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ORIGINAL PAPER

Comparison of long-term up-regulated genes during induction of freezing tolerance by cold and ABA in bromegrass cell cultures revealed by microarray analyses

Toshihide Nakamura · Junshi Yazaki · Naoki Kishimoto · Shoshi Kikuchi · Albert J. Robertson · Lawrence V. Gusta · Masaya Ishikawa

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Abstract A suspension culture of bromegrass (*Bromus inermis* Leyss cv. Manchar) provides a unique system for studying cold hardiness mechanism, where freezing tolerance can be independently induced by two different stimuli, cold and exogenous abscisic acid (ABA). Freezing tolerance was induced in bromegrass cells by exposure to low temperatures for 7 days (LT_{50}: −8.1 °C) and also by incubation with ABA for 7 days at 25 °C (LT_{50}: −12.3 °C) compared to the control cells grown without ABA at 25 °C (LT_{50}: −3.9 °C). To characterize freezing tolerance mechanisms involved in both systems, bromegrass genes up-regulated after 7 days of low temperature or ABA treatment were analyzed using rice cDNA microarrays. In total, 300 and 479 clones were identified as cold- and ABA-inducible genes, respectively. Among them, 149 clones were induced both by cold and ABA treatments. Several genes were newly found to be cold-inducible from our microarray results. The increased expression of 8 selected genes in the microarray results was confirmed by Northern blot analyses. Northern blot analyses of three of these genes during 13 days of exposure to 4 °C confirmed a gradually increased and long-term up-regulated expression during cold acclimation. In agreement with physiological and protein studies, these two cold hardiness induction systems exhibited fairly different transcriptome profiles: cold stress was characterized by triggering numerous genes involved in protein degradation/synthesis and RNA maintenance in addition to cold stress-related genes whilst ABA treatment was characterized by inducing numerous genes involved in seed formation and functional genes related to biotic and abiotic stresses.

Keywords ABA · Bromegrass · Cold acclimation · Cold hardiness · Gene expression · Freezing tolerance · Microarray

Introduction

Freezing tolerance is an extremely complex trait involving many physiological and morphological processes and is usually induced or increased by exposure to non-lethal low temperatures in cold hardy plants (cold acclimation). To understand the mechanisms, alterations during cold acclimation have been extensively studied at physiological, cellular and molecular levels (Thomashow 1999). Many of the processes and molecular mechanisms, however, still remained ambiguous. One instance is the role of abscisic acid (ABA).

Physiological studies have shown that endogenous ABA levels increase in plants during cold acclimation (Daie and Campbell 1981; Chen et al. 1983; Lalk and Dörffling 1985; Lång et al. 1994), which allowed to hypothesize that ABA may work as a second messenger in cold acclimation process. Exogenous application of ABA increased freezing

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T. Nakamura · M. Ishikawa (✉)
Divsion of Plant Sciences, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
e-mail: isikawam@affrc.go.jp

J. Yazaki · N. Kishimoto · S. Kikuchi
Agrogenomics Research Center, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan

A. J. Robertson · L. V. Gusta
Department of Plant Sciences, University of Saskatchewan, Saskatoon, SK S7N5A8, Canada

tolerance in some plants (Lång et al. 1989; Mäntylä et al. 1995; Veisz et al. 1996) and suspension or callus cultures (Chen and Gusta 1983; Keith and McKersie 1986; Orr et al. 1985; Ishikawa et al. 1990; Galiba et al. 1993; Dallaire et al. 1994).

In accordance with this hypothesis, ABA-deficient mutant (*aba1*) and ABA-insensitive mutant (*abi1*) of Arabidopsis plants following exposure to cold-acclimating conditions were found to be less cold hardy compared to wild type plants (Heino et al. 1990; Gilmour and Thomashow 1991; Mäntylä et al. 1995). But the results have to be interpreted with caution as these mutants have much less vigor than wild-type plants, which may result in lower capability to cold-acclimate (Thomashow 1999). Comparison of COR gene expression in these mutants and wild-type plants revealed that some COR genes were highly responsive to exogenous ABA but their expression by low temperature was not necessarily mediated by ABA (Gilmour and Thomashow 1991; Nordin et al. 1991). More recently, molecular analyses of low temperature-responsive genes have demonstrated that there are ABA-dependent and ABA-independent transcriptional pathways (Yamaguchi-Shinozaki and Shinozaki 1994) and even cross-talks between these pathways (Ishitani et al. 1997). The role of ABA in activation of low temperature responses is considered to be minor than it was thought (Thomashow 1999). Yet, question still remains unanswered as to how simple exogenous application of ABA alone can induce high levels of cold hardiness in some plant systems and how it should be interpreted.

Physiological characterization has revealed that in many systems, ABA best induces freezing tolerance at an ambient temperature and does not require lengthy exposure to low temperatures and does not necessarily accompany growth cessation. In suspension cultures of bromegrass (*Bromus inermis* Leyss), which has been extensively studied, high levels of ABA-induced freezing tolerance were best achieved at 25–30 °C and only marginally at 5–15 °C (Ishikawa et al. 1990). Interestingly, cultures incubated with ABA showed high growth rates equivalent to the control cultures at 25–30 °C. The levels of ABA-induced freezing tolerance were linearly correlated with the amount of culture growth attained in the presence of ABA at 5–30 °C. This is contrary to cold acclimation process in cold hardy plants where the growth is usually suppressed during induction of freezing tolerance. When ABA-treated cells were transferred to an ABA-minus medium and incubated at 4 °C, they quickly lost the attained freezing tolerance (Ishikawa et al. 1990). These observations allowed us to consider that freezing tolerance induction by ABA is fairly different from the one by cold. ABA-induced cold hardiness has attracted attention as a novel system where mechanisms directly involved in freezing tolerance could be discriminated from those involved in metabolic adaptation to low temperatures.

In bromegrass cell cultures, freezing tolerance can also be induced by exposure to low temperatures (Ishikawa et al. 1990). Comparison of cold-induced freezing tolerance and ABA-induced freezing tolerance has been considered to provide a unique approach to understand cold hardiness mechanisms. Protein analysis revealed that ABA induced accumulation of specific, unique polypeptides (Robertson et al. 1987, 1988, 1995; Wilen et al. 1996), most of which were not induced or increased by cold stress. From physiological and morphological analyses, Ishikawa et al. (1990) considered that behavior of bromegrass cells during ABA-induced cold-hardening was similar to that of seed formation process. Several genes responsive to ABA (Lee and Chen 1993; Robertson et al. 1994; Wu et al. 2004) and to cold (Nakamura et al. 2008) were isolated from bromegrass. However, these genes represented only a part of gene expression induced by ABA or cold and it remains unknown how they contribute to freezing tolerance induced and whether ABA-induced cold hardiness involves similar mechanisms as low temperature-induced cold hardiness or entirely different mechanisms. To understand these, more global approach on gene expression analysis during cold and ABA-induced cold-hardening is required.

Microarray technology has emerged as a powerful tool that allows simultaneous monitoring of the expression levels of numerous genes and is useful for analyzing genome-wide regulatory networks of a stress response. Arabidopsis microarrays were also applied for gene expression analysis during salt stress in salt cress (*Thellungiella halophila*), a halophyte closely related to Arabidopsis (Taji et al. 2004). Rice cDNA microarrays were used to analyze mRNA expression in Fe-deficient barley roots (Negishi et al. 2002) while barley cDNA-macroarrays were successfully used to study cold-induced transcriptome in wheat (Kocsy et al. 2010). In heterologous microarray analyses, highly conserved gene sequences between the related probe and target plant species allow the heterologous probes (microarray spots) to hybridize the target mRNAs to identify and analyze the expression of orthologs in the target plant. This has been shown to be a valid approach for cross-species transcriptional profiling of rather distantly related species: a *Pinus* array for *Picea* (van Zyl et al. 2002) and *Arrabidopsis* arrays for poplar and oat (Horvath et al. 2003). Since bromegrass and rice are phylogenically related in the recent molecular analysis of Poaceae (Kellogg, 2001), rice microarrays seemed useful for analyzing gene expressions in bromegrass.

The objective of this study is to roughly characterize and compare transcriptome profiles involved in two physiologically different systems of freezing tolerance induction, stimulated by cold and ABA using bromegrass cell

cultures, which may provide a unique approach. For this purpose, the use of rice microarrays for bromegrass mRNA (heterologous system) was likely a reasonable compromise. Our focus was on long-term expressed genes that may be involved in freezing tolerance mechanisms rather than transiently expressed genes. Therefore microarray analyses were done with cells exposed to cold or ABA for 7 days. We attempted to identify up-regulated genes that were cold-specific, ABA-specific, or common to both treatments and characterize these systems. Furthermore, several cold- and ABA-responsive genes were isolated from bromegrass suspension cells.

Materials and methods

Plant material and culture conditions

A non-embryogenic cell suspension culture of smooth bromegrass (*Bromus inermis* Leyss cv. Manchar) was used in this work and was subcultured biweekly in ER medium (pH 5.8, 0.5 mg/mL 2,4-D) at 25 °C as described previously (Ishikawa et al. 1990; Nakamura and Ishikawa 2006).

Cold treatment was conducted by exposure of 3 day-old cultures (initiated from 1.5 g of cell inoculum in 50 mL of ER medium and grown at 25 °C) to 15 °C for a day and then to 4 °C for 6 days. Bromegrass cells (1.5 g) were inoculated in 50 mL of ER medium in the presence (ABA treatment) and absence (control) of 50 μM ABA for 7 days at 25 °C.

Freezing tolerance determination

Following each treatment, the cells were aseptically harvested by filtering on an 80 μm mesh and washed with 250 mL of sterile water to remove extracellular sugars that may affect freeze survival. About 0.26 g fresh weight of cells (control, ABA- or cold-treated) were placed in plastic centrifuge tubes (15 mL, IWAKI) with 0.2 mL of sterile water. After equilibration at −3 °C for 30 min, freezing was initiated by touching the tubes with a liquid nitrogen-cooled steel rod. The cells were held at −3 °C overnight, then cooled at 2 °C/h to −12 °C and then 5 °C/h to −35 °C. The cells were removed at designated temperatures and thawed at 4 °C before being incubated in 15 mL of ER medium at 25 °C for 7 days to assess the viability by regrowth capacity. After 7 days of reculture, the cells were harvested and washed with distilled water prior to determination of dry weight by oven-drying at 70 °C for 2 days. Viability of cells was calculated from the regrowth data (average of three determinations) and freezing tolerance was expressed as LT_{50}, the temperature at which 50 % of the cells were killed as described previously (Ishikawa et al. 1995).

Isolation of mRNA

Total RNA extraction from cells grown under various conditions was described previously (Nakamura et al. 1997). Poly(A)$^+$ RNA was purified using oligo(dT) latex beads (Oligotex dT30 Super, Takara) according to the method specified by the supplier.

Microarray analysis

A DNA chip with 8987 randomly selected ESTs prepared by the microarray project in Japan (Kishimoto et al. 2002), with a system developed by Amersham Bioscience, was used for microarray analysis. The identity and the accession number of each of the EST clones are listed at http://microarray.rice.dna.affrc.go.jp/. Preparation of DNA chips, and microarray analysis methods (experimental procedures and data analysis) were previously described by Yazaki et al. (2000, 2003). Briefly, 8987 cDNA clones

Table 1 Numbers of the functional categories of transcripts in bromegrass cultured cells increased by cold and/or ABA treatments

Functional categories	Cold	ABA	Cold and ABA
Transcription	10 (6.6 %)	15 (4.5 %)	9 (6.0 %)
Signal transduction	2 (1.3 %)	19 (5.8 %)	5 (3.4 %)
Stress, cell rescue and defence	15 (9.9 %)	38 (11.5 %)	21 (14.1 %)
Protein synthesis	46 (30.5 %)	5 (1.5 %)	5 (3.4 %)
Proteolysis	14 (9.3 %)	12 (3.6 %)	6 (4.0 %)
Transporter	6 (4.0 %)	8 (2.4 %)	8 (5.4 %)
RNA function	10 (6.6 %)	5 (1.5 %)	3 (2.0 %)
Metabolism	12 (7.9 %)	32 (9.7 %)	17 (11.4 %)
Others	26 (17.2 %)	65 (19.7 %)	22 (14.8 %)
Unknown	10 (6.6 %)	131 (39.7 %)	53 (35.6 %)
Total	151	330	149

Table 2 Cold-inducible genes identified by microarray analyses

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
Transcription				
23	OsNAC3 protein	D21888, AU075972	2.20	0.215
1866	OsNAC (nam-like protein)	C28162, AU166955	2.13	0.228
174	SPF1 protein (WRKY)	D22355	2.12	0.160
505	SAR DNA binding protein	C26339, AU108271	3.12	0.172
863	SAR DNA binding protein	AU062680, AU166544	2.22	0.244
1440	Zinc finger, RING-type domain containing protein	C27309, AU108846	2.23	0.322
4161	Transcription factor (CONSTANS-like)	C74270, AU075710	2.09	0.107
6803	GAMYB-binding protein	AU162546, AU032332	2.04	0.019
7216	GAMYB-binding protein	AU056013, AU056014	2.26	0.120
8722	Leucine zipper protein (OSE2)	AU108443, AU174305	3.15	0.039
Signal transduction				
1720	Guanine nucleotide-binding protein	AU063182, AU161073	2.10	0.170
8569	Cell division cycle protein 48	D40314, AU097105	2.11	0.005
Stress, cell rescue and defence				
155	Cytosolic glutathione reductase	D22281, AU102120	2.03	0.145
1164	Aluminum-induced protein	AU068708, AU166745	2.30	0.061
1818	Endo-1,4-beta-glucanase (Cel3)	C28145, C97417	4.22	0.428
6993	Class I endochitinase (CHT1)	AU077766, AU077767	2.68	0.251
1200	p23 co-chaperon	AU068730, AU166780	2.10	0.200
1409	Mitochondrial chaperonin HSP60-2	AU166836	3.28	0.226
7157	HSP70	AU055968, AU055969	2.60	0.117
1631	Abscisic stress ripening protein 1	C27651, AU100845	2.05	0.163
1883	Alternative oxidase (AOX1)	AU161214, AU161215	3.88	0.428
3331	Luminal binding protein	C19292, AU094398	2.45	0.211
7185	Protein kinase-like domain containing protein	AU056090, AU101713	2.74	0.011
7126	Stress up-regulated Nod 19 family protein	AU055916, AU055917	2.33	0.114
8589	Germin-like protein 2 (GER2)	D40492, AU174256	2.84	0.019
8799	Germin-like protein 1 (GER1)	D41881, AU102006	2.97	0.018
8662	Tau class GST protein 4	D41191, AU101971	2.08	0.011
Protein synthesis				
124	Elongation factor 1-gamma	D22238, C96682	3.48	0.235
159	Elongation factor 1-gamma	AU102118, C96770	2.74	0.096
370	Elongation factor G	C26157	2.05	0.080
1306	Elongation factor 1-alpha	AU068886, AU068887	2.47	0.287
145	eIF-2 beta subunit	D22267, C96749	2.05	0.167
360	Translation initiation factor eIF-4F	C26116, AU108206	2.20	0.153
362	Initiation factor 2B epsilon	AU068057, AU068058	2.19	0.170
7680	Translation initiation factor 5A (eIF-5A)	AU057661, AU057662	2.11	0.161
166	Ribosomal protein L10 family protein	D22265, C96747	2.68	1.652
312	60S acidic ribosomal protein P0	AU062501, AU092216	2.71	0.135
423	60S ribosomal protein L5	AU062537, AU092295	3.71	0.254
668	60S acidic ribosomal protein P0	AU062621, AU108592	2.32	0.147
1235	Ribosomal protein L15	C26906, AU108726	3.08	0.310
1243	60S ribosomal protein L21	AU062795	2.30	0.315
1370	60S ribosome protein L19	AU166818	3.74	0.455
1373	60S ribosomal protein L7A	C27171	2.82	0.307

Table 2 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
1493	Ribosomal protein L44	C27363, AU166873	2.23	0.269
1517	60S ribosomal protein L36	C27416, AU100817	2.33	0.180
1535	Ribosomal protein L7A	AU069044	3.57	0.456
1554	Ribosomal protein L14	AU160837, AU160836	2.25	0.161
1632	60S ribosomal protein L28	C27632, AU166910	3.53	0.233
5098	60S ribosomal protein L5	AU030880	2.80	0.052
5445	60S ribosomal protein L23	AU164921, AU164922	2.33	0.212
5869	60S ribosomal protein L24	AU031656, AU031657	2.43	0.151
6974	60S ribosomal protein L18	AU071053, AU162616	3.28	0.029
7027	60S acidic ribosomal protein P1a	AU078049	3.78	0.050
8837	60S ribosome protein L9	AU161725, AU033203	2.07	0.053
1178	40S ribosomal protein S3	C26823, C96939	2.25	0.220
1213	40S ribosomal protein S9	C26867	3.83	0.571
1214	40S ribosomal protein S19	AU068715, AU166751	2.29	0.300
1255	40S ribosomal protein S15	AU166787	2.48	0.176
1286	40S ribosomal protein S11	C26971, AU082622	2.26	0.176
1400	40S ribosomal protein S6	AU166837	2.64	0.282
1633	40S ribosomal protein S13	AU160917, C97150	2.89	0.000
1758	40S ribosomal protein S30	AU175083	2.16	0.085
1769	40S ribosomal protein S14	C27962, AU092781	2.20	0.201
1875	40S ribosomal protein S4	C28221, C97561	4.46	0.522
2066	40S ribosomal protein S4	AU063511, AU093083	2.51	0.238
3674	40S ribosomal protein S18	C20368, AU164049	2.15	0.172
4917	40S ribosomal protein S4	AU065658, AU030276	2.46	0.499
4977	40S ribosomal protein S4	AU162297, AU030395	2.10	0.225
5059	40S ribosomal protein S4	AU065166, AU095133	3.40	0.330
5946	40S ribosomal protein S9	D39008, AU164577	2.07	0.114
6692	40S ribosomal protein S3A	AU065343, AU095507	3.19	0.002
8498	40S ribosomal protein S8	D40030, AU174222	3.56	0.147
1141	Ribosome-associated protein p40 (laminin receptor protein)	C26793, C96897	2.39	0.206
Proteolysis				
150	Oryzain gamma chain	D22275, C96775	2.15	0.195
153	26S proteasome subunit RPN3a	AU166288, C96736	2.13	0.176
303	26S proteasome regulatory particle	C25958, AU075361	2.29	0.159
7928	26S proteasome subunit RPN7	AU162969, AU032728	2.21	0.115
178	Peptidase M1	D22330, AU166299	2.02	0.182
182	Proteasome subunit alpha type 7	D22304, C96795	2.14	0.074
8512	Alpha 1-2 subunit of 20S proteasome	D40057, AU101938	2.35	0.026
254	Aspartic proteinase oryzasin 1	AU166323, AU166324	4.01	0.305
8800	Aspartic proteinase oryzasin 1	AU174342	2.39	0.024
6483	GCN5-related N-acetyltransferase domain containing protein	D24912, AU031945	2.22	0.081
8548	Cullin-like proteins	D40303, AU108298	2.54	0.125
1273	Ubiquitin-conjugating enzyme	C26934, AU092416	2.50	0.079
4500	Methionine aminopeptidase-like protein	AU064619, AU081312	2.23	0.049
8568	Peptidase A1, pepsin family protein	D40297, AU078162	2.39	0.473
Transporter				
318	ADP/ATP carrier protein	C25988, AU092221	3.48	0.258

Table 2 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
470	Cystinosin	C26318, AU108261	2.39	0.144
870	Plasma membrane H + -ATPase	AU062677	2.87	0.213
956	Cation-transporting ATPase	AU068485	2.55	0.280
2784	ADP,ATP carrier protein	C72701	2.37	0.245
6013	Mitochondrial import receptor	D24014	2.89	0.040
RNA function				
52	Threonyl-tRNA synthetase	D22042	3.57	0.238
59	Glu-prolyl-tRNA aminoacyl synthetase	AU166273, AU166274	2.63	0.023
753	Arginyl-tRNA synthetase	AU068279, AU166497	2.69	0.240
1397	Lysyl-tRNA synthetase	AU068952	3.73	0.086
1724	Aminoacyl-tRNA synthetase	C27922, AU092761	2.03	0.252
6793	Phenylalanyl-tRNA synthetase	AU032328, AU173815	2.29	0.223
1271	Fibrillarin (Sb21)	C97002, C97003	3.72	0.350
1905	Splicing factor 3A	C28224, AU166966	2.04	0.080
2715	RNA helicase	AU173553, AU172520	3.52	0.318
5018	Poly(A)-binding protein	AU065739, AU030550	2.93	0.062
Metabolism				
10	NAD-dependent epimerase/dehydratase family protein	AU067845	2.01	0.650
85	Pyruvate kinase	C25794, AU166272	2.08	0.113
118	Acyl-CoA independent ceramide synthase	D22135, AU092070	2.24	0.187
120	ADP glucose pyrophosphorylase	D22125, AU092067	2.01	0.083
137	Branched-chain amino acid aminotransferase	AU176396	2.53	0.233
666	Aldehyde dehydrogenase	C96862, AU108608	2.05	0.142
1499	Pyruvate dehydrogenase E1 alpha subunit	C27394	2.17	0.376
4429	Glucose/ribitol dehydrogenase family protein	AU166136, AU172869	2.55	0.019
4501	Nucleoside diphosphate kinase (NDPK)	AU064629, AU088640	2.27	0.219
5149	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	AU162325, AU030945	2.06	0.296
5248	Succinyl CoA ligase	AU065269, AU164718	2.05	0.091
8484	Ribonucleoside-diphosphate reductase	D39682, AU174212	2.56	0.013
Others				
51	AMP deaminase	D22037, AU092025	2.08	0.162
75	Casein kinase II, alpha chain (CK II)	D22035, AU075984	2.04	0.158
189	Cellulose synthase catalytic subunit (Ath-B)	AU176398	2.41	0.151
400	COG complex component, COG2 family protein	C26184, AU100672	7.85	1.334
1236	Hydroxyproline rich glycoprotein PsHRGP1	AU062789, AU108719	2.68	0.191
1247	Histone H2A	C26889, AU092411	2.14	0.246
7692	Histone H2A	AU082124	2.04	0.010
1375	DNA polymerase V family protein	C27179	2.26	0.262
2827	Cytochrome C reductase-processing peptidase subunit I	AU163596, AU172551	2.27	0.406
3342	Alpha-tubulin	C19295	2.22	0.230
6768	Cyclophilin-40	AU070213, AU173793	2.55	0.026
1426	Aux/IAA protein	C27204, AU108818	2.08	0.221
1510	Chemocyanin precursor	C27387, AU166879	3.90	0.359
2860	Seed imbibition protein (Sip1)	C98710	5.16	0.451
3177	Ankyrin repeat containing protein	C73464, AU090558	2.08	0.103

Table 2 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
4451	Alpha-expansin OsEXPA5	C91869	2.27	0.493
4478	ATP synthase delta chain	AU172874, AU172875	2.58	0.159
6253	Vacuolar ATP synthase subunit B isoform 1	D24374, AU173232	2.13	0.256
6787	Proliferating cell nuclear antigen (PCNA) (Cyclin)	AU032312, AU032313	2.54	0.026
7084	NAD-dependent epimerase/dehydratase family protein	AU078717, AU055794	2.18	0.071
7122	T-complex protein 1, epsilon subunit	AU055805, AU055806	2.20	0.172
7200	Prefoldin domain containing protein.	AU056030, AU056031	2.04	0.131
7086	Adaptin ear-binding coat-associated protein 1 NECAP-1 family protein	AU055841, AU055842	2.26	0.014
7991	Translocon-associated protein (TRAP)	D48174, AU174044	2.38	0.129
8543	Putative 5-3 exonuclease domain containing protein	D40189, AU174241	3.83	0.082
1343	U box domain containing protein	C27098, AU166815	2.37	0.309

from japonica rice (cv. Nipponbare) were printed on an aluminum-coated, DMSO-optimized glass slide in duplicate. Target mRNAs purified from bromegrass samples were labeled by reverse transcription with Cy5 and hybridized with the microarrays using Atlas Glass Fluorescent Labeling Kit (Clontech). Images of the microarrays scanned were analyzed using Fujifilm ArrayGauge software. Following an appropriate background subtraction and global normalization, the signal intensities of different samples (cold or ABA-treated vs. untreated control) were compared. Data presented are the mean of three determinations, each of which was the average of duplicated spots. Genes that were up-regulated more than twofold (the means with SD) after 7 days of cold or ABA treatments compared to the untreated control were shown in Tables 1, 2, 3 and 4.

Preparation of a cDNA library and screening procedure

Complementary DNA libraries of cold-stressed and ABA-treated bromegrass cells were constructed using Lambda ZAP II XR Library Construction kit (Stratagene) according to the method specified by the supplier. For screening the cDNA library, rice cDNAs listed in Table 5 were obtained from the Rice Genome Research Program (RGP: http://rgp.dna.affrc.go.jp/) and used as probes.

Northern blot analysis

Northern blot analysis was performed as described previously (Nakamura et al. 1997). Rice full-length cDNAs and gene specific regions of bromegrass cDNAs were used as probes for Northern blot analysis.

Results

Effects of low temperature and ABA treatments on the freezing tolerance of bromegrass cells

Freezing tolerance was measured for bromegrass cells grown at low temperature (15 °C for 1 day and 4 °C for 6 days without ABA) and for cells grown at 25 °C for 7 days with 50 μM ABA or without ABA (control). Fig. 1 shows the viability of cells frozen from −3 to −35 °C as determined by the regrowth method. LT_{50} for control, cold and ABA-treated cells were −3.9, −8.1 and −12.3 °C, respectively. Low temperature and ABA treatments increased freezing tolerance by more than 4 and 8 °C, respectively, as compared to the control.

Although the experimental conditions were not exactly the same (e.g., the size of cell culture inoculum), essentially similar levels of increase in freezing tolerance were obtained with cold- and ABA-treated bromegrass cells in our previous studies (Ishikawa et al. 1990, 1995, 2006).

Microarray analyses

To reveal global gene expression changes during these increases in freezing tolerance, we isolated mRNA from low temperature-treated (7 days), ABA-treated (25 °C for 7 days) and untreated (25 °C for 7 days) bromegrass cells and used for analysis with rice cDNA microarrays containing 8987 cDNAs of rice EST. In preliminary experiments to check the hybridization efficiency of bromegrass mRNA, we observed only marginal differences in the signal intensities between bromegrass mRNA and rice mRNA following hybridization with the rice cDNA

Table 3 ABA-inducible genes identified by microarray analyses

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
Transcription				
958	Zinc finger, RING-type domain containing protein	AU062731, AU100769	3.22	0.102
1056	Zinc finger, RING-type domain containing protein	AU166689, AU166688	2.54	0.098
1083	Zinc finger, RING-type domain containing protein	AU068650	2.15	0.093
1429	Zinc finger, RING-type domain containing protein	C27234, AU166841	2.26	0.593
4403	Zinc finger transcription factor	C91783, AU098331	3.40	1.158
5619	Zinc finger protein (constans)	AU174681, AU174680	2.01	0.166
5626	Zinc finger, U1-type domain containing protein	AU174726, AU174725	2.97	0.286
1097	Growth-regulating factor 3	AU175045, AU175082	2.06	1.236
1500	WRKY transcription factor 68	AU069036	2.08	0.540
7561	BZIP protein	AU173935	2.05	0.166
1109	Basic-leucine zipper (bZIP) transcription factor domain containing protein	AU108123, AU108122	2.59	0.064
1148	Basic helix-loop-helix dimerisation region bHLH domain containing protein	AU068689	2.17	0.100
4140	DRE binding factor 2	C74267, AU091364	2.02	0.082
7806	EREBP1	C23567, AU032440	2.32	0.205
8820	Homeodomain leucine zipper protein	AU181051	3.84	0.455
Signal transduction				
944	Calmodulin-binding protein	AU062718, AU100766	2.22	0.028
3109	Calcium-binding protein	AU176459	2.26	0.113
1317	Calmodulin-domain protein kinase CDPK	C97040	2.22	0.072
5707	Calmodulin-binding diacylglycerol kinase	AU174835, AU174834	3.39	0.323
1430	Protein kinase domain containing protein	C27237, AU166842	2.60	0.115
3105	Serine/threonine-protein kinase PBS1	C22520, C22521	2.07	0.109
3312	Protein kinase GhCLK1	C19228, AU101305	8.38	0.130
3411	Protein kinase-like domain containing protein	AU172707	2.47	0.043
5621	Protein kinase (OSK1)	AU174720, AU174721	2.06	0.148
5769	Calreticulin	AU174939, AU174938	2.21	0.418
5803	Calcineurin B protein	AU174993, AU174992	2.35	0.215
8586	NPK1-related protein kinase 1L	D40523	2.30	0.256
4260	SNF1 kinase complex anchoring protein	AU162269, AU029543	2.56	0.125
4437	RIO kinase	C74522, AU094812	2.28	0.027
6581	Calmodulin-binding receptor-like kinase	AU032002, C22606	4.31	0.389
3156	RabGAP/TBC domain containing protein	AU082491, AU082492	2.20	0.068
3310	Phospholipase D p1	C19214, AU091665	2.56	0.125
6742	Phospholipase D	AU032201, AU032202	2.09	0.060
5690	Response regulator 7	AU174811, AU174810	2.10	0.168

Table 3 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
Stress, cell rescue and defence				
196	Thaumatin-like protein	C25827, AU166305	3.11	0.156
671	Peroxidase precursor	C22706, C19120	2.59	0.110
4264	Peroxidase 1	AU029511, AU029512	2.45	0.128
4367	Peroxidase	C91753, AU029760	2.27	0.110
819	Phenylalanine ammonia-lyase	AU108112, AU172307	2.16	0.016
1195	Universal stress protein (Usp) family protein	AU166753, AU166754	3.07	0.008
1196	Avr9 elicitor response protein-like	C26847, AU166758	2.05	0.086
3477	Embryo-specific protein 1 (ATS1)	C19796, AU172721	3.04	0.158
1031	Class III chitinase RCB4	AU062762	2.16	0.019
5676	Phosphatidylinositol transfer protein-like, N-terminal domain containing protein	AU174771	2.15	0.184
6061	Probenazole-inducible protein PBZ1	AU162395	2.05	0.141
6773	Plant disease resistance response protein family protein	AU032324, AU032325	2.79	0.254
8158	Wound-induced protein	AU175148	2.55	0.253
385	HSP protein	AU062530, AU166382	3.60	0.108
1467	20 kDa chaperonin, chloroplast precursor	AU108867, AU108868	2.40	0.137
5870	Copper chaperon (CCH)	D38996, AU164533	2.09	0.192
8533	Chaperone protein dnaJ	D40141, AU174229	2.39	0.216
918	Water stress protein	AU166557	3.61	0.120
1024	Drought induced 19 family protein	AU166643, AU166642	2.14	0.016
2379	Group 3 LEA (Type I) protein	AU174470, AU174471	5.85	0.271
2388	Late embryogenesis abundant protein repeat containing protein	AU174486, AU174487	6.38	0.222
3528	Late embryogenesis abundant (LEA) group 1 family protein	C19868, AU163829	2.07	0.031
5716	Embryogenesis-associated protein (EMB8)	AU174838, AU174839	2.23	0.138
4319	ABA/WDS induced protein family protein	C91707	2.63	0.126
3017	Thioredoxin domain 2 containing protein	AU172638, AU172639	2.20	0.096
6984	UVB-resistance protein UVR8	AU071037, AU101703	2.02	0.126
1169	Phytochelatin synthase	AU108710, C96924	2.89	0.114
1293	Acetone-cyanohydrin lyase	AU176423	2.33	0.091
7948	Allene oxide cyclase precursor	AU032719, AU175145	2.19	0.230
1408	1-Cys peroxiredoxin	C27238, AU166843	2.94	0.014
1952	1-Cys peroxiredoxin	C28576, AU161332	4.51	1.248
1718	Germin-like protein subfamily 2 member 4 precursor	C97263, AU161109	2.16	0.075
3130	Germin-like protein subfamily T member 1 precursor	C73414, AU101261	2.18	0.070
1772	Hydroxyanthranilate hydroxycinnamoyltransferase 3	C97340, C97341	2.49	0.138
8615	Hydroxyanthranilate hydroxycinnamoyltransferase	D40780	2.33	0.272

Table 3 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
4433	Desaturase/cytochrome b5 protein	AU070006, AU172864	2.81	0.106
7003	Zinc induced protein	AU078041, AU078042	2.15	0.199
8698	Copper/zinc-superoxide dismutase	D41409, AU174303	2.03	0.196
Protein synthesis				
1009	Oligosaccharyltransferase	AU166641, AU166640	2.82	0.080
1309	Protein translation factor SUI1 homolog	AU068878, AU068879	2.51	0.025
3266	60S ribosomal protein L10	AU162207, C99106	2.21	0.104
5732	50S ribosomal protein L28	AU174942	2.71	0.260
4373	Elongation factor 1B gamma	AU064476, AU172839	2.48	0.107
Proteolysis				
139	C13 endopeptidase NP1	D22244	4.19	0.258
1911	Cysteine protease	C28244, C97587	4.50	0.360
1099	Metalloendopeptidase	AU068654, AU068653	3.09	0.065
4244	Serine proteases	AU029523, AU029522	2.73	0.126
5266	Ubiquitin ligase protein mib	C74925, AU173032	2.82	0.012
5730	Lipoprotein signal peptidase	AU174904	2.02	0.166
5915	Aminopeptidase N	AU173095, AU173096	2.12	0.125
7394	Nitrogen fixation protein nifU	AU162670, AU056311	2.09	0.163
7487	C13 endopeptidase NP1 precursor	AU056660, AU056661	3.50	0.296
7615	Peptidase C19, ubiquitin carboxyl-terminal hydrolase 2 family protein	AU057332, AU057333	2.45	0.138
8483	Peptidase A1, pepsin family protein	D39673, AU161608	2.78	0.864
8505	LON1 protease (LON1)	D40049, AU161627	2.45	0.242
Transporter				
1046	Mitochondrial substrate carrier family protein	AU166658, AU166657	2.37	0.051
1900	Amino acid transporter	AU063280, AU166959	3.32	0.144
3338	Sodium/hydrogen exchanger family protein	C19314, AU172696	2.43	0.117
3340	Phosphonates transport ATP-binding protein	C19349	2.55	0.148
4049	Major intrinsic protein	C74098	2.40	0.090
4372	Aquaporin	AU166095, AU029746	2.51	0.106
4384	Protein transport protein Sec61	C91764, AU029774	2.12	0.071
8723	Phospholipid transfer protein	D41439, AU174307	2.11	0.226
RNA function				
1008	RNA binding protein	AU166699, AU166698	2.46	0.060
1050	SC35-like splicing factor SCL30	AU166707, AU166706	2.31	0.081

Table 3 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
1242	DNA-directed RNA polymerase II 23 kDa polypeptide	AU166789, AU166790	2.31	0.057
5638	DNA-directed RNA polymerase beta chain	AU174743, AU174742	2.21	0.255
3354	Glutaminyl-tRNA synthetase	C19360	2.06	0.075
Metabolism				
356	Citrate synthase, glyoxysomal precursor	C26105, AU166357	2.09	0.020
439	ATP-sulfurylase	AU166392, AU166393	5.43	0.229
878	6-phosphogluconolactonase domain containing protein	AU166587, AU166586	2.94	0.139
1010	Short-chain dehydrogenase/reductase SDR family protein	AU166649, AU166648	2.65	0.110
3336	Dormancy related protein	C19333, AU172698	3.97	0.148
1037	Cinnamyl alcohol dehydrogenase	AU166661	2.32	0.003
1092	NADH-dependent hydroxypyruvate reductase	AU108700, AU108699	2.58	0.012
1094	Short-chain dehydrogenase Tic32	AU166716, AU068664	2.46	0.049
1189	Glutamine synthetase	C26815, AU166743	2.06	0.035
1307	Alpha-amylase isozyme 3E precursor	AU068895, AU166807	2.10	0.093
1413	NAD-dependent epimerase/dehydratase family protein	AU062891, AU108816	2.39	0.120
1392	Phosphoglycerate kinase	C97058	2.72	0.101
1393	UDP-glucose 6-dehydrogenase	AU176429	2.85	0.122
1878	24-methylenesterol C-methyltransferase 2	C28197, C97533	2.03	0.077
1904	S-adenosylmethionine decarboxylase	C28220, C97560	2.24	0.225
2137	Aldose reductase-related protein	AU101007, AU101008	4.39	2.320
3108	Avr9/Cf-9 rapidly elicited protein 14	AU064146, AU101257	2.11	0.092
3990	Lipase	C74025	2.35	0.101
5701	Glucose-6-phosphate isomerase	AU174825	2.52	0.153
5738	Glycine cleavage system H protein, mitochondrial precursor	AU174906, AU174905	2.27	0.200
5843	Formate dehydrogenase, mitochondrial precursor	D23770, AU031643	2.20	0.102
5894	UDP-glucuronosyl/UDP-glucosyltransferase family protein	AU173084	2.11	0.534
5934	Shikimate kinase	D23883, AU101616	2.07	0.150
6009	Glycerol kinase	D23993, AU031715	2.10	0.167
6054	Cystathionine beta-lyase, chloroplast precursor	D39050, AU082558	2.46	0.199
6125	Hydroxymethylglutaryl-CoA lyase	AU173159	2.04	0.131
6735	Enoyl-CoA hydratase/isomerase domain containing protein	AU032193, AU173532	2.92	0.212
7630	Pyrophosphate-fructose-6-phosphate 1-phosphotransferase-like protein	AU057478, AU057479	2.22	0.004
8306	Fructose-bisphosphate aldolase	AU032962, AU032963	2.39	0.244
8352	Sucrose synthase 2	D39447, AU174154	2.20	0.238
8519	Lipolytic enzyme, G-D-S-L family protein	D40132, AU174224	2.41	0.045
8719	4-coumarate-CoA ligase	D41418, AU108438	2.17	0.234

Table 3 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
Others				
11	Ricin B-related lectin domain containing protein	D21910, AU088561	2.66	0.083
973	osr40c1	AU166616	2.98	0.031
4364	osr40c1	AU166094, AU029738	3.97	0.023
4947	osr40c1	AU166220, AU058403	2.09	0.214
1065	PGPD14 protein	AU100776, AU100775	2.60	0.006
5025	Ricin B-related lectin domain containing protein	AU030666, AU030667	2.32	0.068
361	Histidine acid phosphatase family protein	C26121, AU100661	2.06	0.056
858	Circadian oscillator component	AU108670, AU068377	2.64	0.011
894	Mitochondrial substrate carrier family protein	AU166569, AU166568	2.54	0.000
905	Cell elongation protein	AU166556	2.11	0.015
985	Chromatin complex subunit	AU062733, AU166638	3.62	0.094
1055	FK506-binding protein 2 precursor	AU166673, AU166672	2.20	0.079
1088	Phox-like domain containing protein	AU068680, AU068679	2.74	0.147
1166	Cell cycle control protein	C26838, C96954	2.05	0.041
1187	Serpin	C26836	2.51	0.026
1246	Ferredoxin III	C26892, AU092413	2.22	0.036
1308	Proline-rich protein	C27063, AU166808	2.13	0.079
1366	CEL5 = CELLULASE 5	C27142, AU166823	2.40	0.086
1396	EL3 protein	AU068948	2.05	0.072
1509	Beta-expansin precursor	AU062937, AU166877	2.60	0.119
3233	Alpha expansin 26	AU172681, AU172682	2.44	0.123
4235	Alpha-expansin OsEXPA7	AU029531, AU029532	2.25	0.060
1528	ECA1 protein	C27411, AU108898	2.14	0.100
1941	Aromatic-ring hydroxylase family protein	AU166971	2.09	0.054
2129	Embryo globulin (BEG1)	AU063570, AU163383	4.34	0.216
3493	Cupin 1 domain containing protein	C73575, AU163793	4.02	0.153
3653	Globulin	C20268	2.06	0.076
3248	Rapid alkalinization factor family protein	AU162202, C99048	2.14	0.078
3432	16 kDa oleosin (ole16)	C19519, C99259	4.34	0.187
3662	16 kDa oleosin (ole16)	C20257, AU164022	3.30	0.091
4344	Clathrin light chain family protein	AU094751, AU029687	2.02	0.888
7800	Clathrin coat assembly protein AP47	C23569, AU032444	2.34	0.170
4408	Amino acid-binding ACT domain containing protein	AU101437, AU101438	2.44	0.079
4247	Glycoside hydrolase, family 10 protein	AU029565, AU029564	2.78	0.126

Table 3 continued

Element no.	Putative gene identification	Accession no.	Induction ratio	SD
4254	Fasciclin-like arabinogalactan protein	AU029566, AU029567	2.32	0.164
4279	Auxin-induced protein PCNT115	C91647	2.08	0.078
5706	Auxin responsive protein IAA-Re	AU174821	3.00	0.294
8654	Auxin-induced SAUR-like protein	D41219, AU097301	2.32	0.257
4347	Virulence factor, pectin lyase fold family protein	C74462, AU172834	2.46	0.106
8547	Pectin methylesterase isoform alpha	D40279, AU101942	2.40	0.288
4355	Chlorophyll a/b binding protein	C74463, AU029720	2.15	0.105
7964	ASCAB9-A (ASCAB9-B)	D48016, AU174030	2.14	0.245
4358	hsr203 J	AU029715, AU029716	2.50	0.135
4507	CAJ1	AU064660, AU094877	3.25	0.156
4511	Pollen allergen Lol p2 family protein	AU064639, AU089708	2.10	0.032
5620	DCL protein, chloroplast precursor	AU174689, AU174688	2.54	0.216
5667	Cuticle protein	AU174778	2.17	0.190
5668	Vesicle-associated membrane protein (VAP27)	AU174785, AU174784	2.32	0.221
5672	Ankyrin-1	AU174770, AU174769	2.09	0.188
5698	Heavy metal transport/detoxification protein domain containing protein	AU174873, AU174872	2.09	0.176
5700	Carotenoid cleavage dioxygenase	AU174820, AU174819	2.04	0.193
5733	Cytochrome P450 family protein	AU174952, AU174951	2.13	0.190
7034	Cytochrome P450 family protein	AU090603	2.38	0.215
5859	Early flowering 5 (ELF5)	AU091985, AU091986	2.16	0.182
6407	External rotenone-insensitive NADPH dehydrogenase	D24680, AU031879	2.62	0.206
6858	BZR1, transcriptional repressor family protein	AU076221, AU095568	2.01	0.204
7062	Actin-depolymerizing factor (ADF)	AU071255, AU162637	5.25	0.489
7207	Seed maturation protein	AU162647, AU056054	2.34	0.226
7382	Membrane related protein-like	AU057227, AU057228	2.52	0.101
7925	Vacuolar ATP synthase	AU174019	2.23	0.216
7934	Rubredoxin (Rd)	D47972, AU174021	2.60	0.282
8021	Tubulin alpha-1 chain	D48151, AU162997	2.23	0.278
8525	UDP-glucuronosyl/UDP-glucosyltransferase family protein	D40163, AU174232	2.29	0.271
8658	DNA-3-methyladenine glycosidase I	AU175161	2.57	0.289
8688	Amidase family protein	D41262, AU174296	2.27	0.262

microarrays (data not shown). This allowed us to conclude that rice microarrays were applicable to bromegrass gene expression analysis. Yet, down-regulated genes might not possibly be properly evaluated due to lower hybridized signals. These genes might include not only down-regulated genes but also orthologous genes with low homology.

Table 4 Cold- and ABA-inducible genes identified by microarray analyses

Element no.	Putative gene identification	Accession no.	Cold		ABA	
			Induction ratio	SD	Induction ratio	SD
Transcription						
1525	No apical meristem (NAM) protein domain containing protein	AU069046, AU100819	2.59	0.156	3.61	0.192
1650	No apical meristem (NAM) protein domain containing protein	C93510, AU082206	2.03	0.074	2.16	0.053
1851	Myb, DNA-binding domain containing protein	C28158	3.26	0.127	2.94	0.144
1519	HAHB-7	C27434, AU166884	2.78	0.158	3.09	0.153
4267	Dehydration responsive element binding protein 2B (DREB2B protein)	C74402, AU172818	2.20	0.088	3.06	0.161
1705	Zinc finger, CCCH-type domain containing protein	C27927	2.02	0.689	2.16	0.148
5196	Zinc finger protein (ZFP2)	AU057491, AU031091	10.03	1.007	7.35	1.001
8715	Zinc finger, A20-type domain containing protein	D41407, AU108433	2.17	0.020	3.21	0.352
8557	Ethylene response factor 2	D40269, AU161649	2.45	0.050	3.56	0.441
Signal transduction						
1294	Phytosulfokines 4	AU068854, AU068855	2.78	0.154	3.73	0.191
1470	SNF1-like protein kinase (OSK1)	C27342, AU166864	2.27	0.178	2.26	0.085
4271	Cyclin-like F-box domain containing protein	AU162267, AU029519	2.04	0.407	3.14	0.803
5586	Calcium-dependent protein kinase	AU174641, AU174640	2.00	0.204	2.29	0.194
8716	Skb1 methyltransferase family protein	D41447, AU101987	3.67	0.015	2.27	0.230
Stress, cell rescue and defence						
494	Class III chitinase homologue (OsChib3H-c)	C26281, AU100685	3.65	0.197	3.82	0.140
1188	Peroxidase	C96956, C96957	2.27	0.120	2.91	0.035
1502	Peroxidase ATP21a	C22707, C19121	2.39	0.203	2.75	0.155
1754	Cationic peroxidase	AU173783, AU173784	2.34	0.104	2.98	0.147
4336	Peroxidase 40 precursor	C91676, AU029674	2.42	0.111	4.19	0.231
4380	Haem peroxidase family protein	C91728, AU172837	3.05	0.152	4.70	0.271
8383	Peroxidase	AU181036	2.26	0.006	3.31	0.384
8571	Peroxidase	D40488, C20510	2.27	0.006	3.20	0.354
1268	Remorin, C-terminal region domain containing protein	C26933, C97012	3.83	0.606	3.66	1.211
8646	Remorin, C-terminal region domain containing protein	AU174290	2.89	0.223	3.58	0.113
1316	Phi-1 protein	C27064, AU100790	2.37	0.125	2.96	0.163
1336	Erg1	C27104, AU108769	3.05	0.129	3.44	0.177
1789	Erg1	AU161184, C97348	2.40	0.172	2.27	0.100
1706	Thaumatin-like protein precursor	AU166929	2.01	0.431	2.21	0.288
1486	Heat shock protein 26 (HSP26)	C27332	2.68	0.151	3.71	0.200

Table 4 continued

Element no.	Putative gene identification	Accession no.	Cold		ABA	
			Induction ratio	SD	Induction ratio	SD
3134	17.5 kD class II heat shock protein	C73383, AU093461	2.05	0.105	2.75	0.121
1703	1-aminocyclopropane-1-carboxylate oxidase	C27913, AU161082	2.61	0.103	2.62	0.114
6760	1-aminocyclopropane-1-carboxylate synthase	AU065421, AU173534	2.19	0.019	2.10	0.201
3355	Endosperm lumenal binding protein	C19371	2.36	0.128	3.59	0.175
6714	Beta-1,3-glucanase-like protein	AU032158, AU175073	2.22	0.003	2.64	0.306
8593	Ankyrin repeat containing protein	D40521, AU033076	5.98	0.009	6.31	0.810
Protein synthesis						
1328	Auxin amidohydrolase	C27041, AU108745	2.87	0.102	2.95	0.133
976	60S ribosomal protein L13	AU068495, AU068494	2.96	0.098	2.19	0.097
7769	40S ribosomal protein S14	AU075866, AU057926	3.77	0.028	2.28	0.232
7932	40S ribosomal protein S4	AU032725	6.79	0.230	2.23	0.236
4393	Protein prenyltransferase domain containing protein	C91778, AU172848	2.71	0.772	4.05	0.824
Proteolysis						
378	Cysteine protease	C26130, AU092283	2.45	0.136	2.17	0.090
1447	L-asparaginase	AU108840, AU108841	2.42	0.139	3.09	0.191
1757	L-asparaginase	AU161119, AU161120	2.50	0.166	2.86	0.109
4257	Peptidase A1, pepsin family protein	AU162268, AU029542	3.04	0.131	3.61	0.229
4295	GDP-mannose pyrophosphorylase (GMP1)	C91653	3.17	0.148	4.68	0.286
5802	UbcM4 interacting protein 48	AU174987	2.21	0.017	3.49	0.361
Transporter						
1376	Amino acid transporter c	C97059, AU108790	2.19	0.131	2.38	0.120
2955	Amino acid/polyamine transporter II family protein	AU076047, AU076048	2.05	0.086	2.51	0.112
2735	Monosaccharide transporter 1	AU063886, AU172500	2.02	0.047	2.40	0.069
4397	MscS Mechanosensitive ion channel family protein	C91799, AU164313	2.84	0.514	4.73	1.512
4440	Auxin influx carrier protein	C74506, AU094807	2.61	0.140	2.93	0.156
7011	Peptide transporter	AU071088, AU095606	3.22	0.052	2.86	0.313
7106	TGF-beta receptor, type I/II extracellular region family protein	AU173859, AU075839	2.31	0.009	2.27	0.253
8655	Delta-type tonoplast intrinsic protein	D41197, AU097295	2.12	0.003	2.63	0.294

Table 4 continued

Element no.	Putative gene identification	Accession no.	Cold		ABA	
			Induction ratio	SD	Induction ratio	SD
RNA function						
879	Possible metal-binding region in RNase L inhibitor, RLI domain containing protein	AU166542, AU166541	2.33	0.598	2.41	0.027
1367	Poly(A)-binding protein (wheatpab)	C27111	4.64	0.293	2.33	0.059
1418	RNA binding protein-like protein	C27216, AU166839	2.73	0.686	2.71	0.696
Metabolism						
115	Glyceraldehyde-3-phosphate dehydrogenase	AU062402	2.51	0.121	2.30	0.076
126	Choline phosphate cytidyltransferase-like protein	C96677	3.73	0.578	2.04	0.531
296	Isovaleryl-CoA-dehydrogenase	AU181069	2.73	0.105	2.23	0.086
922	Malate dehydrogenase	AU166583, AU068406	3.11	0.177	2.94	0.072
1168	Phosphoribosylformylglycinamidine cyclo-ligase, chloroplast/ mitochondrial precursor	AU108708, C96917	2.18	0.112	2.37	0.103
1371	Succinyl CoA ligase	C27132	3.17	0.218	2.04	0.064
1406	Aconitate hydratase	C27218	5.93	0.443	2.68	0.101
8697	Aconitate hydratase, cytoplasmic	D41402, AU108431	7.01	0.158	2.46	0.257
4321	Esterase/lipase/thioesterase domain containing protein	C91665, AU029663	2.43	1.360	3.34	0.538
4345	GTP pyrophosphokinase	C74445, AU172829	2.98	0.169	4.14	0.208
4381	Cinnamoyl-CoA reductase	C91739, AU029748	2.13	0.070	2.97	0.134
6055	Acyl-CoA synthetase	D24049, AU031730	2.54	0.068	2.04	0.152
6578	UDP-glucuronic acid decarboxylase	AU173448	2.14	0.021	2.24	0.194
6701	ATP sulfurylase	AU065356, AU162482	2.00	0.015	2.14	0.078
7173	(S)-2-hydroxy-acid oxidase, peroxisomal	AU055906, AU091712	2.61	0.026	2.34	0.238
7236	S-adenosylmethionine decarboxylase proenzyme	AU056203, AU056204	2.08	0.089	2.41	0.075
8700	Fructokinase	D41344, AU174298	2.45	0.010	2.89	0.315
Others						
58	GRAM domain containing protein	AU067856, AU166271	2.21	0.104	2.06	0.071
106	Protein-L-isoaspartate O-methyltransferase	AU166279, AU166280	2.56	0.134	3.56	0.174
187	MRP protein	D22347, AU100632	2.33	0.024	2.63	0.018
661	AAA ATPase domain containing protein	AU068257, AU068258	3.21	0.584	2.64	0.651
729	Frigida-like family protein	AU166472, AU166473	2.03	0.106	2.13	0.089
1252	Alba, DNA/RNA-binding protein family protein	C26898, AU100786	2.24	0.653	2.20	1.567
1254	LOB domain protein	C26871, AU100784	2.37	0.119	2.27	0.021

Table 4 continued

Element no.	Putative gene identification	Accession no.	Cold		ABA	
			Induction ratio	SD	Induction ratio	SD
1263	Legumin type B alpha chain	AU092414, C96985	2.76	0.148	4.28	0.220
1434	CUC1	C27235, AU108824	2.93	0.183	3.62	0.205
1489	XYPPX repeat containing protein	C27356, AU166869	2.11	0.085	2.01	0.010
1526	Dynein light chain, type 1 family protein	C27428, AU100822	2.74	0.144	2.63	0.122
1895	AAA type ATPase	C97563, AU081005	2.78	0.154	2.83	0.133
3015	Beta-D-xylosidase	AU172626	2.44	0.099	3.22	0.169
3236	SAH7	AU172680	2.03	0.090	2.28	0.082
4357	(1-4)-beta-mannan endohydrolase-like protein	AU162268, AU029542	2.42	0.089	3.66	0.225
6684	Surface antigen	AU065355, AU173508	2.05	0.007	2.95	0.318
7953	Photosystem II type II chlorophyll a/b binding protein	AU174022	2.56	0.011	2.12	0.254
8538	DREPP plasma membrane polypeptide family protein	D40192	2.12	0.015	2.36	0.248
8613	Xyloglucan endotransglycosylase	D40760, AU174270	2.89	0.060	2.16	0.217
8620	ADP-ribosylation factor 1 (Os-ARF1)	D40795, AU081605	2.21	0.018	2.27	0.235
8642	Pistil-specific extensin-like protein family protein	D40952, AU174289	3.82	0.004	5.11	0.577
8808	Selenium binding protein	AU174343, AU174344	3.15	0.010	2.14	0.176

These considerations allowed us to agree with the idea that expression data obtained with heterologous microarray analysis can be used for identifying up-regulation of genes (Taji et al. 2004). Therefore, we focused on only up-regulated genes. Table 1 summarizes functional categories of the genes up-regulated more than twofold by 7 days of cold and ABA treatments compared to the untreated control. The functions of 79 % (237/300) of the cold-responsive genes were known, whereas those of 61.6 % (295/479) were known for ABA-responsive genes.

Cold-inducible genes

The genes up-regulated more than twofold by 7 days of cold acclimation as compared to the untreated control are listed in Tables 2 and 4. A total of 300 genes were identified as long-term or later-stage cold-inducible genes, out of which 149 genes were also increased by 7 days of ABA treatment. Homologues of several genes in Tables 2 and 4 were reported to be cold-inducible in plants, such as zinc finger protein, phospholipase, calcium-dependent protein kinase, chitinase, heat shock protein, alternative oxidase, superoxide dismutase, elongation factor and ribosomal protein (Kim et al. 2001; Thomashow 1999; Yeh et al. 2000; Ito et al. 1997; Seki et al. 2002b). These results show

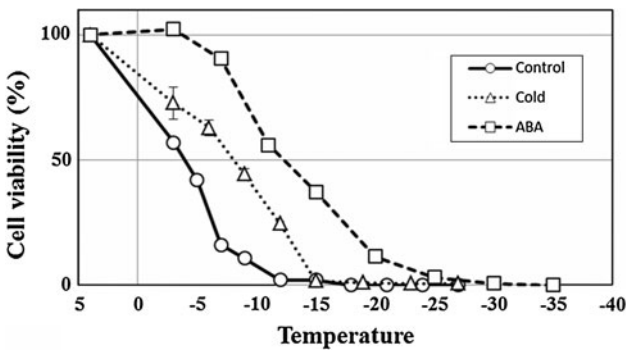

Fig. 1 Effect of freezing temperatures on the viability of untreated control (*circle*), cold-acclimated (*triangle*) and ABA-treated (*square*) bromegrass cells. Cold treatment was conducted by incubating 3 day-old bromegrass cell cultures at 15 °C for a day and then at 4 °C for 6 days. Bromegrass cell cultures were incubated in culture medium in the presence or absence of 50 μM ABA for 7 days at 25 °C. Data were the mean ± SE of three determinations (SE smaller than the symbols were not shown)

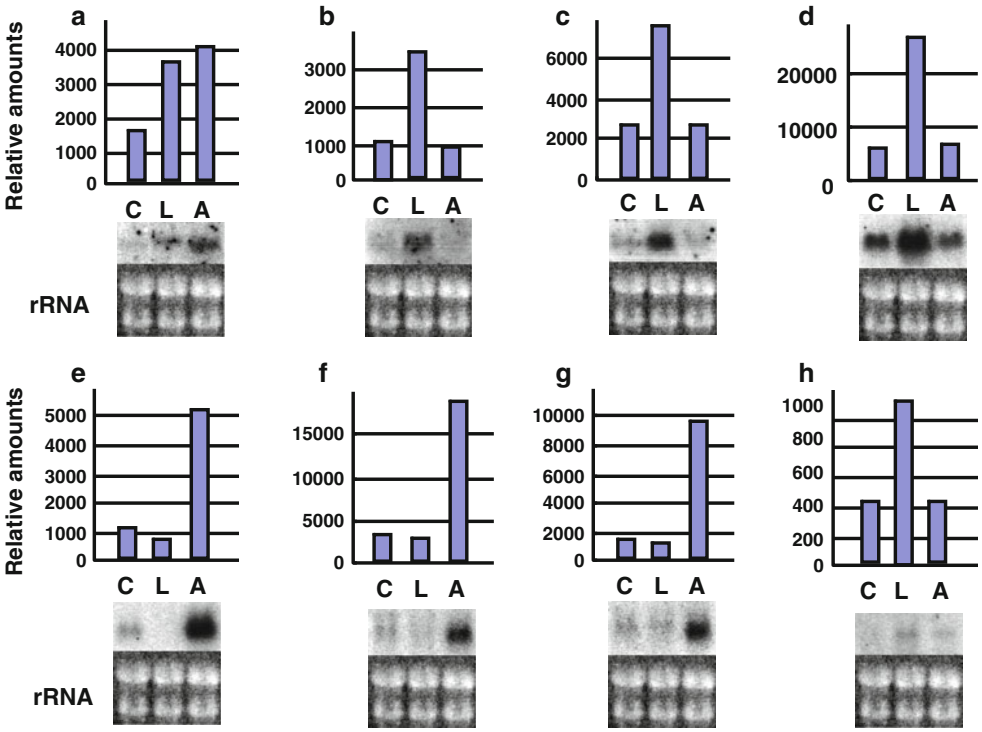

Fig. 2 Comparison between microarray and Northern blot analyses showing the expression patterns of 8 selected genes. The *graphs* show the relative amounts of mRNA observed in the microarray analysis. In Northern blot analyses, each lane was loaded with 15 μg of total RNA isolated from bromegrass cells treated with cold (L) and ABA (A), and untreated bromegrass cells (C). Microarray results show the expression of element No. 187 (a), 505 (b), 1236 (c), 1818 (d), 1952 (e), 2379 (f), 2388 (g) and 6993 (h) listed in Table 5. Rice full-length cDNAs corresponding to the element numbers were used as probes for Northern blot analyses

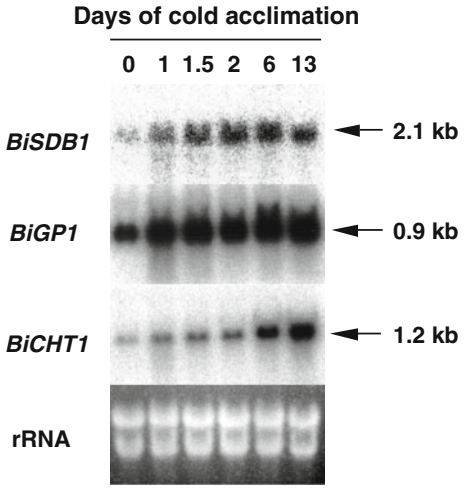

Fig. 3 Time course of mRNA accumulation during cold acclimation as detected by RNA blot analysis. Cold treatment was conducted by incubating 3 day-old bromegrass cell cultures at 15 °C for a day and then at 4 °C for up to 12 days. Gene specific regions of bromegrass cDNAs, listed in Table 5, were used as probes. Ribosomal RNA was visualized by ethidium bromide staining

that the rice cDNA microarray system functioned properly to detect cold-inducible genes in bromegrass.

ABA-inducible genes

Tables 3 and 4 show the genes that were up-regulated by ABA treatment. The transcripts of 479 genes increased after ABA treatment, with inducibilities of more than twofold compared to those of control. The lists (Tables 3, 4) include many genes whose homologues were reported to be ABA-inducible (Skriver and Mundy 1990; Giraudat et al. 1994; Busk and Pages 1998; Seki et al. 2002a). Several genes have been previously reported as responsive to ABA in bromegrass (Lee and Chen 1993). Some of these genes such as germin, aldose reductase and embryo globulin were also shown to be ABA responsive in our microarray results.

Expression analyses of selected genes

To evaluate the validity of the microarray data, the expression patterns of 8 selected clones (listed in Table 5) were further evaluated by Northern blot analysis using heterologous probes. In Fig. 2, the results of microarray analyses were compared with those of Northern blot analyses. For each of 8 clones, the expression patterns under cold and ABA treatments were fairly consistent between the two methods, showing that microarray and Northern blot analyses gave similar quantitative results (Fig. 2). Through this process, we cloned 6 bromegrass genes, three of which were cold-responsive (*BiSDB1*, *BiGP1*, *BiCHT1*) and three of which were ABA-responsive (*BiPOR1*, *BiLEA1*, *BiWSI1*) (Table 5).

Expression of cold-responsive genes during the periods of cold acclimation

To investigate expression patterns of the cold-responsive genes, mRNA accumulation of 3 selected clones during the

Table 5 List of genes used for Northern blot analyses as probes

Element no.	Clone name	Gene annotation	Gene cloning	Bromegrass gene	Homology (%)[a]
187	CB0776	MRP protein	No	ND	ND
505	CB2134	SAR DNA binding protein	Yes	BiSDB1	85.5
1236	CG0327	Hydroxyproline rich glycoprotein PsHRGP1	Yes	BiGP1	92.1
1818	CH0143	Endo-1,4-beta-glucanase (Cel3)	No	ND	ND
1952	CH1659	Peroxiredoxin	Yes	BiPOR1	85.5
2379	EA0201	LEA protein	Yes	BiLEA1	77.6
2388	EA0326	WSI18 protein	Yes	BiWSI1	75.7
6993	RB0638	Class I endochitinase (CHT1)	Yes	BiCHT1	83.8

ND not determined

[a] Homology in the nucleotide sequences of coding regions between a bromegrass gene and the corresponding rice gene

course of cold acclimation was examined. Northern blot analyses of total RNA extracted from cells cold-acclimated for 0, 1, 1.5, 2, 6 and 13 days were performed using bromegrass cDNAs as probes (Fig. 3). *BiSDB1* mRNA, encoding a putative SAR DNA binding protein, accumulated after 1 day of exposure to 15 °C and continued to increase with 5 days of treatment at 4 °C, then slightly decreased after 13 days. RNA of *BiGP1*, encoding a putative glycoprotein, was detected in the control cells and was expressed at elevated levels during cold acclimation. *BiCHT1* transcript, encoding a putative chitinase, accumulated within 1 day of cold treatment and continued to increase during the course of cold acclimation. This cold-inducible bromegrass chitinase had a high homology (89 %) at the protein level with rye chitinase (*CHT9*), which has been reported to be an antifreeze protein (Yeh et al. 2000). The product of *BiCHT1* had chitinase activity but no antifreeze activity under any conditions tested (Nakamura et al. 2008).

Discussion

The development of microarray technology for monitoring the expression of thousands of genes at a time has enabled global analysis of the responses of gene expression to abiotic stresses and to plant hormones. This has been successfully applied to analyses of Arabidopsis responding to cold stress (Seki et al. 2001, 2002b; Fowler and Thomashow 2002; Hannah et al. 2005; Oono et al. 2006; Kilian et al. 2007) and ABA (Seki et al. 2002a; Takahashi et al. 2004). More recently, it has been applied to cold acclimation process of agriculturally important grasses such as barley and wheat (Svensson et al. 2006; Monroy et al. 2007; Winfield et al. 2010; Laudencia-Chingcuanco et al. 2011).

In this study, we analyzed gene expressions during induction of cold hardiness in bromegrass cell cultures exposed to cold and ABA using rice cDNA microarrays (heterologous system). In preliminary experiments, we found that bromegrass mRNA had a hybridization efficiency with the rice cDNA microarrays almost comparable to rice mRNA. This may arise from the tendency that genes in cereal crops are highly conserved at the DNA level (Devos and Gale 2000). Bromegrass has a genome 30 times larger than rice (Tuna et al. 2001) and probably contains more genes (perhaps with more gene families) than rice in the genome. In this heterologous analysis, our rice microarrays may not necessarily distinguish cross hybridization of gene families of bromegrass. Yet, individual hybridization data of cold or ABA treatment were always presented as the ratio compared to hybridization of mRNA from control cells. Each data set may contain possible cross hybridization of gene families. Thus, our analyses likely represent the overall responses (compared to the control) of target genes plus their gene families (if there are any) or approximate estimation of gene expression tendencies rather than collection of pinpoint expression of individual genes. Eventually, northern blot analyses of 8 selected genes (listed in Table 5) using heterologous probes revealed that the expression patterns of these genes under cold and ABA treatments were quantitatively consistent between the microarray and Northern blot analyses (Fig. 2). This indicates that our microarray analyses worked properly. The up-regulation of three genes was further confirmed by Northern blots using gene specific sequences of bromegrass cDNA (orthologous probes) (Fig. 3). This also indicated the highly conserved sequences in the genes of the grass family (Table 5). These things allowed us to consider that rice microarrays were useful for approximate estimation of gene expression tendencies in bromegrass. Similar successful heterologous microarray analyses have been reported between distantly related species (van Zyl et al. 2002; Horvath et al. 2003) and between species differing in the genome size: barley and rice (10 times larger) or wheat and barley (4 times larger)

(Negishi et al. 2002; Schweizer 2008: Kocsy et al. 2010). And yet, for the reasons detailed in the microarray section of the Results, we only focused on up-regulated genes as heterologous microarrays were used (Taji et al. 2004).

Many of the genes detected to be up-regulated by the rice microarrays in response to ABA or cold (Tables 2, 3, 4) have been previously reported to be ABA or cold inducible in Arabidopsis (e.g., Fowler and Thomashow 2002; Seki et al. 2002a, b; Takahashi et al. 2004; Oono et al. 2006) as briefly summarized in the Results and detailed below. Commonalities of our results with Arabidopsis studies also imply that rice cDNA microarray is useful for roughly characterizing cold hardiness induction systems by two different stimuli in bromegrass, which is the purpose of this study. Yet, a problem in this approach may be that rice is not a freezing-tolerant plant and the rice microarray may not contain freezing tolerance related genes. Such cautions have to be paid in interpreting the results that bromegrass-specific genes which have no homologous genes in rice or on the used microarray and genes expressed at low levels are not readily detectable in this system. With these limitations (discussed in the present and previous paragraph) in mind, we compared gene expression profiles of cold and ABA-treated bromegrass cell cultures to characterize the two cold hardiness induction systems.

Cold-inducible genes

In this study where the gene expression profiles of bromegrass cells that were cold-hardened for 7 days were analyzed, 300 genes were identified as long-term or later-stage cold-inducible (Tables 2, 4) and whose products may confer freezing tolerance (LT_{50}: −8.1 °C as compared to −3.9 °C of control cells in Fig. 1). Several genes were common when the expression profiles of cold-hardened bromegrass were compared with those of cold-hardened Arabidopsis (Fowler and Thomashow 2002) which had LT_{50} of −6 to −8 °C after 7 days exposure to 4 °C compared to −4.5 °C of non-hardened control (Gilmour et al. 2000). These genes included pyruvate decarboxylase, zinc finger protein, xyloglucan endotransglycosylase, peroxidase, myb protein, extensin, SNF1 like protein kinase, cysteine proteinase, fibrillarin and chitinase. Some of other genes such as SAR DNA binding protein, nucleoside diphosphate kinase and histone H2A were newly identified as cold-inducible genes in our microarray results (Tables 2, 4).

One newly found cold-inducible gene is *BiSDB1*, encoding a putative SAR DNA binding protein. Scaffold attachment regions (SARs) are AT-rich sequences and are known to bind specifically to components of the nuclear scaffold (Hall et al. 1991). The function of SARs is to enhance transgene expression and normalize it by protection against surrounding chromatin, called position effects (Breyne et al. 1992). In plants, a typical SAR DNA binding protein was purified and whose cDNAs (MARBP-1 and MARBP-2) were isolated from pea (Hatton and Gray 1999). MARBP-1/MARBP-2 genes were expressed in all the tissues, and the function of these products was predicted as housekeeping. Expression of *BiSDB1* was up-regulated after 1 day of exposure to 15 °C and gradually increased throughout the cold acclimation period (Fig. 3). This implies that *BiSDB1* may have a housekeeping role in the normalization of gene expression under cold conditions and/or that *BiSDB1* may possibly regulate cold-inducible genes at the chromatin level.

When bromegrass suspension cells were grown at 4 °C for 0–3 weeks, they showed slow steady growth in the second and third weeks: the fresh weight of cultured cells increased to 118 and 136 % of the initial cell weight, respectively. In contrast, they showed only marginal culture growth (104 % of the initial weight) in the first 7 days at 4 °C (Shinkawa et al. manuscript in preparation), which was the target condition of this study. During this period, bromegrass cells most likely spent most of the energy to adjust their metabolism to cold conditions (a part of housekeeping) and to scrap/rebuild many enzymes and protein machinery, which may have resulted in a limited culture growth.

In agreement with this, numerous genes involved proteolysis, RNA stabilization and protein synthesis were up-regulated after 7 days at 4 °C as compared to untreated control and ABA treatment (Table 2). For instance, expression of protein synthesis related genes such as elongation factors, translation initiation factors, and ribosomal proteins, were increased in cold-acclimated bromegrass (Tables 2, 4). Protein synthesis is necessary for cold response and the accurate translation machinery is an important factor for properly processing cold acclimation. In Arabidopsis, the *LOS1* gene encodes translation elongation factor 2 and the *los1-1* mutant is impaired in its ability to cold-acclimate and defective in protein synthesis in the cold (Guo et al. 2002). Three ribosomal protein genes induced by low temperatures in soybean show long-term up-regulated expressions (Kim et al. 2004). In yeast, ribosomal proteins stimulate cell growth (Warner 1999). Thus ribosomal proteins expressed at low temperatures may be required for bromegrass cells to grow under cold stress.

Cold up-regulated genes involved in proteolysis included proteasome related genes and various proteases whilst those involved in RNA function included various t-RNA synthetases, RNA binding proteins, RNA helicase, etc. (Tables 2, 4). Some RNA binding proteins are known to be cold responsive and work as RNA chaperones whilst RNA

helicases are considered to be involved in the removal of inhibitory secondary structures of mRNA formed at low temperatures and may contribute to maintaining protein synthesis and increased survival under cold conditions (Kwak et al. 2011; Vashisht and Tuteja 2006).

Increased expression of some genes by cold such as plasma membrane ATPase and alternate oxidase (AOX) were consistent with literatures on physiological and molecular studies on alterations during cold acclimation (e.g., Ishikawa and Yoshida 1985; Umbach et al. 2005).

ABA-inducible genes

ABA-treated cells acquired higher freezing tolerance than cold-acclimated cells in 7 days of treatments (Fig. 1). In bromegrass cultured cells, ABA treatment for 7 days induced many stress-related genes such as germin, LEA protein, water stress protein, peroxiredoxin, desaturase and superoxide dismutase (Table 3). These genes were also induced by salt and drought in Arabidopsis (Seki et al. 2001, 2002b; Takahashi et al. 2004) and rice (Kawasaki et al. 2001; Rabbani et al. 2003), but not induced by cold in bromegrass (Table 2). ABA-inducible genes may increase freezing tolerance as a part of ABA-mediated cross-adaptation to various stresses (Ishikawa et al. 1995). Microarray results indicated that freezing tolerance induced by cold or ABA accompanied up-regulation of considerably different sets of genes, and that cold-specific or ABA-specific genes may represent different freezing tolerance mechanisms involved.

ABA has been known to play important roles in seed formation and maturation. In agreement with this, many genes related to storage proteins (e.g., globulin, lectin, allergen), lipid body protein (e.g., oleosin), cell wall proteins (e.g., extension, expansin) were up-regulated in response to 7 days of ABA treatment (Tables 3, 4). The list of ABA-up-regulated genes indicated intensified stress and defense related responses: antioxidation (e.g., peroxidase, peroxiredoxin, superoxide dismutase), biosynthesis of proteins responsive to pathogen, heat, water stress, salt, wound, heavy metals and UV (e.g., heat shock proteins, various types of LEA, embryo specific proteins, water stress protein, osr40c1, germin, chitinase, etc.) and biosynthesis of secondary metabolites such as jasmonic acid (allene oxide cyclase), flavonoid (e.g., phenylalanine ammonia-lyase, 4-coumarate-CoA ligase), lignin (e.g., cinnamyl alcohol dehydrogenase, cinnamoyl-CoA reductase) and phytoalexin (e.g., hydroxyanthranilate hydroxycinnamoyltransferase). Activation of glucose, sucrose and starch biosynthesis was also implied (e.g., fructose biphosphate aldolase, phosphoglycerate kinase, malate dehydrogenase, glucose-6-phosphate isomerase and sucrose synthase).

Since ABA treatment of bromegrass cultures was done at 25 °C, adjustment of metabolism and protein machinery to low temperatures is not required (thus circumventing the process of proteolysis/synthesis/RNA maintenance) and functional stress-related genes may have been expressed rather directly and constitutively, which may partly attribute to the differences in transcriptome profiles between ABA-treated cells and cold-treated cells.

Genes up-regulated by cold and ABA treatments

It is noted that 149 clones were induced by both cold and ABA treatments (Table 4). These genes may possibly be more important for freezing tolerance. Functions of these genes include regulation of transcription, stress and defense related proteins (e.g., heat shock proteins, chitinase, thaumatin-like protein, glucanase, ankyrin repeat containing protein), antioxidants (e.g., various peroxidases), cell wall biogenesis and alteration (e.g., monosaccharide transporter, xyloglucan endotransglycosylase, remorin, extensin, cinnamoyl-CoA reductase), lipid synthesis (e.g., erg1, choline phosphate cytidyltransferase, acyl-CoA synthase), etc. Some transcription factors detected, such as zinc finger protein, may possibly regulate the expressions of downstream genes induced by cold- and ABA. Further understanding of these interactions will become feasible with the availability of regulatory sequences of the target genes. It is likely that common regulatory networks and/or signaling intermediates govern the expression of the genes up-regulated by both treatments. Several of the downstream responses shared by cold and ABA may play important roles in freezing tolerance mechanisms.

One interesting observation is that key enzymes in ethylene biosynthesis (1-aminocyclopropane-1-carboxylate oxidase and 1-aminocyclopropane-1-carboxylate synthase) and ethylene response factor were up-regulated by both cold and ABA in coordination with many disease resistance related genes and a key enzyme in polyamine biosynthesis (S-adenosylmethionine decarboxylase).

Another surmise is that some physiological or metabolic processes are shared in cold- and ABA-treated cell cultures, which results in the expression of common genes, which means that the results in Table 4 have to be interpreted with caution. For instance, phytosulfokine-alpha, a sulfated peptide growth factor, is known to be involved in cell proliferation of cultured rice cells (Yang et al. 1999; Lorbiecke and Sauter 2002). A bromegrass homologue of *OsPSK4* that encodes a precursor of phytosulfokine-alpha was up-regulated both by cold and ABA (Table 4). Interestingly, bromegrass cell cultures showed cell growth in the cold or in the presence of ABA (Ishikawa et al. 1990; Shinkawa et al. manuscript in preparation).

Taken together, bromegrass cell cultures provided a unique system for studying cold hardiness mechanism, where freezing tolerance were independently induced by two different stimuli, cold and exogenous ABA at 25 °C. Microarray analyses of long-term up-regulated genes during freezing tolerance induction by these stimuli revealed that cold stress was characterized by triggering of numerous genes involved in protein degradation/synthesis and RNA maintenance in addition to cold stress-related genes whilst ABA treatment was characterized by induction of numerous genes involved in seed formation and functional genes related to biotic and abiotic stresses. The results were in good agreement with physiological and protein studies of the two systems: proteins induced by cold and ABA treatments were fairly different with more newly induced bands by ABA whilst ABA-treated cells had thicker cell walls, denser cytoplasm with more lipid and protein bodies and starch accumulation in plastids compared to cold-treated cells (Robertson et al. 1987, 1988; Ishikawa et al. 1990). Physiologically, ABA conferred bromegrass cells tolerance to freezing, heat, salt and osmotic stresses simultaneously (cross-adaptation) (Ishikawa et al. 1995). The results may not be conclusive but these two systems had fairly different transcriptome profiles and likely involve different mechanisms of cold hardiness induction. The genes commonly induced or increased by cold and ABA are likely more directly involved in freezing tolerance mechanism and especially transcription factors are of interest for further studies.

More recently, we have found that exogenous ABA can induce freezing tolerance in suspension cells of rice, which is a chilling sensitive plant (Shinkawa et al. manuscript in preparation). Proteomic studies revealed that ABA-treated rice cells had a protein profile similar to that of seed embryos, which shared a common implication with the present study.

The data obtained with heterologous microarrays have to be interpreted with cautions as detailed in the first part of Discussion. A bromegrass gene ROB5, encoding a highly ABA-responsive LEA protein (42 kD) (Robertson et al. 1994), was not detected as ABA-inducible by the rice microarray. This gene has been claimed to confer various stress tolerance to transformed crops without affecting the yield (Robertson et al. 2008). Meanwhile, bromegrass COR (cold-regulated) genes (Thomashow 1999) were not detected as cold-inducible by the rice microarray. These cold hardiness genes most likely specific to bromegrass were not detectable with the rice microarrays. Interestingly, bromegrass transcriptome profiles obtained using such rice microarrays that may lack bromegrass specific freezing tolerance genes still had numerous commonalities with those of Arabidopsis treated with cold or ABA. Moreover, the rice microarray, in spite of being an approximate probe, seems to have successfully characterized two cold hardiness induction systems of bromegrass cells without having bias on well known freezing tolerance genes. This is most likely due to the highly conserved sequences in the genes of the grass family.

In this study, several genes were newly found to be cold-inducible. The expression of three cold-inducible genes (*BiSDB1*, *BiGP1*, *BiCHT1*) was confirmed by Northern blots using the corresponding bromegrass cDNA. We also isolated three ABA-inducible genes (*BiPOR1*, *BiLEA1*, *BiWSI1*) from bromegrass (Table 5). These genes were introduced into rice plants by transformation to see any changes in stress tolerance and performance.

The present study may help understand the nature of ABA-induced freezing tolerance. Yet numerous questions still remain to be unraveled. Why can freezing tolerance induction by ABA be achieved in only limited number of systems? In Arabidopsis suspension cells, for example, ABA treatment only marginally induces freezing tolerance. Why can ABA induce higher levels of cold hardiness in cultured cells than cold treatment (Ishikawa et al. 1990), sometimes irrespective of the frost sensitivity of the examined genotypes (Galiba et al. 1993)? Why can cultured cells achieve only limited levels of freezing tolerance even after prolonged cold treatment compared to the high levels of freezing tolerance acquired in their derived plants after cold acclimation (Ishikawa et al. 1990)?

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