Global identification of potential gene biomarkers associated with ozone-induced foliar injury in rice seedling leaves by correlating their symptom severity with transcriptome profiling

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ABSTRACT

A combined approach of evaluating ozone (O₃)-caused foliar injury symptom and global gene expression profiling was used to identify potential genes associated with severity of injury on leaves of O₃ (200 ppb)-fumigated (1, 12, 24, 48, and 72 h) two-week-old rice (cv. Nipponbare) seedling. Foliar injuries were evaluated up to 72 h using both qualitative visual scale and quantitative RGB (red-green-blue) image analysis methods. The (R-G)/(R+G) was an optimal quantitative RGB parameter to assess foliar injury. Large-scale transcript profiling of leaves identified 270 genes linked with foliar injury. Genes were subjected to Pearson’s correlation test between their expression changes and O₃ concentrations. Correlation coefficient below -0.80. Expression of 146 genes was found to increase with increased foliar severity up to 72 h, showing a positive, tight correlation between a subset of gene expression and commonly observed O₃-triggered symptom of foliar injury with correlation coefficient below -0.80. Of 146 genes, genes involved in metabolism (25%) formed a major functional category. Metabolic networks with identified metabolic genes provided insight into cellular responses such as photorespiration, biosynthesis of secondary metabolites, and detoxification. The O₃ effect on these cellular responses has been previously reported based on physiological and biochemical studies, validating our approach used in this study to globally identify O₃-responsive biomarkers tightly linked with foliar injury symptom. This study provides evidence for presence of large number of genes associated with foliar injury symptom than thought before, serving as a resource of potential biomarkers to study mechanisms of visible injury development by O₃.

Key words: DNA microarray; Foliar injury; Metabolic pathways; Ozone; Rice

INTRODUCTION

Tropospheric ozone (O₃) is one of the notorious environmental gaseous pollutants, produced by photochemical reactions between volatile organic compounds and nitrogen oxides (Varotsos et al., 2004). Since late 1970s, the annual increase in nitrogen oxides due to combustion of fossil fuels for industrialization has been a major factor in global O₃ increment (Fusco and Logan, 2003). Climate models forecast that average level of ground O₃ will reach phytotoxic range in the future with greater increase in its concentration in Asia, Africa and USA, where major crops such as rice, wheat, corn and soybean are cultivated (Dentener et al., 2006; Emberson et al., 2009; Wang and Mauzerall, 2004). Currently, O₃ concentrations sometimes exceed a threshold level to cause visible foliar injury by diurnal fluctuations of O₃ concentration (Kley et al., 1999; Yoshikado, 2004). For example, according to a database of NIES (Japan) maximum hourly oxidant concentrations exceeded 200 ppb at 29 monitoring sites in the Kanto region surrounding Tokyo in 2005.

Acute O₃ exposure in plants causes foliar symptoms such as leaf necrosis and chlorosis (terms for foliar injury), accompanied with damage to cellular membranes and accumulation of pigments in injured/dead cells (Omasa et al., 2002). The foliar injury results in reduction of photosynthesis leading to reduced plant growth rate and productivity in some cases (Omasa et al., 2002). On the other hand,
the ever-increasing concentration of atmospheric O₃ poses a great threat to yield and quality of the global socio-economic crops, chronically. Indeed, the yield of crops such as rice, wheat, maize, barley, soybean, bean and potato is remarkably reduced depending on elevating O₃ concentrations compared to the basal O₃ level (Feng and Kobayashi, 2009; Feng et al., 2008; Heagle, 1989; Kobayashi and Okada, 1995; Morgan et al., 2006; Wang and Mauzerall, 2004; Yonekura et al., 2002). A previous study reported that O₃ sensitivity of rice cultivars evaluated by visible injury in open-top chambers in the field did not coincide with that evaluated by the reductions in grain yield, but the results are preliminary and need further validation (Sawada and Kohno, 2009). However, to understand mechanisms of O₃ effects on plants, it would be worth clarifying plant responses related to visible injury formation among various molecular responses caused by O₃. Furthermore, it would be worth evaluating the extent of O₃-induced visible foliar injury precisely in the well-controlled experimental conditions using an indoor growth cabinet, since symptoms similar to O₃-induced injury could appear without O₃ in the field.

Recently, high-throughput omics approaches (transcriptomics, proteomics and metabolomics) were applied to catalog the O₃-responsive gene, protein or metabolite components in aspen (Bohler et al., 2007; Gupta et al., 2005), birch (Kontunen-Soppela et al., 2007; Ossipov et al., 2008), pepper (Lee and Yun, 2006), Arabidopsis (Mahalingam et al., 2006; Tamaoki et al., 2003), bean (Torres et al., 2007), maize (Torres et al., 2007), rice (Agrawal et al., 2002; Cho et al., 2008) and wheat (Sarkar et al., 2010). Although these, along with targeted studies, continue to refine our understanding on the O₃-constitutive and -triggered signaling and metabolic responses, global identification of genes tightly associated with the symptoms of foliar injury in natural and cultivated plants remain largely unknown. The need to address this ‘black-box’ of hidden gene information is critical as it will further our knowledge on link between O₃-triggered morphological change (i.e., foliar injury) and gene expression in plants. We believe that if foliar injuries uniquely progressed by O₃ exposure time in all the cultivars, we might be able to investigate biomarkers based on the time point of O₃ exposure. Undesirably, the severity of O₃-induced foliar injury is different depending on various conditions including rice cultivar-type, O₃ concentration, exposure time and pattern, etc. Therefore, we selected and performed transcriptomics analysis (via DNA microarray array) based on the severity of O₃-induced foliar injury in a model crop rice (Oryza sativa L.).

Keeping in mind the rationale of our study, we have embarked on global identification of genes associated with foliar injury symptoms caused by acute O₃ exposure with the following objectives: (i) to establish a reproducible quantitative method to evaluate the degree of O₃-caused foliar injury symptoms in rice leaf; (ii) to perform a large-scale time-index study of gene expression in leaves manifesting foliar injury as per their increased severity; (iii) to apply a robust correlation test to select those genes whose expression is tightly associated with increased severity of foliar injury; and (iv) to exploit identified potential gene biomarkers to begin reconstructing the metabolic networks. For this purpose, we have selected rice japonica cv. Nipponbare as a model system with socio-economic importance (Agrawal and Rakwal, 2006). Briefly, two-week-old rice seedlings were exposed to O₃ (200 ppb) and leaves collected at multi-time points were used for evaluating the severity of foliar injury and large-scale transcript profiling. This study reports major achievements toward identification of potential gene biomarkers associated with O₃-triggered foliar injury in rice seedling with dynamic expression profiles of 146 genes positively linked with increased foliar injury symptoms through 72 h and metabolic networks associated with O₃-triggered foliar injury.

MATERIALS AND METHODS

Plant materials and O₃ fumigation

Rice (cv. Nipponbare, Japonica-type) seeds were sterilized with four-fold-diluted sodium hypochlorite solution (WAKO, Tokyo, Japan) and imbibed in water for three days at 25°C to germinate. Germinated seeds were grown in soil (nursery soil for rice seedling growth and transplantation, JA Zen-Noh, Japan; http://www.zennoh.or.jp/) in a growth chamber with 12 h photoperiod white fluorescent light with 400 µmol photosynthetic photon flux density (PPFD) m⁻²s⁻¹ at 25°C and 70% relative humidity. Two-week-old rice seedlings were exposed to O₃ (200 ppb) or filtered air in an O₃ fumigation chamber equipped with charcoal filters up to 72 h. The conditions of O₃ fumigation chamber were at 25°C with a relative humidity of 70% and a wind velocity of 0.22 m/s under the continuous light from metal halide lamps with 400 µmol PPDF m⁻²s⁻¹. The 3rd leaves were collected precisely after O₃ exposure for 0, 1, 3, 6, 12, 24, 48 and 72 h. These leaves were used immediately to assess the O₃-induced foliar injury.
injury symptoms. For the pure-air (filtered air control) 1, 12 and 24 h samples were also collected. To profile gene expression, the collected leaves were frozen immediately with liquid nitrogen and stored at -80°C. A total of three biological replicates (n=3) were performed for the O₃ fumigation and corresponding control experiments.

**Assessment and scoring of foliar injury**

The O₃-induced foliar injury was observed on 3rd leaves and scored from 0 through 5 (Figure 1) according to the qualitative visual scale method used previously in maize and soybean crop plants (Torres et al., 2007). Besides this, a quantitative RGB (red-green-blue) image analysis (Fei et al., 2008, 2009; Karcher and Richardson, 2003; Kawashima and Nakatani, 1998) was also performed on the same leaves by taking their digital images with a professional digital camera (Sony, Tokyo, Japan) (Figure 1). A fixed distance of 15 cm was kept between the camera and leaf during acquisition of all images as JPEG format with 1200 dpi resolution. These images were used to determine the RGB color intensities from the average histogram value of an acquired leaf digital images using Adobe Photoshop Elements 7 software (Fei et al., 2008, 2009). The RGB color intensities were then employed to calculate the value of G/R, G/L (luminosity), (R-G)/(R+G), R/(R+G+B) and G/(R+G+B) parameters alone or in relation with the score assigned using the qualitative visual scale method (Supplementary Table 1, Figure 2A) and the time of O₃ fumigation (Supplementary Table 2, Figure 2B). For both qualitative and quantitative evaluation methods of the foliar injury, a total of five 3rd leaves per biological replicate were used at each time point [0, 1, 3, 6, 12, 24, 48 and 72 h O₃ (or filtered air at 1, 12 and 24 h) fumigation].

**Total RNA extraction and transcriptomics analysis**

Samples collected from each biological replicate were pooled according to same treatment condition. Total RNA was extracted from the pooled samples, which were exposed to O₃ for 0 (reference set), 1, 12, 24, 48 and 72 h or to filtered air for 1, 12 and 24 h, using the QIAGEN RNeasy Plant Mini Kit (QIAGEN, Maryland, USA) according to the manufacturer’s instruction. To verify the quality of the isolated RNA, the yield and purity were determined spectrophotometrically (NanoDrop, Wilmington, DE, USA) and confirmed using formaldehyde-agarose gel electrophoresis. Microarray experiment at each time point was designed for using a dye-swap method (Figure 3) and a rice 22K custom oligo DNA microarray chip (G4138A, Agilent Technologies, Palo Alto, CA, USA) containing 21,475 oligonucleotides. The same total RNA (400 ng) samples were labeled twice with Cy3 or Cy5 according to a dye-swap procedure using an Agilent Low RNA Input Fluorescent Linear Amplification Kit. As shown in Supplementary Figure 2, a Cy5-labeled treatment (T⁵Cy) and a Cy3-labeled reference (C⁵Cy) was hybridized on a slide and then a Cy3-labeled treatment (T³Cy) and a Cy5-labeled reference (C³Cy) were reversely hybridized on another to revise the dye bias associated with unequal incorporation of two Cy dyes into cRNA (Martin-Magniette et al., 2005). The hybridized microarray slides were scanned using a GenePix microarray scanner (Molecular Devices, Sunnyvale, CA, USA). Signal ratios of each spot on all slides were normalized by using LOWESS (locally weighted linear regression) correction in the Genepix ver. 4.0 quantitative microarray analysis application program (Axon Instruments, Union City, CA, USA; Kimura et al., 2006). Finally, log ratio of each spot for technical replicates was averaged to give a representative value for each pooled sample and to adjust remaining gene-specific dye bias (Zieker et al., 2005). For the filtered-air exposure for 1, 12 and 24 h experiment, the Agilent microarray scanner G2565BA was used along with the Agilent Feature Extraction ver. 8.1.1.1 software (Agilent Technologies, Palo Alto, CA, USA) for image analysis and generation of datasets.

The data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus (GEO) and are accessible through GEO Series accession number GSE11157 (see also Cho et al., 2008; where the data, 1 to 24 h was used for a different analysis and study) and GSE34256(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE34256).

**Correlation of the O₃-responsive genes with O₃-induced foliar injury**

To find potential candidate genes tightly linked with the O₃-induced foliar injury, genes meeting two criteria were selected. RGB image analysis showed no visible injury at 1 h fumigation of O₃ and 1, 12 and 24 h filtered-air fumigation, and severe foliar injury at 12, 24, 48, and 72 h fumigation of O₃ (Figure 1). Based on the results, we made first criterion: expression change of a gene after O₃ fumigation for 1 h and filtered-air for 1, 12 and 24 h is not observed [-1 < average log ratio of a gene (M) > 1],
and that after fumigation of O$_3$ for 12, 24, 48 and 72 h is observed [-1≥M or M≥+1] (Supplementary Table 3). Secondly, mean of M values of a gene at O$_3$ fumigation for 12, 24, 48 and 72 h is significantly different compared to that of the gene at O$_3$ for 1 h and filtered air for 1, 12 and 24 h (Supplementary Table 4). Further, under the second criterion, every gene was subjected to correlation test to select only those genes whose expression level is positively or negatively linked with increased severity of the foliar injury (Supplementary Table 4). The correlation test was performed to calculate the correlation coefficient (R) between the transcript level (M value) of each gene and the relative (R-G)/(R+G) value of foliar injury at each time point (at fumigation of O$_3$ for 1, 12, 24, 48 and 72 h, and filtered air for 1, 12 and 24 h) using EXCEL program (Supplementary Table 4, and see example in Supplementary Figure 1). Genes with high correlation coefficient (≥ 0.8 or ≤ -0.8) were selected.

### Bioinformatics analyses

The selected genes were functionally categorized using NCBI database and a MapCave tool (http://mapman.gabipd.org) linked with *Oryza sativa* TIGR5 annotation database. Based on the annotated function of genes, metabolite pathway were drawn using rice pathway database (Rice Cycver 3.2, http://www.gramene.org/pathway) and enzyme database (http://www.brenda-enzymes.info/index.php4) linked with KEGG pathway database (http://www.genome.jp/kegg/pathway.html) and Meta Cyc database (http://metacyc.org/).

### RESULTS AND DISCUSSION

**Determining a reproducible, quantitative RGB parameter suitable for assessing O$_3$ triggered foliar injury**

The O$_3$-induced foliar injury was investigated on 3rd leaves of rice seedlings up to 72 h (Figure 1). In general, the leaf tip was turned to curl up after 6 h O$_3$ fumigation. Silvery speckles started to appear near veins on leaf surface at 12 h, followed by clearly visible symptoms of tiny reddish-brown spots, accompanied with chlorosis and yellowing. These symptoms progressed gradually with increased O$_3$-exposure time, covering almost the entire leaf surface by 72 h. The severity level of observed foliar injuries was scored from 0 through 5 as per the commonly used qualitative visual score method (Figure 1). Previously, the same index scale was used for the assessment of O$_3$-triggered foliar injury in maize and soybean plants (Torres et al., 2007). As the qualitative visual scale method is usually prone to significant deviation and observer specific, a robust and quantitative RGB image analysis was applied to evaluate the foliar injury on leaves. The RGB image analysis is mainly based on the measurement of color intensities in the image of sampled material. The RGB image analysis has previously been applied in natural and cultivated plants to measure physiological symptoms of damaged leaves (Adamsen et al., 1999; Fei et al., 2008, 2009; Karcher and Richardson, 2003; Kawashima and Nakatani, 1998). Yet, the RGB image analysis has not been used to quantitatively assess the O$_3$-caused foliar injury in rice leaf, to the best of our knowledge. The G/R, G/L, (R-G)/(R+G), R/(R+G+B) and G/(R+G+B) parameters of RGB images were determined using the Photoshop program. All RGB parameters showed high correlation coefficient and linear relationship with the assigned index scale (0 to 5 scales) using the qualitative visual scale method (Supplementary Table 1).

However, the (R-G)/(R+G) parameter had the widest relative range with high linear correlation ($R^2$ = 0.97, Figure 2A). Similar results were obtained when the calculated values of each RGB parameter were drawn against the O$_3$ fumigation time.
In this relationship, relative (R-G)/(R+G) values showed the widest range and good curve-fitting with an exponential function of $Y = 0.9881e^{-0.021X}$ and $R^2=0.9711$ (Figure 2B). The result represents that change of (R-G)/(R+G) parameter is not observed in rice leaves exposed to filtered air through 72 h and $O_3$ through 6 h but in $O_3$-fumigated rice leaves for 12 h, 24, 48 and 72 h (Figure 2B), suggesting that (R-G)/(R+G) is the optimal and well-suited RGB parameter for quantitative assessment of the foliar injury symptoms of rice seedlings.

Identification of genes with expression profiles directly proportional to the severity level of foliar injury

To analyze the expression patterns of all genes according to foliar injury in rice seedlings fumigated with $O_3$ or filtered air, microarray experiment was designed as Figure 3. The expression changes of the genes are provided as Supplementary Table 3. Their quantitative expression data were subjected to two rigorous selection criteria to identify only those genes that were tightly associated with increased foliar injury symptoms during progression of $O_3$ fumigation. In other words, transcriptional analysis was strictly performed based on severity of $O_3$ fumigation. RGB analysis showed that there is no phenotypic difference between control and 1 h $O_3$-exposed leaves. Hence, the first criterion was applied to screen genes whose expression is matched with foliar injury derived at each $O_3$ exposure time. The first criterion was that expression levels of genes must be down- or up-regulated over 2-fold by $O_3$ fumigation for 12, 24, 48, and 72 h but should not change at 1 h of $O_3$ fumigation and after 1, 12, and 24 h of filtered air (i.e. control), and this resulted in selection of 270 genes (Supplementary Table 3). It should be mentioned that no foliar injury was observed under filtered air condition through 72 h. Given this, the microarray control experiment was still performed up to 24 h to obtain confident and significantly up- and down-regulated $O_3$-responsive genes over control (filtered air). Further, these 270 genes were subjected to the correlation test, 147 genes (Table 1) passed the test with high correlation coefficients (below-0.80 or above 0.80) (Supplementary Table 4). The correlation test was performed between changes in the average transcript level ($1/2[\log_2 F_{1st} – \log_2 F_{2nd}]$) of each gene and the relative value of (R-G)/(R+G) image parameter at each time point (second criterion). Out of 147 genes, expression levels of 146 genes showed negative correlation with the relative (R-G)/(R+G) value at each time point, indicating that 146 genes are positively correlated with foliar injury severity. These genes were grouped into 11 functional categories (Figure 4, Table 1).

Despite our search of annotated gene functions against the NCBI database and Oryza sativa TIGR5 annotation database, genes with no known function (through writing of this paper) constituted the largest category (unknown; 32%), followed by metabolism (25%), transport (8%), protein synthesis and turnover (6%), defense (6%), signaling (6%), mitochondrial electron transport (4%), protein...
secretory pathway (4%), antioxidant (4%), transcription (4%), and others (3%). This result indicates that functionally diverse genes are associated with the foliar injury symptoms of rice seedlings. Although defense-related genes were not the major gene category among the 146 genes here, two genes (AK061606, AK071613) were identified similar to PBZ1 (probenazole-inducible protein 1; Kim et al., 2011, and references therein), which is well known as one of the most highly O₃-responsive genes in the dataset (see also Supplementary Table 4). The PBZ1 gene has been usually annotated as major pollen allergen Bet v 1-D/H or pathogenesis-related protein 10 (PR10) in various databases to date. Recently, a PR10 family protein with RNase activity was shown to play a key role in cell death in rice and other plants (Kim et al., 2011).

**The O₃ stress relays molecular events causing visible foliar injury symptoms**

To obtain insight into the molecular events linked with the O₃ foliar injury, 146 genes were broadly grouped as per their functional annotation into three major classes: signal transduction, transcription, and metabolism. The signal transduction group include done gene encoding a calcium binding protein, caltractin (AK074019), two genes encoding a gibberellin receptor (AK109437) and a glutamate receptor (AK072337), and six genes encoding a protein phosphatase 2C isoform epsilon (AK071637), a receptor serine/threonine kinase (PR5K, AK110482), a lectin receptor-type protein kinase (AK105111), a BRASSINOESTEROID INSENSITIVE 1-associated receptor kinase 1 (AK070798), an inositol 1, 3, 4-trisphosphate 5/6-kinase family protein (AK102571) and a calcium-dependent protein kinase (AK066615) involved in regulating relevant responses and developmental processes through dephosphorylation and phosphorylation event of target proteins. Five genes belonged to the transcription category; a splicing factor (AK069120), two bZIP transcription factors (AK106988, AK067919), a DNA-binding protein (AK068593) possessing a basic helix-loop-helix motif, and one unclassified transcription factor (AK109494). Furthermore, one gene encoding a translation initiation factor (AK103270) and eight genes encoding a proteasome subunit (AK061383), five components of ubiquitination pathway (AK508717, AK064106, AK071849, AK105227, AK111781), one proteinase (AK100351), and a beclin-1-like protein (AK099664) involved in protein degradation process were categorized into the translation and protein turnover group (Table 1).

Gene expression of several transporters including amino acid and peptide transporters were up-regulated along with the increased severity of visible foliar injury. Transcript levels of glutathione-S-
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Glycolate oxidase (AK068638) was identified as a foliar injury-associated gene. The glycolate oxidase (AK068638) has 96% similarity to maize glycolate oxidase 1, which prevents the accumulation of toxic glycolate rather than to lead photosynthetic pathway (Peterhansel et al., 2010; Zelitch et al., 2009). A deficiency of maize glycolate oxidase 1 caused the accumulation of glycolate and seedling death (necrosis) in normal air condition.

Out of the selected genes related to cell wall and membrane modification and starch mobilization, two genes were found to encode proteins for genes for polysaccharide degradation such as alpha-glucosidase (AK105449), releasing glucose from alpha-(1,4)-linked glucose polymers (Chang et al., 2000) and chitinase (AK070067), hydrolyzing beta-(1,4)-N-acetyl-D-glucosamine linkages in chitin, a main component of cell wall (Nakazaki et al., 2006). Moreover, we identified three genes encoding proteins involved in cell wall biosynthesis and glycosylation of proteins or lipids. The beta-1,6-N-acetyl glucosaminyltransferase (AK070134) is similar to a core-2 branching enzyme family, which participates in forming crucial side-chain branches in the O-glycan biosynthesis (Brockhausen et al., 2009; Williams and Schachter, 1980). Fucosyltransferases encoded by AK099681 and AK060562 have similarities with fucosyltransferases 12 (Q9FX97: 73%) and galactoside 2- alpha-L-fucosyltransferases (Q9MSQ1: 59%), respectively in Arabidopsis, which add the terminal fucosyl residue on glucan side chains in cell wall biosynthesis (Bakker et al., 2001; Faik et al., 2000; Wilson et al., 2001).

Among three lipid metabolism-related genes, the cyclopropane-fatty-acyl-phospholipid synthase (AK111684) might be involved in cyclopropanated fatty acid biosynthesis. Acyl-CoA oxidase 2 (AK066934) and 3-ketoacyl-CoA thiolase 2 (AK069768) have high similarity with acyl-CoA oxidase 3 (Q9LH9: 82%) and 3-ketoacyl-CoA thiolase 2 (Q56WD9: 89%), respectively, and those genes are known to be involved in lipid beta-oxidation in Arabidopsis (Cruz Castillo et al., 2004; Froman et al., 2000; Germain et al., 2001). Besides, six genes encoding proteins in the mitochondrial electron transport chain such as NADH-ubiquinone oxidoreductase complexI-18 kDa (AK105064) and 51 kDa subunits (AK010730), ubiquinol-cytochrome c reductase complex III-6.7 kDa subunit (AK063800)

Metabolic networks for foliar injury symptoms in rice

It appears that genes involved in primary and secondary metabolism might be one of the primary targets of O3 stress at the stage of visible injury development (Figs. 1 and 4). Fortunately, a high percentage of such genes within the dataset of 146 genes allowed us to reconstruct the metabolic networks of genes involved in foliar injury symptoms to enrich our knowledge on predominantly expressed genes and their corresponding metabolic pathway (Table 1, and Supplementary Table 5).

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# Table 1. Functional categorization of 146 potential biomarker genes associated with O3-induced fetal injury.

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**Annotations:**
- **FC:** Functional Category
- **Gene Name:** Gene symbol
- **C-14, C-15, C-24, C-25, O-15, O-16, O-24, O-25, O-35, O-36, O-37, O-38, O-39, O-40:** Expression levels

**References:**
- Cho et al. (2012)

**Implications:**
- The data suggest a comprehensive understanding of O3-induced fetal injury mechanisms.
- Further investigation is necessary to validate these findings.

**Conclusion:**
- O3 exposure significantly alters the expression of these genes, indicating potential targets for future research in prenatal health.
and cytochrome c oxidase complex VI-5C (AK072527), 5B (AK071340) and 6A (AK059576) subunits were found to be associated with the O$_3$-induced visible foliar injury.

Putative participants in the biosynthesis of phenylalanine-derived secondary metabolites such as chorismatmutase (AK068983), caffeic acid 3-O-methyltransferase (AK069960) and dihydroflavonol-4-reductase (AK065264) were also found to correspond with O$_3$-induced foliar injury in this study. Chorismatmutase catalyzes the conversion of chorismate to prephenate in the pathway for the biosynthesis of phenylpropanoids and flavonoids. Caffeic acid 3-O-methyltransferase and dihydroflavonol-4-reductase are implicated in the production of phenylpropanoids and flavonoids, respectively (Winkel-Shirley, 2001). Flavonoids derived from 3-malonyl-CoAs and 4-coumaroyl-CoA are known to play a role as major pigments in plants (Winkel-Shirley, 2001), indicating that the flavonoid pathway could be implicated in the foliar image changes derived from O$_3$ exposure (Figure 1). Moreover, the phenolic metabolites have free radical-scavenging properties behaving as antioxidant agents in defense/stress responses (Grace and Logan, 2000; Lin et al., 2002; Rice-Evans et al., 1997). The polyamines conjugated with the phenolic compounds (e.g., cinnamic acid) also have scavenging properties comparable to that of ascorbate as a radical scavenger (Bouchereau et al., 1999; Langebartels et al., 1991). Among these is a polyamine biosynthesis-related gene (AK065153), spermidine synthase, catalyzing the conversion of putrescine to spermidine. Polyamines such as spermidine have been observed to accumulate in O$_3$-damaged diverse plants (reviewed in Dudareva et al., 2004), suggesting that inducible phenolic metabolites and their polyamine conjugates not only mop up free radicals derived from O$_3$, but may serve as an indicator of O$_3$-induced foliar injury in several plant species.

Furthermore, six terpene biosynthesis-related genes were also identified and linked with O$_3$-induced foliar injury. One gene (AK067589) is annotated as putative 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase, which may be involved in the non-mevalonate pathway in plastids of plants. The non-mevalonate pathway in plastid leads to the conversion of pyruvate and glyceraldehyde 3-phosphate to isopentenylphosphate (IPP) and dimethylallyldiphosphate (DMAPP), which condense to form geranyldiphosphate, farnesylidiphosphate, and geranylgeranyldiphosphate as the precursors for monoterpenes, sesquiterpenes, and diterpenes, respectively (Tholl, 2006). In this study, genes encoding terpene synthase 10 (AK108761) with unknown function, ent-kaurene synthase (KA072928, AK068310, AK072461) for kaurene, and putative 10-deacetylbaccatin III 10-O-acetyltranferase (AK064755) of unknown function were identified. Kaurenediterpene class is known as the precursor for gibberellin phytohormone involved in plant growth and development (Tholl, 2006). Out of metabolism-categorized genes (Table 1), there are six genes (AK059089, AK060722, AK067458, AK067847, AK107084, AK108382) encoding cytochrome P450, whose functions are unclear. Numerous cytochrome P450 enzymes are known to be involved in diverse metabolic pathways such as the biosynthesis of terpenes, phenylpropanoids, flavonoids, and volatiles derived from fatty acids.

The overview of Table 1 and Supplementary Table 5 indicates that O$_3$ stress causes degradation of cellular components such as protein, lipid, and polysaccharide, probably using energy produced by oxidative phosphorylation in the mitochondria, with visible injury formation. Additionally, these processes may run parallel to the induced biosynthesis of secondary metabolites such as phenylpropanoid and terpene. The degraded cellular components are probably translocated to younger leaves as a process of senescence, since O$_3$ is known to accelerate senescence of older leaves (Torres et al., 2007). The mapped genes onto the metabolic pathway and the corresponding cellular responses having tight link with foliar injury symptom have never been the subject of study at this scale. Nevertheless, the physiological and biochemical studies have previously shown the influence of O$_3$-stress on such cellular responses including the well-characterized example of photosynthetic apparatus (reviewed in Cho et al., 2011), validating our applied approach in this study in identifying potential biomarker genes of foliar injury.

**CONCLUSION**

In conclusion, we present convincing evidence to fulfill the objectives of this study. The quantitative RGB image analysis method was successfully applied for the first time to evaluate the degree of severity of foliar injury in rice leaves. The RGB image analysis could be coupled with the global
transcript profiling and robust selection criteria, resulting in identification of 146 genes positively associated with the foliar injury symptoms (its severity). The reconstruction of metabolic networks based on their annotated functions suggests how O₃ stress dramatically up-regulates the gene expression of metabolic pathways controlling the cellular activities of starch mobilization, cell wall and membrane modification, photosynthesis, biosynthesis of secondary metabolites including flavonoid, terpene and polyanine, detoxification, protein turnover & protein modification leading to the cellular responses as manifestation of foliar injury symptoms. A considerable portion of unknown genes (46 out of 146 genes) linked with foliar injury is of great interest, and those genes are the subject of our future studies to reveal their function in rice and other model plants. It appears that 146 genes could be exploited as potential biomarkers of O₃-triggered foliar injury to better understand the underlying mechanisms regulating the foliar injury symptom. It is also likely that a similar approach could be applicable in identifying genes linked with other visual symptoms caused by environmental factors in plants.

Acknowledgments

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Additional/Supplementary data

Supplementary data (Figures and Tables) associated with this article are deposited on our webpage, which can be accessed at https://fzp.nies.go.jp/proself/login/login.go?AD=init, using the user – supplement, and password – h0j#fyAO.

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