Characterization of Expressed Genes Under Ozone Stress in Soybean

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ABSTRACT To identify the genes specifically or predominantly expressed in ozone-fumigated leaves of two soybean cultivars: Jinpumkong and Cheongjakong, expression levels of mRNA were investigated using differential banding patterns on agarose gel. A total of 408 bands differently expressed after ozone fumigation was identified; 153 of which were up-regulated while 225 were down-regulated. Using BLASTx, the putative functions of the expressed sequence tags were determined. The 178 ozone-regulated differentially expressed genes (DEGs) matched with the previously known genes with high significance. The putative functional classes of these DEGs were categorized by two databases: Gene Ontology and MIPS. Based on the Gene Ontology database, majority of the DEGs have molecular function related to transferase activity. Most of them are involved in the cellular and metabolic processes. Cytoplasmic part and cell part were the primary types of cellular component in the ozone-responding DEGs. Whereas findings using the MIPS database revealed the function distribution of up-regulated DEGs across all classes. Most of the ozone-regulated genes identified in this study are related to biotic and abiotic stresses. The characterized ESTs will serve as useful data to provide a better understanding of the molecular basis and transcript profiles.

Keywords Differentially expressed gene, Ozone, Soybean, Transcripts

INTRODUCTION

One of the best characterized types of oxidative stress is exposure to high concentrations of ozone. Anthropogenic hydrocarbons and oxides of nitrogen (NO, NO₂) and sulfur (SO_x) react with solar UV radiation to generate ozone (O₃). Stratospheric ozone is beneficial because it shields the earth from UV irradiation; however, tropospheric ozone is harmful to life because it is a highly reactive oxidant. The negative effects of ozone on plants include decreasing photosynthesis rates, leaf injury, reducing growth of shoots and roots, accelerating senescence, and reducing crop yield. ROS damaging free radicals and their products react with proteins, DNA, and membrane lipids to reduce photosynthesis, increase electrolyte leakage, and accelerate senescence and cell death (Rao *et al.* 2000; Sharma and Davis 1997). Elevated levels of ROS induce the biosynthesis

of antioxidant molecules, including ascorbates, polyamines, and glutathione (Blokina *et al.* 2003; Conklin and Last 1995; Creissen *et al.* 1999). Oxidative stress increases the activity of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase, catalase, and glutathione S-transferase (Blokina *et al.* 2003; Ludwikow *et al.* 2004; McClung 1997; Mittler 2002; Tognolli *et al.* 2002). The transcription and activity of several ROS change drastically in response to ozone stress. In addition to antioxidant genes, a number of other genes are regulated by ozone stress at the transcript and protein levels in *Arabidopsis* and rice (Agrawal *et al.* 2002; Ludwikow *et al.* 2004).

The effects of ozone resemble characteristics of systemic acquired resistance (SAR) (Greenberg *et al.* 1994) and hypersensitive resistance (HR) (Pasqualini *et al.* 2003; Tenhaken *et al.* 1995). In addition, its effects are similar to responses to ethylene, salicylic acid, and jasmonic acid

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(Overmyer *et al.* 2003; Rao *et al.* 2002; Tamaoki *et al.* 2003a).

Soybeans are a major food crop worldwide, and can supply edible vegetable oil and high quality protein. The other domains and derivatives of the seed have substantial economic importance for a wide range of industrial, food, pharmaceutical, and agricultural products. In many studies, soybeans have been the target of various stress effects. Using meta-analytic techniques, Morgan *et al.* (2003) quantitatively summarized the responses of soybeans to chronic ozone exposure of 70 ppb. At maturity, the average shoot biomass was reduced to 34%, while the yield decreased to 24%. In previous studies, chronic ozone exposure has caused serious biomass and seed production losses even at low concentrations, and often in the absence of visible symptoms Fiscus *et al.* (1997). Furthermore, Renaud *et al.* (1997) reported that ozone stress can decrease root biomass in alfalfa.

To understand the molecular basis of the stress response, the differentially expressed genes (DEGs) in stress-induced leaves must be identified. DEGs are only expressed at low levels and their transcripts are difficult to detect in the abundant mRNA within tissues. To detect DEGs transcribed at low levels, highly specific polymerase chain reaction (PCR) amplification is required. Novel annealing control primer (ACP)-based DDRT-PCR technology has been used to identify differentially expressed genes in tissues Hwang *et al.* (2004). This method specifically targets sequence hybridization to the template via a polydeoxy-inosine linker Hwang *et al.* (2003).

The objective of this study was to isolate and characterize the differentially expressed genes of soybean leaves treated with ozone. ACP-based DDRT-PCR was used to identify the up-regulated and down-regulated DEGs in the tissue. And the putative functions of the DEGs were categorized using Gene ontology and the Munich Information Center for Protein Sequences (MIPS) databases.

MATERIALS AND METHODS

Plant materials and ozone treatment

In order to select plants that could be used for ozone stress test, this study used the previous data on physiological characteristics (Lee *et al.* 2006) and selected two cultivars of soybean [*Glycine max* (L.) Merr]: Jinpungkong and Cheongjakong. The plants were grown in a greenhouse at the National Institute of Crop Science (NICS) in Korea, and the ozone stress was treated at the V5 stage. Ozone-sensitive (cv. Jinpungkong) and ozone-insensitive (cv. Cheongjakong) soybeans were fumigated with 200 ppb of ozone for 4 h in 5 consecutive days in an airtight greenhouse (Fig. 1).

Total RNA extraction and annealing control primer (ACP)-based differential display

The total RNA was isolated from 200 mg of soybean leaves using TRIZOL reagent (Invitrogen), as instructed by the manufacturer, which was then employed in the synthesis of first-strand cDNA by reverse transcriptase, as described

Fig. 1. The soybean leaves treated with 200 ppb of ozone for 4 h in 5 consecutive days.

in Hwang *et al.* (2003). First-strand cDNA synthesis was performed using the dT-ACP1 primer (GeneFishing™ DEG kits, Seegene, Korea); and 3 µg of total RNA was incubated with 2 µl of dT-ACP1 (10 µM) at 80°C for 3 min. Subsequently, reverse transcription was performed with a total volume of 20 µl containing purified total RNA, 4 µl of 5x first strand buffer (Invitrogen), 5 µl dNTPs (each 2 mM), 0.5 µl of RNasin Plus RNase Inhibitor (40 U/µl, Promega), and 1 µl of SuperScript™ II RNase H-Reverse Transcriptase (200 U/µl, Invitrogen) for 1.5 h at 42°C. The first-strand cDNA samples were diluted with 80 µl of DNase-free water and were used as template for second-strand cDNA synthesis by random PCR amplification using dT-ACP2 and one of 120 arbitrary ACPs (GeneFishing™ DEG kits, Seegene, Korea) as primers. The second-strand cDNA synthesis was performed in a final reaction volume of 20 µl containing diluted first-strand cDNA (50 ng), 2 µl of 5 µM arbitrary ACP primer, 1 µl of 10 µM dT-ACP2, and 10 µl of 2x SeeAmp™ ACP™ Master Mix (GeneFishing™ DEG kits, Seegene, Korea). The PCR protocol for second-strand synthesis is consisted of one cycle at 94°C for 1 min, followed by 50°C for 3 min, and 72°C for 1 min. A total of 40 cycles of amplification which include denaturation at 95°C for 20 sec, annealing at 65°C for 40 sec, extension at 72°C for 40 sec, and a final extension step of 5 min at 72°C was used. The amplified products were separated on 2% agarose gels, and then stained with ethidium bromide.

The differentially expressed transcripts were eluted and cloned into a T&A cloning vector (Real Biotech Corp., Taiwan). To confirm the identities of the insert DNAs, isolated plasmids were sequenced (Bionics, Korea).

Annotation of soybean EST genes and function

The EST sequences were decoded to FASTA format by the PHRED program, using default parameters Ewing and Green (1998). These sequences were filtered to remove contaminants such as vector and bed quality sequences, using the LUCY2 tool Li and Chou (2004). The CAP3 program was used to cluster the ESTs so that they directly assemble to contig and siglet Huang and Madan (1999). The sequence homologies were determined by BLASTx of the NCBI nr database (<http://www.ncbi.nlm.nih.gov>),

using the default options. Non-significant results were removed using a cut-off e-value of 10^{-4} . Functions were assigned using the Gene Ontology database (<http://www.geneontology.org>) and the Munich Information Center for Protein Sequence (MIPS) database (<http://mips.gsf.de/proj/funcatDB>). All the EST data are publicly available through the NCBI, with GenBank dbEST accession nos. EG702072 - EG702322.

RESULTS AND DISCUSSION

To identify the genes specifically or predominantly expressed in ozone-treated soybean leaves, differential expression levels of the mRNA fragments of treated and untreated leaves were investigated using differential banding patterns in agarose gels. The differentially expressed gene (DEG) bands were detected in the ozone-treated and -untreated leaves using a combination of 120 arbitrary primers and two anchored oligo (dT) primers from GeneFishing™ DEG kits (Seegene, Korea). A total of 408 DEGs

Table 1. Patterns of up- and down-regulated differentially expressed genes in ozone treated soybean.

Cultivar Regulated	Jinpumkong (sensitive)	Cheongjakong (insensitive)
Up	61	92
Down	225	30

Table 2. Summary of ESTs from soybean leaves treated with ozone.

	Number	Percentage (%)
Total clones	408	
Sequenced clones	382	93.6
Non-sequenced clones	26	6.4
Non-redundant sequence	223	54.7
Cluster	20	
Redundancy	179	43.9
Singlet	203	
Blast match	178	79.8
Blast no-match	45	20.2

were expressed in the ozone-treated soybean leaves (cv. Cheongjakong and Jinpungkong); of which 153 DEGs were up-regulated while 225 were down-regulated (Table 1).

The nucleotide sequences of 382 DEGs in the ozone-fumigated treatments were analyzed; and their putative identification was conducted using BLASTx. Result of homology searches revealed that 178 (79.8%) of the identified DEGs after ozone treatment matched well with the previously known genes. The sequenced clones of the ESTs from the ozone-treated soybean leaves included 223 unique clones (54.7%), which in turn consisted of 20 assembled sequences and 203 singletons. The sequences of 179 clones were assembled into 20 clusters with a redundancy of 43.9% (Table 2).

The observed DEGs, such as those encoding glutathione S-transferase, copper-binding protein, ascorbate peroxidase, ribulose-1,5-bisphosphate carboxylase/oxygenase activase, lipoxygenase, Cu/Zn superoxide dismutase, chlorophyll a/b-binding protein, and photosynthesis and antioxidant enzymes, were previously reported as ozone responsive (Conklin and Last 1995; Glick *et al.* 1995; Kliebenstein *et al.* 1998; Kubo *et al.* 1995; Maccarrone *et al.* 1992; Miller *et al.* 1999; Tamaoki *et al.* 2003b; Tamaoki *et al.* 2003a).

Glutathione S-transferase was up-regulated in response to ozone treatment. Kawasaki *et al.* (2001) reported the up-regulation of many genes encoding glycine-rich proteins, ABA and stress-induced proteins, metallothionein-like proteins, glutathione S-transferase, and ascorbate peroxidase in salt-tolerant rice.

Seki *et al.* (2002) identified cold-, drought-, and salinity-induced target genes as well as stress-related transcription factor family members such as helix-loop-helix using *Arabidopsis* cDNAs. These results suggest cross-talk

between the salt and drought stress signaling processes. Helix-loop-helix was down-regulated in response to ozone treatment, inferring cross-talk between ozone stress and other stress signaling processes.

For the identified DEGs, 11 up-regulated and 2 down-regulated DEGs overlapped in the sensitive and insensitive cultivars of the ozone-treated leaves (Fig. 2). These overlapping genes were subsequently categorized with a binding molecular function based on the Gene Ontology database.

Plastidic aldolase, MSS4 (*Saccharomyces cerevisiae* protein), and metalloendoproteinase were shown to overlap in the up-regulated DEGs by ozone response. The ribulose-1,5-bisphosphate carboxylase small subunit and chlorophyll a/b binding protein overlapped in the down-regulated DEGs by ozone response (Fig. 3).

Plastidic aldolase (AldP) overlapped in the up-regulated DEGs by ozone response. AldP is involved in the photosynthetic carbon reduction cycle and catalyzes the cleavage of fructose-1,6-bisphosphate (FBP) into D-glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP). Two forms of aldolase are present in higher plants: cytoplasmic and plastidic. The cytoplasmic aldolase (AldC) gene is inducible under anaerobic conditions and is considered to have an important role in producing ATP by stimulating glycolysis under such conditions (Andrews *et al.* 1994; Umeda and Uchimiya 1994). Its expression is regulated by light Kagaya *et al.* (1995). The isolation of two AldP genes from a salt-tolerant *Nicotiana* species, *N. paniculata*, and their expression regulation among three *Nicotiana* species were compared, showing varied tolerant reaction to salt stress Yamada *et al.* (2000).

Putative functions of the identified DEGs were determined using the Gene Ontology database. Majority of the ozone-

Fig. 2. Distribution of the DEGs according to type of ozone response.

responsive DEGs identified have molecular function related to transferase activity, while the down-regulated DEGs in insensitive cultivar fell under the class possessing structural molecule activity. Most of them are involved in the cellular and metabolic processes. However, stimulus response and the developmental process were the major classes of the up-regulated DEGs in the ozone-insensitive cultivar. Furthermore, cytoplasmic and cell parts were the primary cellular component types involved in response to ozone.

The functional classes of the up-regulated DEGs in the ozone sensitive and insensitive cultivars were distributed across all classes within the MIPS database (Fig. 3). Biogenesis of cellular components (31.7%) and protein with binding function of cofactor requirement (27.9%) were the major classes in the up-regulated DEGs. The other functions

identified include protein fate (23.4%), metabolism (22.3%), cellular communication/signal transduction mechanism (21.4%), unclassified proteins (21.1%), regulation of metabolism and protein function (20.9%), transport facilities and transport routes (20.9%), subcellular localization (19.8%), energy (17.3%), cellular transport, cell rescue, defense and virulence (14.6%), cell cycle and DNA processing (12.1%), transcription (11.6%), interaction with the environment (11.4%), cell fate (10.7%), protein synthesis (7.7%), and transposable elements, viral and plasmid proteins (4.0%). Fourteen classes overlapped in the up-regulated DEGs, while two classes overlapped in the down-regulated DEGs.

Approximately half of the ozone-responsive down-regulated DEGs were unclassified proteins (52.2%). The down-regulated DEGs responding to ozone application were categorized according to protein fate (19.4%), meta-

Fig. 3. Functional distribution of the ozone-regulated DEGs. The putative functional categories of the DEGs were determined using the MIPS database. A: Up-regulated, B: Down-regulated.

bolism (17.3%), protein with binding function of cofactor requirement (15.6%), subcellular localization (15.4%), cellular transport, transport facilities and transport routes (15.2%), cellular communication/signal transduction mechanism (14.9%), cell rescue, defense and virulence (11.5%), regulation of metabolism and protein function (11.0%), transcription (10.8%), protein synthesis (8.9%), cell fate (7.9%), interaction with the environment (5.4%), cell cycle and DNA processing (4.7%), and transposable elements, viral and plasmid proteins (0.7%).

In a previous study using pepper, the common types of functions for up- and down-regulated ozone stress-regulated genes were related to metabolism and defense Lee and Yun (2006). The ESTs of O₃-exposed *Arabidopsis* were categorized as cell rescue/defense genes for the up-regulated genes, and for energy category in the down-regulated genes Tamaoki *et al.* (2003a).

CONCLUSION

This study was conducted to identify and characterize the genes that predominantly responded to ozone stress in soybean. Of the 408 differentially expressed bands, 153 were up-regulated while 225 were down-regulated. Results further demonstrated various functions of the DEGs, majority of which were implicated to have molecular function related to transferase activity, and are involved in cellular and metabolic processes. DEG profiling by ozone treatment highlights the possible signaling pathways and molecular biological process for future research. More so, this study serves as a benchmark in finding out the regulatory pathways involve in acclimation responses to ozone, as well as relationships among the DEGs responsive to ozone and other abiotic stress.

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