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Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray

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Abstract Full-length cDNAs are essential for functional analysis of plant genes. Recently, cDNA microarray analysis has been developed for quantitative analysis of global and simultaneous analysis of expression profiles. Microarray technology is a powerful tool for identifying genes induced by environmental stimuli or stress and for analyzing their expression profiles in response to environmental signals. We prepared an *Arabidopsis* full-length cDNA microarray containing around 7,000 independent full-length cDNA groups and analyzed the expression profiles of genes. The transcripts of 245, 54, 299 and 213 genes increased after abscisic acid (ABA), drought-, cold-, and salt-stress treatments, respectively, with inducibilities more than fivefold compared with those of control genes. The cDNA microarray analysis showed that many ABA-inducible genes were induced after drought- and high-salinity-stress treatments, and that there is more crosstalk between drought and ABA responses than between ABA and cold responses. Among the ABA-inducible genes identified, we identified 22 transcription factor genes, suggesting that many transcriptional regulatory mechanisms exist in the ABA signal transduction pathways. Electronic supplementary material to this paper can be obtained by using the Springer Link server located at http://dx.doi.org/10.1007/s10142-002-0070-6 or from http://www.gsc.riken.go.jp/Plant/index.html.

Keywords ABA · cDNA microarray · *Arabidopsis thaliana* · Full-length cDNA

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Introduction

Recently, microarray technology has become a useful tool for the analysis of genome-scale gene expression (Schena et al. 1995; Eisen and Brown 1999). With this DNA chip-based technology cDNA sequences are arrayed on a glass slide at a density >1,000 genes/cm^2. These arrayed sequences are hybridized simultaneously to a two-color fluorescently labeled cDNA probe pair prepared from RNA samples of different cell or tissue types, allowing direct and large-scale comparative analysis of gene expression. This technology using ESTs was first reported for analyzing the differential expression of 48 *Arabidopsis* genes in roots and shoots (Schena et al. 1995). Reymond et al.

(2000) analyzed gene expression in response to mechanical wounding and insect feeding, and defense signaling pathways have been analyzed using fungal pathogen and signaling molecules (Schenk et al. 2000).

Plant growth is greatly affected by environmental abiotic stresses, such as drought, high salinity and low temperature. Plants respond and adapt to these stresses in order to survive under abiotic stress. Among these abiotic stresses, drought or water deficit is the most severe limiting factor of plant growth and crop production. Drought stress induces various biochemical and physiological responses in plants. Several genes that respond to drought, salt and cold stress at the transcriptional level have been studied (Ingram and Bartels 1996; Shinozaki and Yamaguchi-Shinozaki 1996, 1997; Bray 1997). The products of the stress-inducible genes have been classified into two groups: those that directly protect against environmental stresses and those that regulate gene expression and signal transduction in the stress response (Shinozaki and Yamaguchi-Shinozaki 1997). The first group includes proteins that likely function by protecting cells from dehydration, such as the enzymes required for biosynthesis of various osmoprotectants, late-embryogenesis-abundant (LEA) proteins, antifreeze proteins, chaperones, and detoxification enzymes (Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997). The second group of gene products includes transcription factors, protein kinases, and enzymes involved in phosphoinositide metabolism (Shinozaki and Yamaguchi-Shinozaki 1997, 1999). Stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer (Holmberg and Bulow 1998; Bajaj et al. 1999; Thomashow 1999; Hasegawa et al. 2000; Zhu 2001). It is important to analyze the functions of stress-inducible genes not only to understand the molecular mechanisms of stress tolerance and the responses of higher plants but also to improve the stress tolerance of crops by gene manipulation. Hundreds of genes are thought to be involved in abiotic stress responses (Shinozaki and Yamaguchi-Shinozaki 1999, 2000).

Previously, we prepared an *Arabidopsis* full-length cDNA microarray using around 1,300 full-length cDNAs, and applied the full-length cDNA microarray to identify drought- or cold-inducible genes, and target genes of DREB1A/CBF3, a transcription factor that controls stress-inducible gene expression (Seki et al. 2001a). The full-length cDNA microarray was useful for analyzing the expression pattern of *Arabidopsis* genes under drought and cold stresses, and the target genes of stress-related transcription factors and potential *cis*-acting DNA elements could be analyzed by combining the expression data with the genomic sequence data.

Recently, we prepared a new full-length cDNA microarray containing around 7,000 independent full-length cDNA groups. In the present study, we applied the 7,000 full-length cDNA microarray to identify new ABA-inducible genes, and analyzed the crosstalk to investigate the time course of gene expression by ABA treatment, differences between ABA-signaling and signaling of drought, cold and high-salinity stresses, and of crosstalk between them. This is the first report on crosstalk and differences of signaling cascades among drought, cold, high-salinity stresses and ABA using a global expression profiling strategy. We also discuss the functions of the ABA-inducible genes in stress responses and tolerance.

Materials and methods

Plant materials, stress treatments and RNA isolation

Arabidopsis thaliana (Columbia ecotype) was germinated and grown on germination medium (GM) containing Murashige and Skoog salts, 3% sucrose (WAKO, Osaka, Japan) and 0.8% Bacto-agar (Difco, Detroit, Mich.). The plants were grown for 3 weeks in a growth chamber kept at 22°C under 16 h light/8 h dark. ABA, dehydration, cold, and high-salinity stress treatments were done essentially as reported previously (Yamaguchi-Shinozaki and Shinozaki 1994). For ABA treatments, plants were transferred and grown hydroponically in 100 μM ABA. For dehydration treatments, plants were removed from the agar and desiccated in plastic dishes at 22°C under dim light. For cold treatments, the plants were grown under dim light at temperatures of 4°C. For high-salinity-stress treatments, plants were transferred and grown hydroponically in a 250 mM NaCl solution under dim light. The plants were subjected to the stress treatments for various periods and then frozen in liquid nitrogen for further analyses. Total RNA was prepared using TRIZOL reagent (Life Technologies, Rockville, Md.), and mRNA was prepared using the mRNA isolation kit (Miltenyi Biotec, Calif.).

cDNA clones

In the cDNA microarray analyses, we used around 7,000 cDNA sequences representing *A. thaliana* full-length cDNA clones isolated from full-length cDNA libraries, and the drought- and cold-inducible genes, *responsive to dehydration* (*rd*) and *early responsive to dehydration* (*erd*) (Taji et al. 1999). As an external control, a PCR-amplified fragment from the λ control template DNA fragment (TX803; Takara, Kyoto, Japan) was used. As negative controls, two DNAs derived from the mouse nicotinic acetylcholine receptor epsilon-subunit (nAChRE) gene and the mouse glucocorticoid receptor homolog gene were used. The RAFL cDNA clones whose full-length cDNA sequences are determined by the *Arabidopsis* SSP sequencing consortium, which comprises the Salk Institute (principal investigator: Dr. Joseph R. Ecker), the Stanford Genome Technology Center (principal investigator: Dr. Ronald W. Davis), and the Plant Gene Expression Center (principal investigator: Dr. Athanasios Theologis; SSP), are available from the RIKEN Bioresource Center (Seki et al. 2002).

Sequence analysis

The cDNA clones were grown in 96-well plates using a microincubator (TAITEC, Saitama, Japan). Plasmid DNA was extracted with a DNA extraction instrument (model Biomek; Beckman Coulter, Calif.) and purified using 96-well MultiScreen filter plates (Millipore, Bedford, Mass.). DNA sequences were determined using the dye terminator cycle sequencing method (Big Dye Terminator Cycle Sequencing Kit; Perkin-Elmer Applied Biosystems, Foster City, Calif.) with a DNA sequencer (model ABI Prism 3700; Perkin-Elmer Applied Biosystems, Foster City, Calif.). Sequence homologies were examined with the GenBank/EMBL database using the BLAST program.

Amplification of cDNA inserts

In the cDNA microarray analyses, we used around 7,000 cDNA clones, and the λ control template DNA fragment (TX803; Takara, Kyoto, Japan) as an external control. As negative controls, DNA derived from the mouse nicotinic acetylcholine receptor epsilon-subunit (nAChRE) gene and the mouse glucocorticoid receptor homolog gene were used. The vectors used for cDNA library construction were modified λZAP (Carninci et al. 1996) and λFLC-1 (Carninci et al. 2001). Inserts of cDNA clones were amplified by PCR using primers that were complementary to vector sequences flanking both sides of the cDNA insert, as described previously (Seki et al. 2001a). We precipitated PCR products in ethanol and resuspended the DNA at around 2 µg/µl in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). One aliquot of each reaction product was electrophoresed on a 0.7% agarose gel to confirm amplification quality and quantity. Two microliters of DNA were mixed with 2 µl 2× polymer (Fuji Photo Film, Kanagawa, Japan) and 4 µl dimethyl sulfoxide (DMSO; Kishida Chemical, Osaka, Japan) at least 10 times using the automatic dispenser (model EDS-384S; Biotech, Tokyo, Japan).

cDNA microarray preparation

PCR products were arrayed from 384-well microtiter plates onto micro-slides (model Super Aldehyde substrates; Telechem International, Sunnyvale, Calif.) using the microarray stamping machine (model SPBIO2000; Hitachi Software Engineering, Tokyo, Japan). The tip loaded 2 µl PCR products (500–1,000 ng/µl) from the 384-well microtiter plates and deposited 0.5 nl per slide on 48 slides with a spacing of 300 µm. Postprocessing of the slides was performed according to the manufacturer's protocol (Telechem International, Sunnyvale, Calif.). The printed slides were dried (relative humidity ≤30%) and subjected to UV cross-linking. They were rocked in 0.2% SDS for 2 min 3 times and then rocked in distilled water for 2 min twice vigorously. The slide racks were transferred into a chamber containing boiling water and left for 2 min. The blocking solution, containing 1 g sodium borohydride, 300 ml phosphate-buffered saline (PBS; Life Technologies, Rockville, Md.), and 90 ml 100% ethanol, was poured into the glass chamber. The slide racks were shaken gently for 5 min, transferred into a chamber containing 0.2% SDS and shaken gently for 1 min 3 times. They were transferred into a chamber containing distilled water, shaken gently for 1 min, and dried by centrifugation for 20 min.

Microarray hybridization and scanning

Each mRNA sample was reverse-transcribed in the presence of Cy3-dUTP or Cy5-dUTP (Amersham Pharmacia, Piscataway, N.J.). The reverse transcription reaction was performed in a 20-µl solution containing 1 µg denatured poly(A)$^+$RNA with 1 ng λ polyA$^+$ RNA-A (TX802; Takara, Kyoto, Japan) for external control, 50 ng/µl oligo-(dT) 12–18 mer (Life Technologies, Rockville, Md.); 0.5 mM each dATP, dGTP and dCTP; 0.2 mM dTTP; 0.1 mM Cy3 dUTP or Cy5 dUTP; 100 units RNase inhibitor; 10 mM DTT; and 200 units Superscript II reverse transcriptase (Life Technologies, Rockville, Md.) in 1× Superscript first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 20 mM DTT; Life Technologies). Following incubation at 42°C for 35 min, 200 units of Superscript II were added and the reaction sample was incubated for an additional 35 min. Following addition of 5 µl 0.5 M EDTA, 10 µl 1 N sodium hydroxide, and 20 µl distilled water to stop the reaction and to degrade the template, they were incubated for 1 h at 65°C. The solution was neutralized with 25 µl 1 M Tris-HCl (pH 7.5). The reaction products of two samples (one with Cy3 labeling and the other with Cy5 labeling) were combined. The samples were placed in a Microcon 30 microconcentrator (Millipore, Bedford, Mass.). Two hundred and fifty microliters of TE buffer were added, spun for 10 min in a bench-top microcentrifuge at a high speed to a volume of 10 µl, and the flow-through product was discarded. This step was repeated 4 times. The probes were then collected by inverting the filter and spinning for 5 min. Several microliters of distilled water were added to the Microcon. The filter was inverted and spun so that the final volume of the collected probes was 18 µl. Then 5.1 µl 20× SSC, 2.5 µl 2 µg/µl yeast tRNA and 4.8 µl 2% SDS were added to the probes. The probe samples were denatured by placing them in a 100°C heat block for 2 min, leaving at room temperature for 5 min, and they were then used for hybridization. The slides were placed in a sealed hybridization cassette (Telechem International, Sunnyvale, Calif.) and submerged in a 65°C water bath for 16–20 h. After hybridization, slides were washed in 2× SSC, 0.03% SDS for 2 min, then in 1× SSC for 2 min, and finally in 0.05× SSC for 2 min. The slides were immediately centrifuged to dry (1 min at 2,500 *g*). Slides were scanned with a ScanArray 4000 (GSI Lumonics, Oxnard, Calif.) as described previously (Seki et al. 2001a).

Data analysis

Image analysis and signal quantification were performed with QuantArray version 2.0 (GSI Lumonics, Oxnard, Calif.). Background fluorescence was calculated on the basis of the fluorescence signal of the negative control genes [the mouse nicotinic acetylcholine receptor epsilon-subunit (nAChRE) gene and the mouse glucocorticoid receptor homolog gene]. Genes showing a signal value <1,000 (which was typically twice the mean background value) in both Cy3 and Cy5 channels were not considered for the analyses. A λ control template DNA fragment (TX803; Takara, Kyoto, Japan) was used as an external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with Genespring (Silicon Genetics, San Carlos, Calif.).

RNA gel blot analysis

Isolated total RNA was also used for RNA gel blot hybridization. The isolated total RNA was denatured with a mixture of 2.15 M formaldehyde and 50% formamide, fractionated by electrophoresis on a 1.0% agarose gel that contained 2.2 M formaldehyde according to the protocol described earlier (Mainatis et al. 1982), and subsequently capillary transferred to nylon membrane using 20× SSC. The membrane was probed with DIG-labeled antisense RNAs prepared by in vitro transcription using an RNA transcription kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. The nylon membranes were washed twice with a mixture of 2× SSC and 0.1% SDS for 5 min at room temperature, then twice with a mixture of 0.1× SSC and 0.1% SDS for 15 min at 68°C and subjected to detection of DIG-labeled RNA probes using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals, Mannheim, Germany).

Results and discussion

Arabidopsis full-length cDNA microarray

Using the biotinylated CAP trapper method, we constructed full-length cDNA libraries from *Arabidopsis* plants under different conditions, such as ABA-treated, drought-treated, cold-treated, and unstressed plants, at various developmental stages from germination to mature seeds (Seki et al. 1998, 2001b, 2002). From the full-length cDNA libraries, we isolated around 7,000 independent *Arabidopsis* full-length cDNAs. We used a method described previously (Eisen and Brown 1999) to array polymerase chain reaction (PCR) -amplified cDNA fragments onto glass slides. We prepared a full-length

cDNA microarray containing around 7,000 *Arabidopsis* full-length cDNAs, including drought-inducible genes *rd* and *erd* (Kiyosue et al. 1994a; Yamaguchi-Shinozaki and Shinozaki 1994; Taji et al. 1999) as positive controls, the PCR-amplified fragment from a λ control template DNA fragment (Takara) as an external control, and the mouse nicotinic acetylcholine receptor epsilon-subunit (nAChRE) gene and the mouse glucocorticoid receptor homolog gene, which have no substantial homology to any sequences in the *Arabidopsis* database, to assess for nonspecific hybridization as negative controls.

Isolation of ABA-, drought-, cold-, or high-salinity-stress-inducible genes by use of the cDNA microarray

The cDNA microarrays were hybridized with Cy3 and Cy5 fluorescently-labeled probe pairs of ABA-treated plants plus unstressed plants, drought-treated plants plus unstressed plants, cold-treated plants plus unstressed plants, and high-salinity-treated plants plus unstressed plants, prepared as described in Materials and methods. Hybridized microarrays were scanned by two separate laser channels for Cy3 and Cy5 emissions from each DNA element. The ratio of the two fluorescent signal intensities of each DNA element was then measured as a relative measure to determine changes in the differential expression of genes represented by cDNA spots on the microarrays. In this study, we used the PCR-amplified fragment from the λ control template DNA fragment (Takara) as an external control gene to equalize hybridization signals generated from different samples.

mRNAs from ABA-, drought-, cold-, or high-salinity-stress-treated plants and wild-type unstressed plants were used for the preparation of Cy3-labeled and Cy5-labeled cDNA probes, respectively. These cDNA probes were mixed and hybridized with the cDNA microarray. To assess the reproducibility of the microarray analysis, we did three repetitive experiments with independent microarray slides from the same biological sample. To compare the data of various microarray experiments, we used the plant materials grown under the same conditions (at 22°C, under a 16/8-h light/dark cycle; light period is from 5:00 a.m. to 9:00 p.m.) and started various stress treatments at almost the same time (starting time of 9:00–10:00 a.m.). Hybridization of different microarrays with the same mRNA samples indicated good correlation (see Supplemental Table 1). As genes with expression ratios (ABA/unstressed, dehydration/unstressed, cold/unstressed, or high-salinity/unstressed) more than fivefold that of the λ control template DNA fragment for at least one time-course point, we identified 245, 299, 54, and 213 genes as ABA-, drought-, cold- and high-salinity-inducible genes, respectively (Fig. 1, Table 1). As genes with expression ratios (ABA/unstressed, dehydration/unstressed, cold/unstressed, or high-salinity/unstressed) more than threefold that of the control at one time-course point or more, we identified 856, 742, 229, and 554 genes as ABA-, drought-, cold- and high-salinity-inducible genes, respectively. In this study,

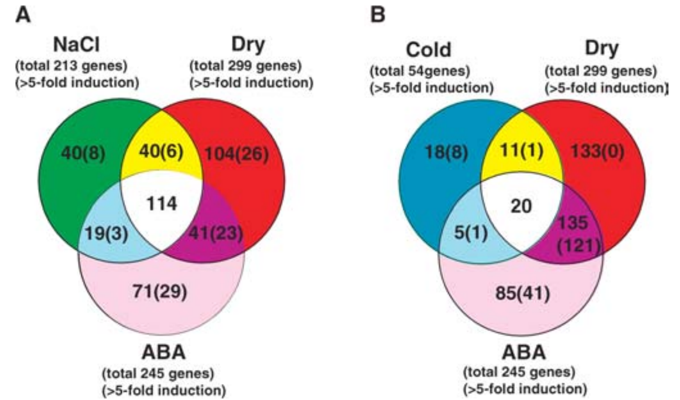

Fig. 1A, B Classification of the abscisic acid- (ABA-), drought-, cold- or high-salinity-stress-inducible genes on the basis of their expression pattern. **A** The ABA-, drought-, or high-salinity-stress-inducible genes identified were put into the following seven groups: (1) ABA-highly-inducible genes, (2) drought-stress-highly-inducible genes, (3) high-salinity-stress-highly-inducible genes, (4) ABA- and drought-stress-highly-inducible genes, (5) ABA- and high-salinity-stress-highly-inducible genes, (6) drought- and high-salinity-stress-highly-inducible genes, and (7) ABA-, drought- and high-salinity-stress-inducible genes. A list of the genes is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 2). **B** The ABA-, drought-, or cold-stress-inducible genes identified were put into the following seven groups: (1) ABA-highly-inducible genes, (2) drought-stress-highly-inducible genes, (3) cold-stress-highly-inducible genes, (4) ABA- and drought-stress-highly-inducible genes, (5) ABA- and cold-stress-highly-inducible genes, (6) drought- and cold-stress-highly-inducible genes, and (7) ABA-, drought- and cold-stress-inducible genes. A list of the genes is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 3)

we focused on the genes with expression ratios more than fivefold that of unstressed plants. A list of these ABA-, drought-, cold-, or high-salinity-inducible genes identified are available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 1). These genes included many reported ABA-, drought-, cold-, and high-salinity-stress-inducible genes, which indicates that our cDNA microarray system functions properly to find ABA-, drought-, cold-, or high-salinity-stress-inducible genes. To evaluate the validity of expression profile analysis of gene expression during ABA treatment using cDNA microarray, we performed RNA gel blot analysis on 16 ABA-inducible genes. The results of expression data obtained by microarray analyses were in good agreement with those obtained by RNA gel blot analyses (data not shown). This is consistent with our previous report (Seki et al. 2001a).

The relationship between each stress

The ABA-, drought-, cold-, or high-salinity-stress-inducible genes were classified on the basis of their expression pattern (Fig. 1). The results of the classification is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Tables 2 and 3). Overlapping on a Venn diagram showed that 20 genes were induced under ABA-, drought-, cold-, and high-salinity-stress treatments.

Table 1 Number of clones involved in different functional groups upregulated[a] or downregulated[b] by ABA treatment

Functional category of cDNA	Gene number	Representative gene names
Upregulated genes		
Transcription factor	22	DREB family transcription factor, two ERF family transcription factors, five zinc finger family transcription factors, WRKY family transcription factor, three MYB family transcription factors, bHLH family transcription factor, four NAC family transcription factors, two homeodomain family transcription factors, two bZIP family transcription factors, other family transcription factor
Osmoprotectant synthesis	8	Two galactinol synthases, P5CS, two raffinose synthases, sucrose synthase, arginine decarboxylase, trehalose-6-phosphate synthase
Osmoprotectant degradation	1	ERD5
Protein degradation	4	ERD1, ubiquitin conjugating enzyme, two cysteine proteinases
Protease inhibitor	3	Cysteine proteinase inhibitor, two trypsin inhibitors
LEA protein	8	ERD10, RD17, Rab18, 5 LEA proteins
Hydrophilic protein	2	RD29A, RD29B
KIN protein	2	KIN1, KIN2
Detoxification enzyme	4	ERD9, two glutathione S-transferases, peroxidase
Heat shock protein	5	Five heat shock proteins
Lipid transfer protein	4	Four lipid transfer proteins
Transport protein, ion channel, carrier	11	Two ABC transporter proteins, oligopeptide transporter protein, potassium transporter protein, two amino acid transporter proteins, two sugar transporters, mitochondrial dicarboxylate carrier protein, nitrate transporter, chloroplast protein import component protein
Membrane protein	3	Three membrane-related proteins
Fatty acid metabolism	5	Two lipases, lysophospholipase, fatty acid elongase, CER1
Cytochrome P450	6	Six cytochrome P450 proteins
Protein kinase	4	Ser/Thr protein kinase, receptor-like protein kinase, two protein kinases
Protein phosphatase	3	ABI1, two protein phosphatase 2C-like proteins
Signaling	5	RD20, Ca^{2+}-binding protein, two calmodulin-related proteins, pseudo response regulator
Aldehyde dehydrogenase	1	Aldehyde dehydrogenase
Plant defense	4	Pathogen-inducible alpha-dioxygenase, nematode-resistance protein, beta-1,3-glucanase, polygalacturonase inhibiting protein
Alcohol dehydrogenase	1	Alcohol dehydrogenase
JA biosynthesis	1	Allene oxide synthase
JA-regulated genes	1	Myrosinase-binding protein
IAA metabolism	1	Indole-3-acetate beta-glucosyltransferase
Senescence-related genes	4	ERD7, SAG29, SEN1, fibrillin
Cellular metabolism	19	p-Hydroxyphenylpyruvate dioxygenase, carboxyesterase, malate oxidoreductase, saccharopine dehydrogenase, alanine:glyoxylate aminotransferase, citrate synthase, aspartate aminotransferase, glutamate-ammonia ligase, alpha-hydroxynitrile lyase, 12-oxophytodienoate-10–11-reductase, tyrosine aminotransferase, isovaleryl-CoA-dehydrogenase, acyl-CoA oxidase, glutamate-dependent asparagine synthetase, glutamate dehydrogenase, 3-ketoacyl-CoA thiolase, glyoxalase, myo-inositol 1-phosphate synthase, branched-chain-amino acid aminotransferase
Carbohydrate metabolism	12	Three glucosyltransferases, UDP-glucose glucosyltransferase, neutral invertase, two beta-amylases, two beta-glucosidases, UDP-glucose: indole-3-acetate beta-D-glucosyltransferase, alpha-L-arabinofuranosidase, beta-fructosidase
Secondary metabolism	13	Two anthocyanidin synthases, two reticuline oxidases, five cinnamyl-alcohol dehydrogenases, flavanone 3-hydroxylase, two cinnamoyl-CoA reductases, anthocyanin 5-aromatic acyltransferase
Respiration	2	alternative oxidase, flavin-containing monooxygenase
Protein synthesis	1	Peptidylprolyl isomerase
Reproductive development	3	Pollen coat protein, two pollen allergen proteins
Cellular structure, organization and biogenesis	12	Pectinesterase, xylosidase, two arabinogalactan proteins, seven endoxyloglucan transferases, blue copper binding protein

Table 1 (continued)

Functional category of cDNA	Gene number	Representative gene names
DNA, nucleus	1	Histone
Photosynthesis	2	Pyruvate-orthophosphate dikinase, thioredoxin
RNA-binding protein	1	RNA-binding protein
Epoxide hydrolase	2	Two epoxide hydrolases
Downregulated genes		
Photosynthesis	10	Three RBCS genes, PsbS gene, carbonic anhydrase, five chlorophyll a/b-binding proteins
Carbohydrate metabolism	3	Glyceraldehyde 3 phosphate dehydrogenases, aldolase, fructose-bisphosphatase
RNA-binding protein	1	RNA-binding protein
Protein phosphatase	1	Protein phosphatase 2C protein
GTP-binding protein	1	GTP-binding protein
Chloroplast protein	1	Peptidyl-prolyl *cis-trans* isomerase
Cytochrome P450	1	Cytochrome P450 protein
Detoxification enzyme	1	Glutathione S-transferase

[a] In this study, we regarded the genes with an expression ratio (ABA-treated/untreated) greater than five times that of the lambda control template DNA fragment in at least one time point as ABA-inducible genes

[b] In this study, we regarded the cDNAs whose expression level was less than one-fifth-fold that of wild-type unstressed plants for at least one time point in ABA-treated plants as genes downregulated by ABA treatment

Among them, we found 6 well-known stress-inducible genes, such as *rd29A/cor78, cor15a, kin1, kin2, rd17/cor47* and *erd10* (Kiyosue et al. 1994b; Bohnert et al. 1995; Ingram and Bartels 1996; Bray 1997; Shinozaki and Yamaguchi-Shinozaki 1997, 1999, 2000; Taji et al. 1999). Seven cDNAs (RAFL04–09-B07, RAFL04–10-D13, RAFL05–10-J09, RAFL05–19-O22, RAFL05–21-F13, RAFL06–16-B22 and RAFL08–11-P07) whose functions are unknown were also included in this group.

We analyzed the Venn diagram for the differences and crosstalk of gene expression among ABA-, drought-, cold- and high-salinity-stress responses. As shown in Fig. 1, 245, 299, 54 and 213 genes were identified as ABA-, drought-, cold- and high-salinity induced genes with more than fivefold induction, respectively. Around 47% (114 genes) of the ABA-inducible genes were induced by both drought and high-salinity stresses. Around 63% (155 genes) were induced by both ABA and drought treatments and around 54% (133 genes) were induced by both ABA and high-salinity-stress treatments, whereas only around 10% (25 genes) were induced by both ABA and cold treatments. These results indicate the existence of greater crosstalk between ABA-, drought- and high-salinity-stress signaling processes than those between ABA- and cold-stress signaling processes. These results are consistent with our previous observation that most of the ABA-inducible genes respond to drought- and high-salinity stresses (Shinozaki and Yamaguchi-Shinozaki 1999, 2000).

ABA-highly-inducible genes

Among the identified ABA-inducible genes, we found 27 genes that were highly induced by ABA (Fig. 1). In this study, we regarded the genes whose expression ratio was more than fivefold for ABA treatment and less than threefold for the other stress treatments as ABA-highly-inducible gene. Information on each gene is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 1). Among them, we found 6 cDNAs (RAFL09–13-A04, RAFL05–12-D20, RAFL05–12-E24, RAFL08–12-D06, RAFL09–17-L13 and RAFL11–09-C14) showing sequence identity with a putative sugar transporter (accession number AJ249968), a nodulin N21-like protein (accession number AF370525), a vegetative storage protein Vsp1 (accession number AF386930), a putative MtN3 protein (AY059108), a putative flavonol glucosyltransferase (accession number AF360262), and a hypothetical protein (accession number AL161495), and 4 cDNAs (RAFL08–18-K18, RAFL09–10-N21, RAFL09–10-L06, and RAFL07–13-L17) showing sequence similarity with a cysteine proteinase (accession number AF454956), a hypothetical protein (accession number AP003315), an EF-hand Ca^{2+}-binding protein CCD1 (accession number AF181661), and an inorganic pyrophosphatase-like protein (accession number T07399).

Characterization of the ABA-, drought-, cold-, or high-salinity-inducible genes

The ABA-inducible genes identified can be classified into two groups (Table 1 and Supplemental Table 1). The first group includes functional proteins, or proteins that probably function in stress tolerance. They are late-embryogenesis-abundant (LEA) proteins, heat shock proteins, KIN proteins, osmoprotectant-biosynthesis-related

proteins, carbohydrate-metabolism-related proteins, sugar transporters, detoxification enzymes, proteases, senescence-related proteins, protease inhibitors, and lipid transfer proteins (Table 1 and Supplemental Table 1). LEA proteins and heat shock proteins have been shown to be involved in protecting macromolecules and membranes (Shinozaki and Yamaguchi-Shinozaki 1999). Proline and sugars probably function as osmolytes in protecting cells from dehydration (Cushman and Bohnert 2000). KIN proteins may have a unique ability to neutralize ice nucleators and inhibit ice recrystallization (Holmberg and Bulow 1998). Sugar transporters are thought to function in transport of sugars through plasma membranes and tonoplast to adjust the osmotic pressure under stress conditions. Detoxification enzymes, such as glutathione S-transferase, are thought to be involved in protecting cells from active oxygens. Proteases, including cysteine proteases, are thought to be required for protein turnover and recycling of amino acids. Drought stress has been shown to accelerate leaf senescence which is characterized by many subcellular changes, including an increase in protease activities (Thomas and Stoddart 1980). The protease inhibitors may have a defensive role against the proteases. Lipid transfer proteins and fatty acid-metabolism-related genes may have a function in repairing stress-induced damage in membranes or changes in the lipid composition of membranes, perhaps to regulate the permeability to toxic ions and the fluidity of the membrane (Torres-Schumann et al. 1992; Holmberg and Bulow 1998).

The second group contains regulatory proteins, that is, protein factors involved in further regulation of signal transduction and gene expression that probably function in the stress response. They are various transcription factors, protein kinases, protein phosphatases, and other signaling molecules, such as calmodulin-related proteins or calcium-binding protein (Table 1 and Supplemental Table 1). Among 22 ABA-inducible genes for transcription factors, we found novel families of transcription factors, such as NAC and WRKY. They may function in regulating some stress-inducible genes. Among 4 ABA-inducible protein kinase genes, we found a receptor-like protein kinase gene. These regulatory proteins are thought to function in further regulating various functional genes under stress conditions.

Various genes involved in the metabolism of auxin and jasmonic acid (JA), and JA-regulated genes were identified as ABA-inducible genes (Table 1 and Supplemental Table 1), suggesting the link between auxin and JA, and ABA-signaling pathways. Also, aldehyde dehydrogenase, genes related to secondary metabolism, genes involved in various cellular metabolic processes, genes encoding membrane proteins and cytochrome P450 were identified as ABA-inducible genes (Table 1 and Supplemental Table 1). At present, the functions of most of these genes are not fully understood. Furthermore, we found many ABA-inducible genes whose functions are unknown.

ABA-inducible transcription factors

In this study, 22 genes for transcription factors were identified as ABA-inducible genes (Table 1 and Supplemental Table 1). This result suggests the existence of many transcriptional regulatory mechanisms in the ABA signal transduction pathways. Among these ABA-inducible transcription factors are one DREB family cDNA, two ERF family cDNAs, five zinc finger family cDNAs, one WRKY family cDNA, three MYB family cDNAs, one bHLH family cDNA, two bZIP family cDNAs, four NAC family cDNAs, and two homeodomain transcription factor family cDNAs. Information on the ABA-inducible transcription factors is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 1). We will study the function of these ABA-inducible transcription factors using knock-out mutants and transgenics, such as overexpression (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999), antisense suppression (Huang et al. 1999; Nanjo et al. 1999), and double-stranded RNA interference (RNAi; Chuang and Meyerowitz 2000; Smith et al. 2000). Furthermore, we will study the target genes of the transcription factors by cDNA microarray analyses of these mutants and transgenic plants.

Various expression profiles of ABA-inducible genes during ABA treatment

Expression profiles of ABA-, drought-, cold-, or high-salinity-stress-inducible genes were classified into several gene groups by the principal components analysis and K-means clustering using the GeneSpring software (Silicon Genetics). The ABA-inducible genes were classified into at least three groups from analysis of their expression profiles during ABA treatment (Fig. 2). In one group (Fig. 2A) containing the *rd22BP1 (AtMYC2)* gene, gene expression was rapid and transient after ABA treatment, reached a maximum at 1 h, and then decreased. In this group, we found cDNAs (RAFL05–12-B21 and RAFL07–07-B15) showing sequence identity with calmodulin-related protein (accession number T02109) and protein kinase AtPP-like protein (accession number T51783), and cDNAs (RAFL05–18-H12 and RAFL09–10-L06) showing sequence similarity with *Petroselinum crispum* transcription factor WRKY4 (accession number AF204925), and wheat EF-hand Ca^{2+}-binding protein CCD1 (accession number AF181661). These genes may function as regulatory protein factors involved in the regulation of ABA signal transduction and gene expression functioning in stress responses. In the second group, containing the *rd26* gene, gene expression increased after ABA treatment within 1 h and the expression level was kept relatively constant. In this group, we found three NAC transcription factor homolog cDNAs (RAFL09–15-E01, RAFL07–07-G15 and RAFL05–21-I22) showing sequence identity with RD26 (Taji et al. 1999), OsNAC6-like protein (accession number AF370617), and ATAF2 protein (accession num-

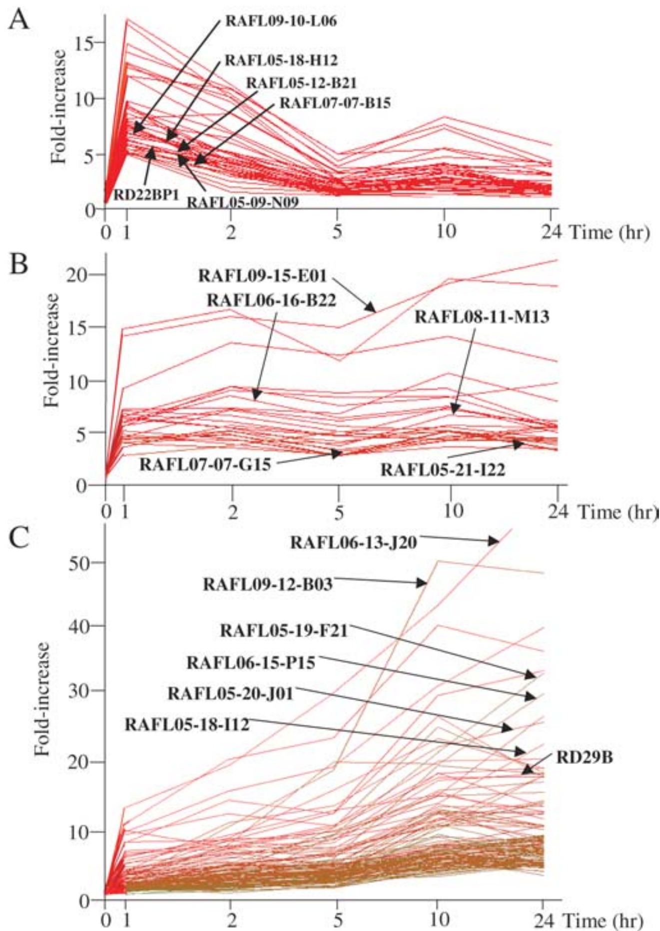

Fig. 2A–C Classification of ABA-inducible genes identified into three groups on the basis of expression pattern under ABA treatment. In one group (**A**) containing RD22BP1 gene, the expression was induced rapidly after ABA treatment, and reached a maximum 1 h after ABA treatment, then decreased. In the second group (**B**) containing the RD26 gene (= RAFL09–15-E01), gene expression increased after ABA treatment within 1 h and the expression level was kept relatively constant. In the third group (**C**) containing the *rd29B* gene, gene expression increased after ABA treatment and reached a maximum at 10 or 24 h. A list of the genes in the groups is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 8)

ber AL590346) and two cDNAs (RAFL06–16-B22 and RAFL08–11-M13) showing sequence identity with cold acclimation protein WCOR413-like protein (accession number AF283006) and putative zinc finger protein (accession number AY050915). In the third group, containing the *rd29B* gene, gene expression increased after ABA treatment and reached a maximum at 10 or 24 h. In this group we found cDNAs (RAFL06–13-J20, RAFL09–12-B03, RAFL05–19-F21, RAFL05–20-J01 and RAFL05–18-I12) showing sequence identity with LEA76 homologue type 1 protein (accession number X91919), beta-glucosidase-like protein (accession number T47838), senescence-associated protein (SAG29; accession number AL391711), and two unknown proteins (accession numbers AY039978 and AC005309) and a cDNA (RAFL06–15-P15) showing sequence similarity with a glyoxalase homolog (accession number AC007591).

Promoter analysis of ABA-inducible genes

As we identified the 5′-end of each mRNA based on the comparison of the full-length cDNAs and genomic sequences (Seki et al. 2002), the promoter sequences and *cis*-acting elements of each stress-inducible gene can be studied on the basis of full-length cDNA sequences. *Cis*-acting elements involved in ABA-inducible gene expression (Busk and Pages 1998; Shinozaki and Yamaguchi-Shinozaki 1999, 2000; Hattori et al. 2002), such as the ABA-responsive element (ABRE; Marcotte et al. 1989; Mundy et al. 1990), coupling elements (Shen and Ho 1995; Shen et al. 1996) and recognition sites of MYB and MYC (Iwasaki et al. 1995; Abe et al. 1997), have been reported. Information on the *cis*-acting elements observed in the promoter regions of the ABA-, drought-, cold or high-salinity-stress-inducible genes identified is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 4). We identified 245 ABA-inducible genes in this study and obtained the promoter sequence for 179 ABA-inducible genes (Supplemental Table 4). Among them, ten genes (RAFL09–10-H19, RAFL09–10-C03, RAFL08–12-H04, RAFL08–08-H23, RAFL07–15-D01, RAFL05–21-L12, RAFL05–20-J01, RAFL05–18-O21, RAFL05–09-N09 and RAFL04–18-B07) did not contain ABRE, coupling elements, and recognition sites of MYB and MYC in their promoters. These results suggest the existence of novel *cis*-acting elements involved in ABA-inducible gene expression. Members of the recently identified family of WRKY transcription factors have been implicated in the control of some stress responses (Eulgem et al. 2000). Most of the WRKY proteins were also shown to bind to W box-binding motif sequence, TTGAC(C/T) in vitro. Among the ten genes mentioned above, three contained the W box-binding motif in their promoter regions, suggesting that the W box-binding motif sequence is a novel *cis*-acting element involved in ABA-inducible gene expression.

ABA-, drought-, cold- or high-salinity-stress-downregulated genes

Analysis of ABA- or stress-downregulated as well as ABA- or stress-upregulated genes is important in understanding the molecular responses to abiotic stresses. In this study, we regarded the cDNAs as ABA- or stress-downregulated genes whose expression levels were less than 1/5 fold that of wild-type unstressed plants for at least at one time point during ABA-, drought- or high-salinity-stress treatment. As for cold-stress-downregulated genes, we found zero and four cDNAs with an expression ratio (cold/unstressed) less than 1/5 and 1/3, respectively, at least at one time point. Therefore, in this study, we regarded the cDNAs as cold-downregulated genes whose expres-

sion level was less than a half that of wild-type unstressed plants for at least at one time-point in cold-treated plants. A total of 34, 77, 79 and 70 genes were identified as ABA-, drought-, high-salinity- and cold-stress-downregulated genes by microarray analysis (Fig. 3 and Table 1). A list of these ABA-, drought-, cold-, or high-salinity-stress-downregulated genes is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 5).

The ABA-, drought-, cold- or high-salinity-stress-downregulated genes were classified into groups on the basis of their expression profiles (Fig. 3, Table 1). The classification is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Tables 6 and 7). Among the ABA-, drought-, cold- or high-salinity-stress-downregulated genes, we found many photosynthesis-related genes, such as ribulose 1,5-bisphosphate carboxylase small subunit (*rbcS*) and chlorophyll a/b-binding protein (*cab*; Supplemental Table 5). These results are consistent with a previous report that water stress inhibits photosynthesis (Tezara et al. 1999).

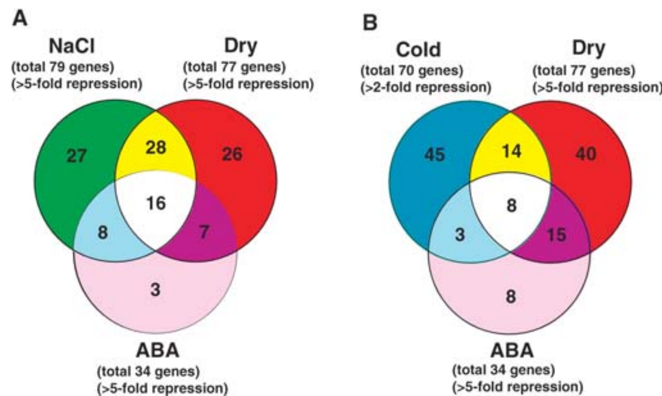

Fig. 3A, B Classification of the ABA-, drought-, cold-, or high-salinity-stress-downregulated genes identified on the basis of their expression pattern. **A** The ABA-, drought-, or high-salinity-stress-downregulated genes identified were put into the following seven groups: (1) ABA-highly-downregulated genes, (2) drought-stress-highly-downregulated genes, (3) high-salinity-stress-highly-downregulated genes, (4) ABA- and drought-stress-highly-downregulated genes, (5) ABA- and high-salinity-stress-highly-downregulated genes, (6) drought- and high-salinity-stress-highly-downregulated genes, and (7) ABA-, drought- and high-salinity-stress-downregulated genes. A list of the genes is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 6). **B** The ABA-, drought-, or cold-stress-downregulated genes identified were put into the following seven groups: (1) ABA-highly-downregulated genes, (2) drought-stress-highly-downregulated genes, (3) cold-stress-highly-downregulated genes, (4) ABA- and drought-stress-highly-downregulated genes, (5) ABA- and cold-stress-highly-downregulated genes, (6) drought- and cold-stress-highly-downregulated genes, and (7) ABA-, drought- and cold-stress-downregulated genes. A list of the genes is available at http://www.gsc.riken.go.jp/Plant/index.html (as Supplemental Table 7)

Conclusions and perspectives

In the present study, we identified 245 ABA-inducible genes, 299 drought-inducible genes, 54 cold-inducible genes, and 213 high-salinity-stress-inducible genes. These results show that full-length cDNA microarray analysis is a powerful tool for the identification of ABA- and stress-inducible genes. Using our full-length cDNA microarray, it is easy to isolate full-length cDNAs for further functional analysis (Seki et al. 2001b). Biochemical characteristics of the gene products are easily analyzed from the overexpression of the full-length cDNAs in bacteria or yeast. Functions of the gene products in plants can be analyzed by the overexpression of full-length cDNAs in transgenic plants. Moreover, promoter sequences and putative *cis*-acting elements of each gene can be predicted by comparing full-length cDNA sequences with the *Arabidopsis* genomic sequence.

In this study, we identified many ABA-inducible genes. However, the functions of many of them remain unknown. It is important to analyze the function of the ABA-inducible genes not only for further understanding the molecular mechanisms of the ABA-signaling cascade, stress tolerance and response of higher plants but also for improving the stress tolerance of crops by gene manipulation. Full-length cDNAs are useful resources for transgenic analyses, such as overexpression, antisense suppression, and double-stranded RNAi. Therefore, we will apply the identified full-length cDNAs to the transgenic analyses and biochemical analyses of the encoded proteins.

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