

Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold and high-salinity stresses using a full-length cDNA microarray

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Summary

Full-length cDNAs are essential for functional analysis of plant genes in the post-sequencing era of the *Arabidopsis* genome. Recently, cDNA microarray analysis has been developed for quantitative analysis of global and simultaneous analysis of expression profiles. We have prepared a full-length cDNA microarray containing ≈ 7000 independent, full-length cDNA groups to analyse the expression profiles of genes under drought, cold (low temperature) and high-salinity stress conditions over time. The transcripts of 53, 277 and 194 genes increased after cold, drought and high-salinity treatments, respectively, more than fivefold compared with the control genes. We also identified many highly drought-, cold- or high-salinity- stress-inducible genes. However, we observed strong relationships in the expression of these stress-responsive genes based on Venn diagram analysis, and found 22 stress-inducible genes that responded to all three stresses. Several gene groups showing different expression profiles were identified by analysis of their expression patterns during stress-responsive gene induction. The cold-inducible genes were classified into at least two gene groups from their expression profiles. DREB1A was included in a group whose expression peaked at 2 h after cold treatment. Among the drought, cold or high-salinity stress-inducible genes identified, we found 40 transcription factor genes (corresponding to $\approx 11\%$ of all stress-inducible genes identified), suggesting that various transcriptional regulatory mechanisms function in the drought, cold or high-salinity stress signal transduction pathways.

Keywords: *Arabidopsis thaliana*, full-length cDNA, cDNA microarray, abiotic stress.

Introduction

Recently, microarray technology has become a useful tool for the analysis of genome-scale gene expression (Eisen and Brown, 1999; Schena *et al.*, 1995). This DNA chip-based technology arrays cDNA sequences on a glass slide

at a density of up to 1000 genes cm^{-2} . These arrayed sequences are hybridized simultaneously to a two-colour, fluorescently labelled 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 demonstrated by analysing 48 *Arabidopsis* genes for differential expression in roots and shoots (Schena *et al.*, 1995). Reymond *et al.* (2000) analysed the expression in response to mechanical wounding and insect feeding, and defence-signalling pathways have been analysed using fungal pathogen and signalling 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. These abiotic stresses are severe limiting factors of plant growth and crop production. These abiotic stresses induce various biochemical and physiological responses in plants to acquire stress tolerance. The mechanism of the molecular response of higher plants against water stress has been analysed by studying a number of genes responding to drought, high-salinity and cold stress at the transcriptional level (Bray, 1997; Hasegawa *et al.*, 2000; Ingram and Bartels, 1996; Thomashow, 1999). The products of the stress-inducible genes can be classified into two groups: those that directly protect against environmental stresses; and those that regulate gene expression and signal transduction in the stress response (Bray, 1997; Hasegawa *et al.*, 2000; Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999). Stress-inducible genes have been used to improve the stress tolerance of plants by gene transfer (Bajaj *et al.*, 1999; Holmberg and Bülow, 1998). It is important to analyse 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. Expression analyses of drought-, cold- and high-salinity-inducible genes have shown the existence of several regulatory systems of stress-responsive gene expression. Some are dependent on abscisic acid (ABA), others are ABA-independent (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999), which indicate the existence of complex regulatory mechanisms between perception of abiotic stress signals and gene expression (Shinozaki and Yamaguchi-Shinozaki, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001).

Previously, we prepared an *Arabidopsis* full-length cDNA microarray using ≈ 1300 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 controlling stress-inducible gene expression (Seki *et al.*, 2001a). Our previous results showed that the full-length cDNA microarray is

useful to analyse the expression pattern of *Arabidopsis* genes under drought and cold stresses, and to identify target genes of stress-related transcription factors and potential *cis*-acting DNA elements by combining the expression data with the genomic sequence data.

Recently, we prepared a new full-length cDNA microarray containing ≈ 7000 independent full-length cDNA groups. In the present study, we applied the 7000 full-length cDNA microarray to identify new drought-, cold- or high-salinity-inducible genes, to analyse the time course of gene expression by drought, cold and high-salinity stresses, and to examine the differences and cross-talk between their signalling cascades. This is the first report on cross-talk of signalling cascades among drought, cold and high-salinity stresses using a global expression-profiling strategy. We also discuss functions of the stress-inducible genes in stress response and tolerance.

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, including drought-treated, cold-treated and unstressed plants, at various developmental stages from germination to mature seeds (Seki *et al.*, 1998; Seki *et al.*, 2001b). From the full-length cDNA libraries, we isolated ≈ 7000 independent *Arabidopsis* full-length cDNAs. We used a method described previously (Eisen and Brown, 1999) to array PCR-amplified cDNA fragments onto glass slides. We prepared a full-length cDNA microarray containing ≈ 7000 *Arabidopsis* full-length cDNAs, including the drought-inducible genes, *responsive to dehydration (rd)* and *early responsive to dehydration (erd)* (Taji *et al.*, 1999) as positive controls; the PCR-amplified fragment from lambda control template DNA fragment (Takara, Kyoto, Japan) as an external control; and the mouse nicotinic acetylcholine receptor epsilon-subunit (*nAChRE*) gene and the mouse glucocorticoid receptor homologue gene (which have no substantial homology to any sequences in the *Arabidopsis* database) to assess for non-specific hybridization as negative controls.

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

cDNA microarrays were hybridized with Cy3 and Cy5 fluorescently labelled probe pairs of drought-treated plants plus unstressed plants; cold-treated plants plus unstressed plants; and high-salinity-treated plants plus unstressed plants, prepared as described in Experimental procedures. Hybridized microarrays were

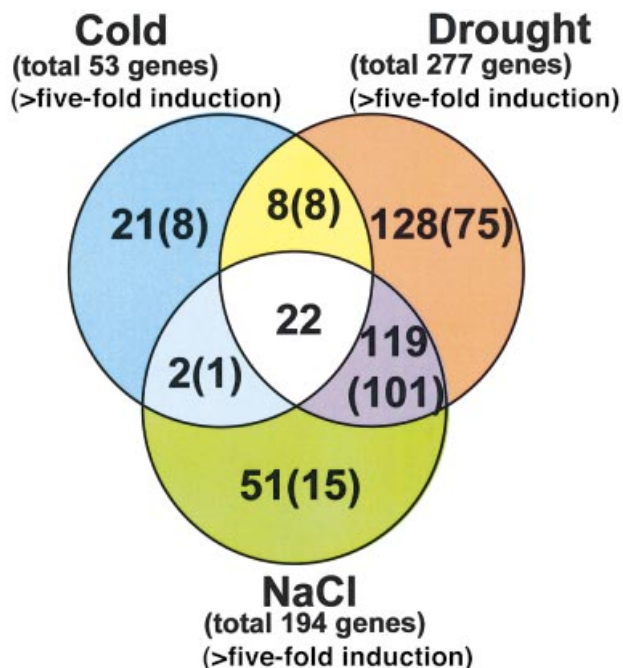


Figure 1. Classification of the drought, cold or high-salinity stress-inducible genes identified on the basis of microarray analyses.

In total, 277 drought-inducible, 53 cold-inducible and 194 high-salinity stress-inducible genes were identified by cDNA microarray analysis. The drought, cold or high-salinity stress-inducible genes identified were grouped into the following seven groups: (1) highly cold-stress-inducible; (2) highly drought-stress-inducible; (3) highly high-salinity-stress-inducible; (4) drought, cold and high-salinity stress-inducible; (5) genes that were highly induced by drought and high-salinity stress; (6) genes that were highly induced by drought and cold stress; (7) genes that were highly induced by cold and high-salinity stress. The number of genes whose expression ratio is more than fivefold for each stress treatment and less than fivefold for the other stress treatments is indicated. Numbers in parentheses represent the number of genes whose expression ratio is more than fivefold for each stress treatment and less than threefold for the other stress treatments. A list of the genes is available as supplementary material (Tables S1 and S2).

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 lambda control template DNA fragment (Takara) as an external control gene to equalize hybridization signals generated from different samples.

mRNAs from drought, cold or high-salinity stress-treated plants and wild-type unstressed plants were used for preparation of Cy3- and Cy5-labelled cDNA probes, respectively. These cDNA probes were mixed and hybridized with the cDNA microarray. To assess the reproducibility of the microarray analysis, we repeated the same experiment three times. Hybridization of

different microarrays with the same mRNA samples indicated good correlation. As for the genes with expression ratios (dehydration/unstressed; cold/unstressed; high-salinity/unstressed) greater than five times that of the lambda control template DNA fragment in at least one time-course point, we identified 277, 53 and 194 genes as drought-, cold- and high-salinity-inducible, respectively. As for the genes with expression ratios (dehydration/unstressed; cold/unstressed; high-salinity/unstressed) greater than three times in at least one time-course point, we identified 742, 229 and 554 genes as drought-, cold- and high-salinity-inducible, respectively. In this study we focused on the genes with expression ratios greater than five times compared with unstressed plants.

Drought, cold or high-salinity stress-inducible genes identified with the full-length cDNA microarray

In total, 277 drought-inducible, 53 cold-inducible and 194 high-salinity-inducible genes were identified by cDNA microarray analysis (Figure 1; Table 1). The number of cold stress-inducible genes was less than one-fifth, and one-third of that of drought-inducible genes and high-salinity stress-inducible genes, respectively, suggesting that drought stress or water deficit is the most severe limiting factor of plant growth. On the other hand, this may be due to the cold stress condition (transfer of plants to 4°C) that were used. The list and expression data for the drought-, cold- or high-salinity-inducible genes identified are available as supplementary material (Table S1). These genes included many reported drought, cold and high-salinity stress-inducible genes, which indicates that our cDNA microarray system functions properly to find drought, cold or high-salinity stress-inducible genes.

Relationship between each stress

The stress-inducible genes were classified into groups on the basis of their expression pattern (Figure 1). The results of the classification are available as supplementary material (Table S2). Analysis of overlapping on the Venn diagram showed that 22 genes were induced under all three stresses. Among these we found six well known stress-inducible genes including *rd29A/cor78*, *cor15a*, *kin1*, *kin2*, *rd17/cor47* and *erd10* (Bohnert *et al.*, 1995; Bray, 1997; Ingram and Bartels, 1996; Kiyosue *et al.*, 1994; Shinozaki and Yamaguchi-Shinozaki, 1997; Shinozaki and Yamaguchi-Shinozaki, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000; Taji *et al.*, 1999). A cDNA (RAFL05-19-G24) that encodes a constans-like protein (GenBank accession number Y10555) and eight cDNAs (RAFL04-09-B07, RAFL04-12-F24, RAFL04-10-D13, RAFL05-10-J09, RAFL05-

Table 1. Number of clones involved in different functional groups upregulated^a or downregulated^b by drought, cold or high-salinity stress

Functional category	Gene number	Representative gene names
Upregulated		
Transcription factor	40	Six DREB family transcription factors, two ERF family transcription factors, 10 zinc finger family transcription factors, four WRKY family transcription factors, three MYB family transcription factors, two bHLH family transcription factors, five NAC family transcription factors, three homeodomain family transcription factors, four bZIP family transcription factors, other family transcription factor
Osmoprotectant synthesis	11	Four galactinol synthases, P5CS, two raffinose synthases, two sucrose synthases, arginine decarboxylase, trehalose-6-phosphate synthase
Protein degradation	3	ERD1, RD21, ubiquitin conjugating enzyme
Protease inhibitor	1	Cysteine proteinase inhibitor
LEA protein	9	ERD10, RD17, Rab18, 6 LEA proteins
Hydrophilic protein	2	RD29A, RD29B
KIN protein	2	KIN1, KIN2
Detoxification enzyme	6	Two glutathione S- transferases, three peroxidases, phytochelatin synthase
Heat shock protein	4	Four heat-shock proteins
Lipid-transfer protein	4	Four lipid-transfer proteins
Transport protein, ion channel, carrier	11	ERD6, 2 ABC transporter proteins, oligopeptide transporter protein, potassium transporter protein, sodium sulfate or dicarboxylate transporter protein, neutral amino acid transport system protein, two mitochondrial dicarboxylate carrier proteins, Na ⁺ -dependent inorganic phosphate co-transporter protein, chloroplast protein import component protein
Water channel protein	1	RD28
Membrane protein	4	ERD4, 3 membrane-related proteins
Fatty acid metabolism	6	Three lipases, lysophospholipase, choline kinase, fatty acid elongase
Cytochrome P450	7	Seven cytochrome P450 proteins
Protein kinase	6	Two Ser/Thr protein kinases, two receptor-like protein kinases, two protein kinases
Protein phosphatase	3	ABI1, two protein phosphatase 2C-like proteins
Signalling	4	RD20, calmodulin-binding protein, calmodulin, two-component response regulator
Aldehyde dehydrogenase	2	Two aldehyde dehydrogenases
Plant defence	7	Endochitinase, disease resistance response protein, harpin-induced protein, nematode-resistance protein, beta-1,3-glucanase, polygalacturonase inhibiting protein, pathogen-inducible alpha-dioxygenase
Alcohol dehydrogenase	2	Two alcohol dehydrogenases
ABA biosynthesis	2	ATNCD3, zeaxanthin epoxidase
Ethylene biosynthesis	2	1-aminocyclopropane-1-carboxylate oxidase, ethylene-forming enzyme
JA biosynthesis	1	Lipoxygenase
JA-regulated genes	1	Myrosinase-binding protein
IAA metabolism	4	Three indole-3-acetate beta-glucosyltransferases, nitrilase
Auxin-regulated genes	2	IAA18, auxin-responsive GH3-like protein
Ionic homeostasis	1	Metallothionein-like protein
Senescence-related genes	2	ERD7, SAG29
Cellular metabolism	24	<i>p</i> -hydroxyphenylpyruvate dioxygenase, polygalacturonase, carboxyesterase, steroid sulfotransferase, 3-methylcrotonyl-CoA carboxylase precursor, saccharopine dehydrogenase, alanine : glyoxylate aminotransferase, tyramine hydroxycinnamoyl transferase, citrate synthase, aspartate aminotransferase, nodulin/glutamate-ammonia ligase, alpha-hydroxynitrile lyase, 12-oxophytodienoate-10-11-reductase, tyrosine aminotransferase, malate dehydrogenase, isovaleryl-CoA-dehydrogenase, two nodulin-related proteins, acyl-CoA oxidase, 3-ketoacyl-CoA thiolase, glyoxalase, succinate dehydrogenase, L-aspartate oxidase, 3-methylcrotonyl-CoA carboxylase non-biotinylated subunit
Carbohydrate metabolism	20	Glucose-6-phosphate/phosphate translocator precursor, two UDP-glucose glucosyltransferases, beta-galactosidase, two neutral invertases, UDP glucose 4-epimerase, UDP-glucose:flavonoid 7-O-glucosyltransferase, three beta-amylases, indole-3-acetate beta-glucosyltransferase, O-linked GlcNAc transferase, two beta-glucosidases, UDP-glucose : indole-3-acetate beta-D-glucosyltransferase, two glucosyltransferases, glucose and ribitol dehydrogenase, alpha-L-arabinofuranosidase
Secondary metabolism	12	Three strictosidine synthases, anthocyanidin synthase, reticuline oxidase, berberine bridge enzyme, mannitol dehydrogenase, four cinnamyl-alcohol dehydrogenases, cinnamoyl-CoA reductase

Table 1 (continued)

Functional category	Gene number	Representative gene names
Respiration	4	Alternative oxidase, mono-oxygenase 1, flavin-containing monooxygenase, flavin-binding monooxygenase
Protein synthesis	2	Eukaryotic translation initiation factor, eukaryotic release factor
Reproductive development	2	Pollen coat protein, pollen-specific protein
Cellular structure, organization and biogenesis	9	Three pectinesterases, extracellular dermalglycoprotein, pectin methylesterase, arabinogalactan, two endoxyloglucan transferases, blue copper-binding protein
DNA, nucleus	5	Topoisomerase, histone, nucleolin, regulator of chromosome condensation-like protein, nucleoid DNA-binding protein
Photosynthesis	1	Pyruvate-orthophosphate dikinase
RNA-binding protein	1	RNA-binding protein
Ferritin	1	Ferritin
Downregulated		
Transcription factor	2	Homeodomain family transcription factor, other family transcription factor
Photosynthesis	37	Four RBCS genes, two PsbS genes, nine photosystem II oxygen-evolving complex proteins, six photosystem I subunit proteins, 13 chlorophyll <i>a/b</i> -binding proteins, ribulose-bisphosphate carboxylase activase, Rubisco subunit-binding protein beta subunit precursor, sedoheptulose-1,7-bisphosphatase
Carbohydrate metabolism	11	Phosphoribulokinase, three glyceraldehyde 3 phosphate dehydrogenases, two aldolases, two fructose-bisphosphatases, three beta-glucosidases
Respiration	2	Two H ⁺ -transporting ATP synthases
RNA-binding protein	3	Three RNA-binding proteins
Lipase	1	Lipase/acylhydrolase
GTP-binding protein	1	GTP-binding protein
DNA-damage repair	1	DNA-damage-repair/toleration protein
Protein synthesis	1	50S ribosomal protein
Amino acid metabolism	1	Asparagine synthetase
Cell wall-related genes	5	Extensin, pectinesterase, three xyloglucan endotransglycosylases
Ethylene biosynthesis	1	S-adenosylmethionine synthase
Chloroplast protein	5	CP12-like protein, three 50S ribosomal proteins, peptidyl-prolyl <i>cis-trans</i> isomerase
Cytochrome P450	2	Two cytochrome P450 proteins
Protein degradation	1	Aspartic protease
Detoxification enzyme	5	Three glutathione S-transferases, 2-cys peroxiredoxin, ascorbate peroxidase
Cytoskeleton	1	Beta-tubulin
Cellular metabolism	4	Isopropylmalate synthase, malate dehydrogenase, hydroxypyruvate reductase, IAA-Ala hydrolase
DNA, nucleus	1	Deoxyribodipyrimidine photolyase

^aIn this study we regarded genes with expression ratios (dehydration/unstressed, cold/unstressed or high-salinity/unstressed) greater than five times that of lambda control template DNA fragment in at least one time-course point as dehydration, cold or high-salinity stress-inducible genes. ^bIn this study we regarded cDNAs whose expression level is less than one-fifth in at least one time-course point in drought-stressed and high-salinity-stressed plants of that in wild-type unstressed plants as genes downregulated by drought stress and high-salinity stress, respectively. We also regarded cDNAs whose expression level is less than one-half in at least one time-course point in cold-

19-O22, RAFL05-21-F13, RAFL08-11-P07 and RAFL08-15-M21) whose function is unknown were also included in this group.

Based on Venn diagram analysis, we analysed differences and cross-talk of gene expression among drought, cold and high-salinity stress responses. As shown in Figure 1, 277, 53 and 194 genes were identified as drought-, cold- and high-salinity-induced genes with greater than five times induction, respectively; 141 genes were induced by both drought and high salinity, whereas only 30 genes were induced by both drought and cold stress; and 24 genes were identified as cold-

and high-salinity-inducible genes. Seventy per cent of the high-salinity-inducible genes were also induced by drought stress, which indicates a strong correlation between drought and high-salinity stress responses. These results indicate the existence of greater cross-talk between drought and high-salinity stress signalling processes than those between cold and high-salinity stress signalling processes. These results are consistent with our previous observation on the overlap of drought- and high-salinity-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki, 1999; Shinozaki and Yamaguchi-Shinozaki, 2000).

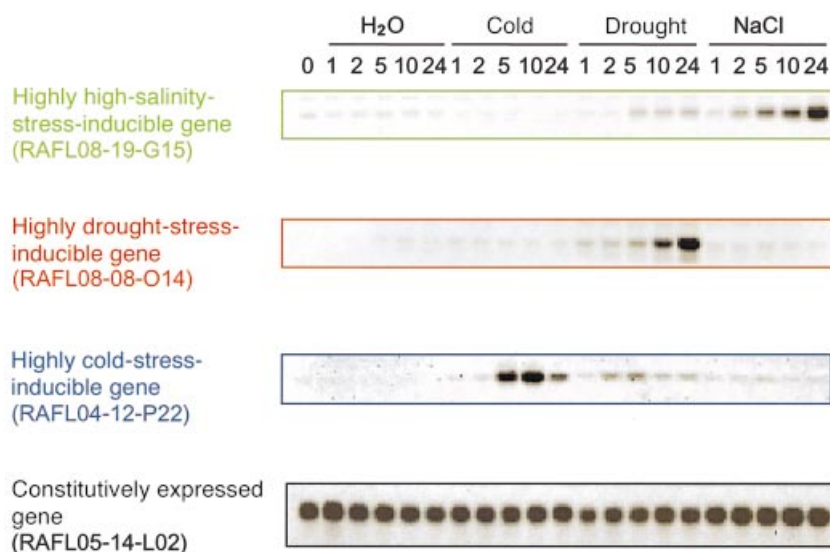


Figure 2. RNA gel-blot analysis of highly drought-, cold- or high-salinity-inducible genes and constitutively expressed genes. Results for a highly high-salinity-stress-inducible gene (RAFL08-19-G15); a highly drought-stress-inducible gene (RAFL08-08-O14); a highly cold-stress-inducible gene (RAFL04-12-P22); and a constitutively expressed gene (RAFL05-14-L02).

Highly stress-inducible genes and constitutively expressed genes

Among the stress-inducible genes identified, we found many genes that were highly induced by each stress. In this study we regard the genes whose expression ratio is more than fivefold for each stress treatment and less than threefold for the other stress treatments as highly stress-inducible genes. We identified 75 highly drought-stress-inducible genes; eight highly cold-stress-inducible genes; and 15 highly high-salinity-stress-inducible genes (Figure 1). Information on each gene is available as supplementary material (Table S2). Among these, we found a cDNA (RAFL08-08-O14) showing sequence similarity with a hypothetical protein (accession number AL035524) as a highly drought-stress-inducible gene; a cDNA (RAFL04-12-P22) showing sequence similarity with a hypothetical protein (accession number AC006193) as a highly cold-stress-inducible gene; and a cDNA (RAFL08-19-G15) showing sequence similarity with putative glucosyl transferase (accession number AC006282) as a highly high-salinity-stress-inducible gene. Expression profiles obtained by microarray analysis were consistent with those obtained by RNA gel-blot analysis (Figure 2).

In the genes that were highly induced by cold stress, genes for DREB1A (accession number AB007787) and beta-amylase (accession number AJ250341) existed (Tables S1 and S2). This result is consistent with our previous report (Seki *et al.*, 2001a). We also found a highly cold-stress-inducible cDNA (RAFL04-12-N15) showing sequence similarity with regulator of chromosome condensation-like protein (accession number T47697). In the genes that were highly induced by drought stress, genes in

the functional categories such as lipid-transfer protein, secondary metabolism-related genes and transport protein existed (Tables S1 and S2). In the genes that were highly induced by high-salinity stress, genes involved in carbohydrate metabolism existed (Tables S1 and S2). However, we could not identify any gene families in which all genes are specifically expressed only in a specific stress condition.

We identified three constitutively expressed genes with almost the same expression level under drought, cold and high-salinity stresses. Among these we found a cDNA (RAFL05-14-L02) identical with RUB1 conjugating enzyme (RCE1; accession number AF202771). This gene may be useful as an internal control gene in cDNA microarray analysis.

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

In this study we identified 277 drought stress-inducible genes, 53 cold stress-inducible genes and 194 high-salinity stress-inducible genes (Table 1; Table S1). These gene products can be classified into two groups. 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 (cold-inducible) proteins; osmoprotectant biosynthesis-related proteins; carbohydrate metabolism-related proteins; water-channel proteins; sugar transporters; potassium transporters; detoxification enzymes; proteases; senescence-related genes; protease inhibitors; ferritin; and lipid-transfer proteins (Table 1; Table S1). LEA proteins and heat-shock proteins have been shown

to be involved in protecting macromolecules such as enzymes and lipids (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 Bülow, 1998). Water-channel proteins and sugar transporters are thought to function in transport of water and sugars through plasma membranes and tonoplast to adjust the osmotic pressure under stress conditions. Potassium transporters may function in the transport of K^+ , which is an essential cofactor for many enzymes (Hasegawa *et al.*, 2000); or control K^+ uptake and regulate Na^+ uptake, which can be an important determinant of salinity tolerance (Bray, 1997). Detoxification enzymes such as glutathione S-transferase are thought to be involved in protection of cells from active oxygens. Proteases including cysteine proteases, CIP protease and ubiquitin-conjugating enzyme 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 perform a defensive role against the proteases. Ferritin may have a function in protecting cells from oxidative damage caused by various stresses, by sequestering intracellular iron involved in the generation of various reactive hydroxyl radicals through a Fenton reaction (Bajaj *et al.*, 1999). Lipid-transfer proteins and fatty acid metabolism-related genes may have a function in repair of stress-induced damage in membranes or changes in the lipid composition of membranes, perhaps to regulate permeability to toxic ions and the fluidity of the membrane (Holmberg and Bülow, 1998; Torres-Schumann *et al.*, 1992).

The second group contains regulatory proteins, that is, protein factors involved in further regulation of signal transduction and gene expression that probably function in stress responses. They are various transcription factors, protein kinases, protein phosphatases, enzymes involved in phospholipid metabolism, and other signalling molecules such as calmodulin-binding protein (Table 1; Table S1). Among 40 stress-inducible genes for transcription factors, we found novel families of transcription factors such as NAC and WRKY. These may function in regulation of some stress-inducible genes. Among six protein kinase genes, we found two receptor-like protein kinase genes. These regulatory proteins are thought to function in further regulating various functional genes under stress conditions.

Various genes involved in the metabolism of ethylene, jasmonic acid (JA) and auxin, and JA- or auxin-regulated genes were identified as drought-inducible genes (Table 1; Table S1), suggesting a link between ethylene, JA and

auxin, and drought stress-signalling pathways. Also, aldehyde dehydrogenase genes, genes related to secondary metabolism, genes involved in various cellular metabolic processes, genes encoding membrane proteins, and cytochrome P450 were identified as drought stress-inducible genes (Table 1; Table S1). At present the functions of most of these genes are not fully understood. We also found many drought stress-inducible genes whose functions are unknown.

Several similar studies have reported gene expression profile analysis under abiotic stress in other plant species such as rice (Bohnert *et al.*, 2001; Kawasaki *et al.*, 2001). They analysed the expression profiles using cDNA microarray including ≈ 1700 cDNAs under salt stress conditions in rice, and similarly reported that transcripts of protease inhibitor, beta-glucosidase, detoxification enzyme, water-channel protein and protein synthesis-related genes are upregulated after salt stress.

Drought, cold or high-salinity stress-inducible transcription factors

In this study, 40 genes (corresponding to $\approx 11\%$ of all stress-inducible genes identified) for transcription factors were identified as drought, cold or high-salinity stress-inducible genes (Table 1; Table S1). This result suggests the existence of many transcriptional regulatory mechanisms in the drought, cold or high-salinity stress signal transduction pathways. Among these stress-inducible transcription factors, there are six DREB family cDNAs, two ERF family cDNAs, 10 zinc finger family cDNAs, four WRKY family cDNAs, three MYB family cDNAs, two bHLH family cDNAs, four bZIP family cDNAs, five NAC family cDNAs, and three homeodomain transcription factor family cDNAs. These transcription factors regulate various stress-inducible genes co-operatively or separately. Information on each stress-inducible transcription factor is available as supplementary material (Table S1). Among these were transcription factors highly induced by each stress (Table S1). We will study the function of these stress-inducible transcription factors using knock-out mutants and transgenics, including overexpression (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu *et al.*, 1998); antisense suppression (Huang *et al.*, 1999; Nanjo *et al.*, 1999); and double-stranded RNA interference (RNAi) (Chuang and Meyerowitz, 2000; Smith *et al.*, 2000). We will also study the target genes of the transcription factors by cDNA microarray analyses of these mutants and transgenic plants.

Various expression profiles of stress-inducible genes during stress treatment

To evaluate the validity of expression profile analysis of gene expression during stress treatment using cDNA

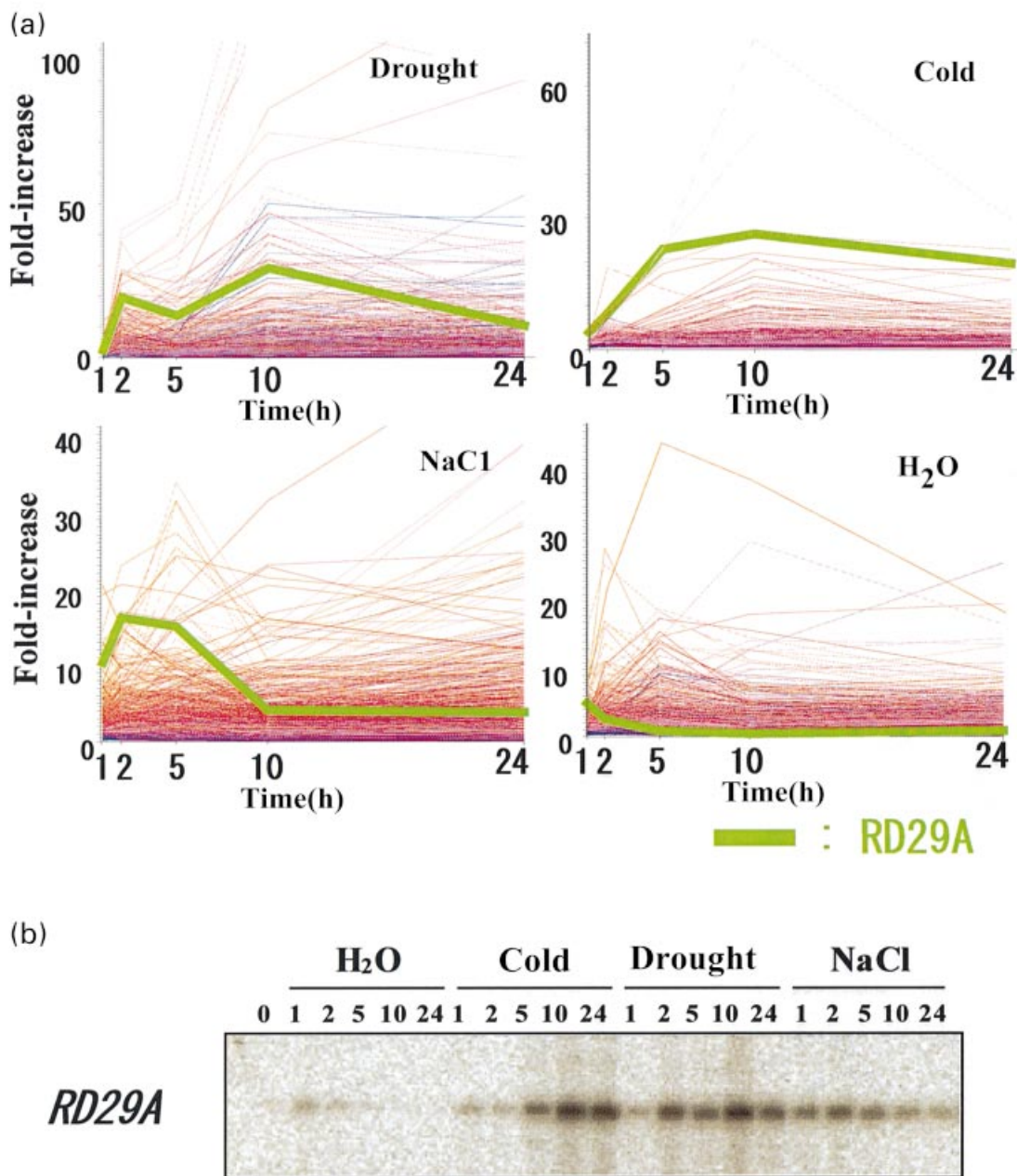


Figure 3. Expression pattern of ≈7000 *Arabidopsis* genes after drought, cold and high-salinity stress treatments. (a) Time course of changes in expression pattern of ≈7000 *Arabidopsis* genes after drought, cold and high-salinity stress treatments, and plants transferred to a plate containing water as control. The x axis shows time after each treatment; the y-axis shows the fold-increase in expression level. The expression pattern of drought-inducible gene RD29A is shown as green, bold bars. (b) RNA gel-blot analysis of the RD29A gene.

microarray, we performed RNA gel-blot analysis on 16 stress-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). An example of a drought-inducible gene, *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1993), is shown in Figure 3. Expression

profiles of drought, cold or high-salinity stress-inducible genes were classified by principal components analysis and *K*-mean clustering using the GENESPRING software. These expression profile analyses demonstrated that there are several gene groups which show different expression profiles.

Analysis of the expression profiles of cold-inducible genes during cold treatment (Figure 4) showed the exist-

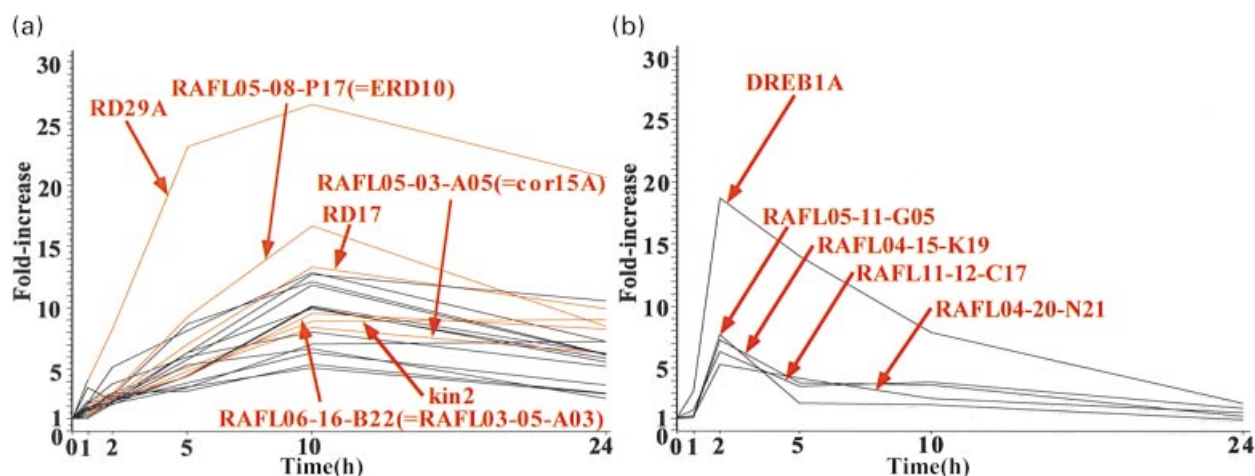


Figure 4. Classification of cold-inducible genes divided into two groups on the basis of expression pattern under cold stress.

In one group (b) containing *DREB1A*, RAFL04-15-K19, RAFL05-11-G05, RAFL04-20-N21 and RAFL11-12-C17, expression was induced rapidly after cold treatment, reached a maximum at 2 h after cold treatment, and then decreased. In the other group (a) containing *DREB1A* target genes such as *RD29A*, ERD10, *cor15A*, *RD17*, *kin2* and RAFL06-16-B22 (= RAFL03-05-A03), expression was induced after cold treatment within 2 h, and strongly expressed after 5 h.

ence of at least two groups that show different expression profiles. In one group containing the *DREB1A* gene, gene expression was rapid and transient in response to cold treatment, reached a maximum at 2 h, and then decreased (Figure 4). In the other group containing *DREB1A* target genes such as *rd29A*, *erd10*, *cor15A*, *rd17*, *kin2* and RAFL06-16-B22 genes, their expression increased slowly and gradually after cold treatment within 10 h (Figure 4). Expression of the *DREB1A* gene during cold stress preceded that of the *DREB1A* target genes. These results support our previous findings that *DREB1A* regulates the expression of *DREB1A* target genes such as *rd29A*, *erd10*, *cor15A*, *rd17*, *kin2* and RAFL06-16-B22 genes (Kasuga *et al.*, 1999; Seki *et al.*, 2001a). Among the genes whose expression was rapid and transient in response to cold treatment, we found cDNAs (RAFL04-15-K19 and RAFL05-11-G05) showing sequence identity with salt tolerance-related zinc-finger protein (accession number X95573) and mitochondrial uncoupling protein (accession number AJ286346) and cDNAs (RAFL04-20-N21 and RAFL11-12-C17) whose function is unknown.

Analysis of expression profiles of drought-inducible genes during drought stress treatment also exhibited the existence of at least two groups showing different expression profiles (data not shown). In one group, containing the *rd22BP1* and *DREB2A* genes, gene expression was rapid and transient after drought stress treatment; reached a maximum at 2 h; and then decreased. In this group we found cDNAs (RAFL04-15-K19, RAFL06-07-B08, RAFL09-12-N16, and RAFL08-16-D06) showing sequence identity with salt tolerance-related zinc finger protein (accession number X95573); SOS2-like protein kinase PKS5 (accession number AF339146); putative bHLH transcription factor

(accession number AC006418); AP2 domain-containing protein RAP2 (accession number NP_173638) and cDNAs (RAFL05-11-M11, RAFL05-18-H12 and RAFL05-14-I17) showing sequence similarity with an AP2 domain-containing protein (accession number AF332422); *Petroselinum crispum* transcription factor WRKY4 (accession number AF204925); and growth factor-like protein (accession number AF325104). These genes may function as regulatory protein factors involved in the regulation of signal transduction and gene expression functioning in stress responses. In the other group containing the *rd22* and *rd29A* genes, gene expression slowly and gradually increased after drought stress treatment within 2 h and reached a maximum at 10 h, then decreased gradually.

Promoter analysis of stress-inducible genes

In this study, 22 genes were identified as drought, cold and high-salinity stress-inducible genes. As we identified the 5'-end of each mRNA based on comparison of full-length cDNAs and genomic sequences, the promoter sequences and *cis*-acting elements of each stress-inducible gene can be studied on the basis of full-length cDNA sequences. Table 2 summarizes ABRE, DRE and CCGAC core sequences observed in the 19 drought, cold and high-salinity stress-inducible genes identified by the cDNA microarray analysis. Among these, 16 genes (84%) contain DRE (TACCGACAT) or DRE-related CCGAC core motif in their promoters (Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999; Yamaguchi-Shinozaki and Shinozaki, 1994), suggesting that the 16 genes were regulated by the *DREB1*/CBF or *DREB2* transcription factors. Also, 15 genes (79%) contained ABRE

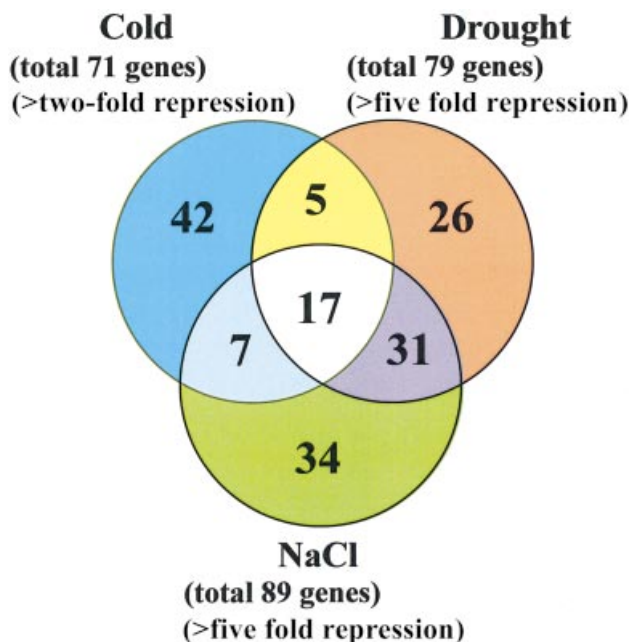


Figure 5. Classification of the drought, cold or high-salinity stress-downregulated genes identified on the basis of microarray analyses. The drought, cold or high-salinity stress-downregulated genes were grouped in the following seven groups: (1) highly cold-stress-downregulated genes; (2) highly drought-stress-downregulated genes; (3) highly high-salinity stress-downregulated genes; (4) drought, cold and high-salinity-stress-downregulated genes; (5) genes that were highly downregulated by drought and high-salinity stress; (6) genes that were highly downregulated by drought and cold stress; and (7) genes that were highly downregulated by cold and high-salinity stress. A list of the genes is available as supplementary material (Tables S4 and S5).

(PyACGTG(T/G)C) in their promoters, suggesting that they were ABA-inducible.

We identified 53 cold-inducible genes in this study and obtained the promoter sequence for 41 genes. Among these, nine genes (RAFL07-18-O08, DREB1A, RAFL06-16-M17, RAFL08-17-G11, RAFL05-14-E16, RAFL05-20-O23, DREB2A, RAFL05-11-G05 and RAFL06-15-O23) did not contain DRE or DRE-related CCGAC core motif in their promoters. These results suggest the existence of novel *cis*-acting elements involved in cold-inducible gene expression.

Among the 351 drought, cold or high-salinity stress-inducible genes, we constructed a promoter database on 279 genes. Data on ABRE, DRE and CCGAC core sequences observed in the promoter regions of the 279 genes are available as supplementary material (Table S3).

Drought, cold or high-salinity stress-downregulated genes

Analysis of stress-downregulated as well as stress-upregulated genes is important in understanding molecular responses to abiotic stresses. In this study, we regarded

the cDNAs as stress-downregulated genes whose expression levels are less than one-fifth in at least one time-course point during drought or high-salinity stress treatment than in wild-type unstressed plants. As for cold stress-downregulated genes, we found 0 and 4 cDNAs with expression ratio (cold/unstressed) less than one-fifth and one-third, respectively, in at least one time-course-point. Therefore, in this study we regarded the cDNAs as cold-downregulated genes whose expression level is less than half in at least one time-course-point in cold-treated plants than in wild-type unstressed plants. A total of 79, 89 and 71 genes were identified as drought, high-salinity and cold stress-downregulated genes by microarray analysis (Figure 5; Table 1). The list and expression data on these drought, cold or high-salinity stress-downregulated genes is available as supplementary material (Table S4). The drought, cold or high-salinity stress-downregulated genes were classified into groups on the basis of their expression profiles (Figure 5). The results of the classification are available as supplementary material (Table S5). Among the drought, cold or high-salinity stress-downregulated genes, we found many photosynthesis-related genes such as ribulose 1,5-bisphosphate carboxylase small subunit (*rbcS*); chlorophyll *a/b*-binding protein (*cab*); and the components of photosystems I and II (Table 1; Table S4). These results are consistent with a previous report that water stress inhibits photosynthesis (Tezara *et al.*, 1999).

Conclusions and perspectives

In the present study we identified 277 drought-inducible, 53 cold-inducible, and 194 high-salinity stress-inducible genes. These results show that full-length cDNA microarray analysis is a powerful tool for the identification of stress-inducible genes. We first compared the signalling cascades of the three abiotic stresses (drought, cold and high-salinity stress) using a global expression-profiling technique. Our results indicated the existence of greater cross-talk between drought and high-salinity stress signalling processes than between cold and high-salinity stress signalling processes.

Using our full-length cDNA microarray, it is easy to isolate full-length cDNAs for further functional analysis. Biochemical characteristics of the gene products are easily analysed from overexpression of the full-length cDNAs in bacteria or yeast. Functions of the gene products *in planta* can be analysed by 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. We are planning to isolate more than 15 000 independent *Arabidopsis* full-length cDNAs and prepare a new cDNA microarray using the cDNA clones for identifying new stress-inducible genes,

Table 2. ABRE, DRE and CCGAC core sequences ^aobserved in the promoter regions of the drought, cold and high-salinity-stress-inducible genes ^bidentified by microarray analysis.

Gene	ABRE (PyACGTG(T/G)C)	DRE (TACCGACAT)	CCGAC Core Motif (CCGAC)
RAFL04-10-D13	GACGTGGC (-99 to -106) ^c		AGCCGACAT (-128 to -120) TTCCGACAC (-65 to -73)
RAFL04-12-F24			AGCCGACAT (-340 to -348) CGCCGACAT (-201 to -209)
RAFL04-17-F01	TACGTGTC (-66 to -59)	TACCGACAT (-226 to -218) TACCGACAT (-169 to -161)	GACCGACTA (-276 to -268) AGCCGACAC (-132 to -124)
RAFL04-20-N09	TACGTGTC (-920 to -913)		GACCGACAT (-996 to -988) AGCCGACCA (-967 to -959) TACCGACTT (-162 to -154)
RAFL05-03-A05	CACGTGGC (-132 to -125)		GGCCGACAT (-361 to -353) GGCCGACCT (-184 to -176) AACCGACAA (-416 to -424)
RAFL05-08-P17	GACGTGGC (-998 to -991) CACGTGGC (-805 to -798)		GACCGACAT (-966 to -958) CACCGACCG (-173 to -165) GACCGACCG (-169 to -161) GACCGACGT (-165 to -157)
RAFL05-10-J09	CACGTGGC (-897 to -904) AACGTGGC (-736 to -743)	TACCGACAT (-754 to -762)	GACCGACAG (-869 to -861)
RAFL05-14-E16	TACGTGTC (-141 to -134)		
RAFL05-19-G24	CACGTGGC (-82 to -89)		GACCGACTT (-674 to -666)
RAFL05-19-O22			GACCGACCC (-114 to -106)
RAFL05-20-O23	CACGTGGC (-90 to -83)		
RAFL05-21-F13	TACGTGTC (-799 to -806)		TGCCGACTC (-71 to -63) AACCGACCG (-224 to -232) GACCGACGT (-132 to -140)
RAFL06-08-N16		TACCGACAT (-120 to -112)	ATCCGACAT (-719 to -711)
RAFL06-16-B22	CACGTGGC (-74 to -67) CACGTGGC (-69 to -76)	TACCGACAT (-415 to -407)	TGCCGACAT (-806 to -798)
RAFL08-11-P07	CACGTGGC (-232 to -239)		CGCCGACAT (-326 to -318) GGCCGACAT (-140 to -132)
RAFL08-15-M21			GACCGACAC (-71 to -63) TGCCGACAT (-155 to -163)
RAFL08-19-H17	AACGTGGC (-990 to -983)		GACCGACCG (-211 to -203) GACCGACAT (-207 to -199)
RAFL04-17-B12 ^d	CACGTGGC (-79 to -72)	TACCGACAT (-138 to -130)	
DREB2A ^d	TACGTGTC (-817 to -824) TACGTGTC (-108 to -115)		

^aABRE, DRE, and CCGAC core sequences observed in 1000 bp upstream regions of the 5' termini of the cDNA clones isolated are listed.

^bThese genes represent those whose expression ratio is more than fivefold for drought, cold and high-salinity stress treatments.

^cNumbers in parentheses indicate the nucleotide beginning at the 5' terminus of the cDNA clone isolated. Minus signs indicate that the nucleotide exists upstream of the 5' terminus of the putative transcription start site.

^dThe promoter sequences of the RAFL04-17-B12 and DREB2A were analysed using the cDNA sequences of kin2 (GenBank accession number: X55053) and DREB2A (GenBank accession number: AB007790), respectively. The promoter sequences of the following cDNA clones were not analysed because we have not obtained the 5'-end sequences as of June 1 2001: RAFL04-09-B07, RAFL05-17-B13 and RAFL08-08-L20.

new hormone-inducible genes, new tissue-specific-expressed genes, and new target genes of stress-related transcription factors.

In this study we identified many stress-inducible genes. However, the functions of many remain unknown. It is important to analyse function of the stress-inducible genes, not only for further understanding of the molecular mechanisms of stress tolerance and responses 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 (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Liu *et al.*, 1998); antisense suppression (Huang *et al.*, 1999; Nanjo *et al.*, 1999); and double-stranded RNA interference (RNAi) (Chuang and Meyerowitz, 2000; Smith *et al.*, 2000). Therefore we will apply the identified full-length cDNAs

to the transgenic analyses and biochemical analyses of the encoded proteins.

Experimental procedures

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, MI). The plants were grown for 3 weeks in a growth chamber at 22°C under 16 h light/8 h dark. Dehydration, cold and high-salinity stress treatments were applied essentially as reported previously (Yamaguchi-Shinozaki and Shinozaki, 1994). For dehydration treatments, plants were removed from the agar and desiccated in plastic dishes at 22°C under dim light (0.7–0.8 $\mu\text{mol sec}^{-1} \text{m}^{-2}$). For cold treatments, plants were grown under dim light (0.7–0.8 $\mu\text{mol sec}^{-1} \text{m}^{-2}$) at 4°C. For high-salinity stress treatments, plants were transferred to and grown hydroponically in water containing 250 mM NaCl under dim light (0.7–0.8 $\mu\text{mol sec}^{-1} \text{m}^{-2}$). The plants were subjected to stress treatments for various periods (1, 2, 5, 10 and 24 h), then frozen in liquid nitrogen for further analyses. Total RNA was prepared using TRIZOL Reagent (Life Technologies, Rockville, MD), and mRNA was prepared using a mRNA isolation kit (Miltenyi Biotec, Auburn, CA, USA).

CDNA clones

In the cDNA microarray analyses, we used ≈ 7000 cDNA sequences representing RIKEN *Arabidopsis thaliana* full-length (RAFL) cDNA clones (Seki *et al.*, 2002) isolated from full-length cDNA libraries (Seki *et al.*, 1998); and the drought- and cold-inducible genes, *responsive to dehydration* (*rd*) and *early responsive to dehydration* (*erd*) (Taji *et al.*, 1999). As external controls, PCR-amplified fragment from lambda control template DNA fragment (TX803; Takara, Kyoto, Japan) was used. As negative control, two DNAs derived from the mouse nicotinic acetylcholine receptor epsilon-subunit (*nAChRE*) gene and the mouse glucocorticoid receptor homologue 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) are available from RIKEN Bioresource Center (Seki *et al.*, 2002).

Sequence analysis

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

Amplification of cDNA inserts

In the cDNA microarray analyses we used ≈ 7000 cDNA clones and the lambda control template DNA fragment (TX803; Takara) as an external control. As negative controls, DNA derived from the mouse nicotinic acetylcholine receptor epsilon-subunit (*nAChRE*) gene and the mouse glucocorticoid receptor homologue gene were used. The vectors used for cDNA library construction were modified lambda ZAP (Carninci *et al.*, 1996) and lambda FLC-1 (Carninci *et al.*, 2001). Inserts of cDNA clones were amplified by PCR using primers complementary to vector sequences flanking both sides of the cDNA insert, as described previously (Seki *et al.*, 2001a). PCR products were precipitated in ethanol and the DNA was resuspended at $\approx 2 \mu\text{g} \mu\text{l}^{-1}$ in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). One aliquot of each finished reaction was electrophoresed on a 0.7% agarose gel to confirm amplification quality and quantity. Two μl of DNA were mixed with 2 μl 2 \times polymer (Fuji Photo Film Co., Kanagawa, Japan) and 4 μl dimethyl sulfoxide (DMSO) (Kishida Chemical Co., Osaka, Japan) at least 10 times using an automatic dispenser (model EDS-384S; Biotech Co., Ltd, Tokyo, Japan).

CDNA microarray preparation

PCR products were arrayed from 384-well microtitre plates onto a micro slide glass (model Super Aldehyde substrates; Telechem International Inc., Sunnyvale, CA) using the microarray stamping machine (model SPBIO2000; Hitachi Software Engineering Co. Ltd, Tokyo, Japan). Of the 2 μl of PCR products (500–1000 $\text{ng} \mu\text{l}^{-1}$) from 384-well microtitre plates, 0.5 nl was deposited per slide on 48 slides with a spacing of 300 μm . The slides were post-processed according to the manufacturer's protocol (Telechem International Inc.). The printed slides were dried (RH < 30%) and subjected to UV cross-linking. They were rocked in 0.2% SDS for 2 min three 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 PBS (Life Technologies) and 90 ml 100% ethanol] was poured into the glass chamber. The slide racks were shaken gently for 5 min, then transferred into a chamber containing 0.2% SDS and shaken gently for 1 min three 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, NJ). The reverse transcription reaction was performed in a 20 μl volume containing 1 μg denatured poly(A)⁺ RNA with 1 ng lambda poly(A)⁺ RNA-A (TX802; Takara) as an external control, 50 $\text{ng} \mu\text{l}^{-1}$ oligo-(dT) 12–18-mer (Life Technologies); 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) in 1 \times Superscript first-strand buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl₂, 20 mM DTT) (Life Technologies). Following incubation at 42°C for 35 min, 200 units of Superscript II was 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 labelling and the other with Cy5 labelling) were combined. The samples were placed in a Microcon 30 microconcentrator (Millipore). TE buffer (250 µl) was added and spun for 10 min in a benchtop microcentrifuge at high speed to a volume of 10 µl, and the flow-through product was discarded. This step was repeated four times. The probes were then collected by inverting the filter and spun for 5 min. Several microlitres of distilled water was 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⁻¹ 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, left at room temperature for 5 min, then used for hybridization. The slides were placed in a sealed hybridization cassette (Telechem International Inc.) 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. Then the slides were immediately dried by centrifugation (1 min at 2500 g). Slides were scanned with a ScanArray 4000 (GSI Lumonics, Oxnard, CA) as described previously (Seki *et al.*, 2001a).

Data analysis

For the microarray data analysis, image analysis and signal quantification were performed with QUANTARRAY version 2.0 (GSI Lumonics). 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 homologue gene. Genes showing a signal value <1000 (typically twice the mean background value) in both Cy3 and Cy5 channels were not considered for the analyses. Lambda control template DNA fragment (TX803; Takara) was used as external control to equalize hybridization signals generated from different samples. Gene-clustering analysis was performed with the GENESPRING software (Silicon Genetics, San Carlos, CA).

RNA gel-blot analysis

Isolated total RNA was also used for RNA gel-blot hybridization. The isolated total RNA was denatured with the mixture of 2.15 M formaldehyde and 50% formamide, then fractionated by electrophoresis on a 1.0% agarose gel that contained 2.2 M formaldehyde according to the protocol described earlier (Maniatis *et al.*, 1982), and subsequently capillary transferred to nylon membrane using 20 × SSC. The membrane was probed with DIG-labelled 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 the mixture of 2 × SSC and 0.1% SDS for 5 min at room temperature, then twice with the mixture of 0.1 × SSC and 0.1% SDS for 15 min at 68°C and subjected to detection of DIG-labelled RNA probes using the DIG Chemiluminescent Detection Kit (Roche Molecular Biochemicals).

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Supplementary Material

The supplementary material is available from <http://www.gsc.riken.go.jp/plant/index.html>.

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