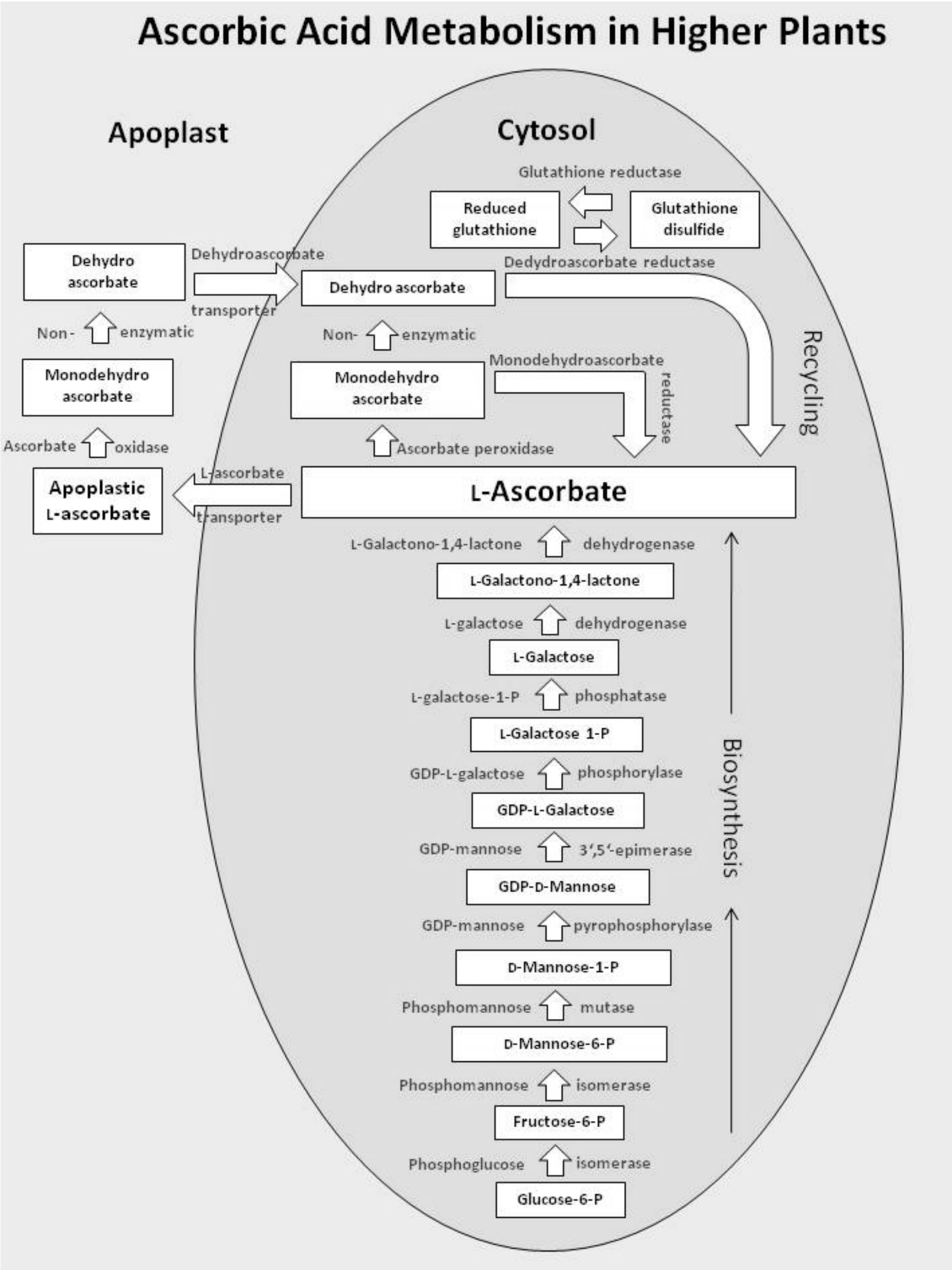

Fig. 6 Diagram of ascorbic acid metabolism in higher plants. Illustration based on pathways described in Noctor and Foyer (1998) , Conklin and Barth (2004), and Dowdle *et al.* (2007); substrates are shown in white boxes and enzymes are shown in gray font; some pathways have been slightly simplified to improve readability.

higher plants as indicated in **Fig. 6** (Wheeler *et al.* 1998; Smirnoff and Wheeler 2000; Conklin 2001; Ishikawa *et al.* 2006).

(ii) Peroxidation and recycling of ascorbic acid in the ascorbate-glutathione cycle, mediated by the enzymes ascorbate peroxidase (APX) monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR). Genes encoding for certain isoforms of these enzymes have been cloned in plants and have been characterized regarding their involvement in ozone tolerance. Although suppression of APX led to increased ozone susceptibility in *Nicotiana tabacum* (Orvar and Ellis 1997), overproduction of APX in the chloroplasts did not confer enhanced tolerance in transgenic tobacco exposed to both chronic and acute ozone stress (Torsethaugen *et al.* 1997). Similarly, overexpression of GR did not improve ozone tolerance in transgenic *Populus tremula* (Strohm *et al.* 1999). In contrast, over-expression of the ascorbate recycling enzymes MDHAR and DHAR did consistently improve ozone tolerance in several experiments with *Arabidopsis thaliana* or *Nicotiana tabacum* (Chen and Gallie 2005; Eltayeb *et al.* 2006; Yoshida *et al.* 2006; Eltayeb *et al.* 2007).

(iii) Transport of ascorbic acid to the apoplast, apoplastic oxidation, and re-transport to the cytosol. There is general agreement that the concentration of apoplastic ascorbic acid, especially in its reduced form, constitutes a crucial ozone tolerance factor in plants (Conklin and Barth 2004). Various authors suggested that apoplastic ascorbic acid level responded to ozone exposure (Conklin and Barth 2004; Frei *et al.* 2010). However, it remains unclear whether adaptations in apolastic ascorbic acid were the result of active transport, diffusion through membranes, or exchange of reduced ascorbate and dehydroascorbate. Moreover, genes controlling cross-membrane transport remain to be characterized in plants. Slightly more information is available on ascorbate oxidase (AO). The function of this apoplastic enzyme remained unclear for a long time, but more recently

AO has been proposed to facilitate leaf expansion by removing reduced ascorbate from the apoplast to enhance cell elongation (Pignocchi *et al.* 2003). Its involvement in ozone tolerance was first demonstrated by Sanmartin *et al.* (2003), who overexpressed the enzyme in *Nicotiana tabacum* and found increased ozone susceptibility in transgenic plants. In agreement with these results, Frei *et al.* (2010) proposed reduced expression of a putative AO gene as a mechanism of ozone tolerance in rice.

Apart from the ascorbic acid metabolism, only a few other antioxidants have been shown to be directly involved in ozone tolerance. Over-expression of superoxide dismutase led to enhanced ozone tolerance in *Nicotiana tabacum* in several independent studies (van Camp *et al.* 1994; Pitcher and Zilinskas 1996). Other antioxidant enzymes such as peroxidases or catalases are responsive to ozone, but direct evidence of their involvement in ozone tolerance using gene suppression or over-expression is lacking to date. The same is true for biosynthesis of non-enzymatic antioxidants such as glutathione (Noctor and Foyer 1998), or tocopherol (Burkey *et al.* 2000) that do not seem to correlate with ozone tolerance.

CONCLUDING REMARKS

To date, there is still a surprising scarcity of literature regarding ozone stress and tolerance in rice, especially at the genetic and molecular level. Currently, only two microarray studies (Cho *et al.* 2008; Frei *et al.* 2010) and two QTL mapping studies (Kim *et al.* 2004; Frei *et al.* 2008) have been published. To our knowledge, no transgenic rice lines have been developed with the particular purpose of improving tolerance to ozone stress. With the completion of the rice genome in 2005, as well as the advances in the various "-omics" and genetic/molecular tools, there is great opportunity for further progress in our understanding of stress tolerance in rice.

However, the question of what defines "tolerance" or "sensitivity" to ozone needs to be given full consideration when studying ozone stress tolerance. The criteria that one chooses to evaluate sensitivity or tolerance is of key importance when planning an ozone fumigation experiment. Studies which have screened rice lines for ozone sensitivity have found that the relative tolerance ranking of lines varies depending on which dependent variable or phenotype is chosen. For example, Frei *et al.* (2008) screened 23 rice lines of different subspecies and regions and found that there was no significant correlation between leaf bronzing score and relative dry weight accumulation; many lines which showed significant visual foliar symptoms did not have a corresponding large decrease in biomass compared to their controls. Therefore, although the use of leaf bronzing scoring is a convenient method of screening large numbers of plants, the results of studies which have characterized ozone tolerance solely on the basis of visual foliar symptoms may not always be applicable to the goal of improving yield of rice grown under season-long high [O_3] conditions.

The type of ozone exposure (duration, intensity, and frequency) is another factor which often differs between studies. Up until recently, short-term, acute O_3 exposures have dominated laboratory-based studies, which sought to elucidate the molecular basis of plant responses to O_3, with the aim of understanding how to adapt plants to rising [O_3] pollution (reviewed by Kangasjarvi *et al.* 2005). However, some studies have indicated that acute and chronic ozone exposure trigger different physiological response mechanisms at different time scales (Pell *et al.* 1997; Long and Naidu 2002; Chen *et al.* 2009). The complex temporal dynamics of tropospheric ozone in the field indicate that we may need data from both long-term and short-term experiments in order to fully unravel the mechanisms of ozone damage in rice and effectively develop stress-tolerant lines in the future.

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