

Genomics Approach to Abscisic Acid- and Gibberellin-responsive Genes in Rice

Junshi YAZAKI,¹ Naoki KISHIMOTO,¹ Yuko NAGATA,² Masahiro ISHIKAWA,¹ Fumiko FUJII,¹ Akiko HASHIMOTO,² Kanako SHIMBO,² Zenpei SHIMATANI,² Keiichi KOJIMA,³ Kouji SUZUKI,³ Makoto YAMAMOTO,³ Sachiko HONDA,¹ Ayano ENDO,¹ Yumiko YOSHIDA,² Yuki SATO,¹ Keiko TAKEUCHI,¹ Kazuko TOYOSHIMA,¹ Chikako MIYAMOTO,¹ Jianzhong WU,² Takuji SASAKI,¹ Katsumi SAKATA,¹ Kimiko YAMAMOTO,² Koh IBA,⁴ Takahiro ODA,⁴ Yasuhiro OTOMO,⁵ Kazuo MURAKAMI,⁵ Kenichi MATSUBARA,^{5,6} Jun KAWAI,⁷ Piero CARNINCI,⁸ Yoshihide HAYASHIZAKI,^{7,8} and Shoshi KIKUCHI^{1,*}

Department of Molecular Genetics, National Institute of Agrobiological Sciences, 2-1-2 Kannon-dai, Tsukuba, Ibaraki 305-8602, Japan,¹ Institute of the Society for Techno-innovation of Agriculture, Forestry and Fisheries, 446-1 Ippaizuka, Kamiyokoba Tsukuba, Ibaraki 305-0854, Japan,² Hitachi Software Engineering Co., Ltd., 4-12-7, Higashishinagawa, Shinagawa-ku, Tokyo 140-0002, Japan,³ Department of Biology, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka City, Fukuoka 812-8581, Japan,⁴ Laboratory of Genome Sequencing and Analysis Group, Foundation of Advancement of International Science (FAIS), 586-9, Akatsuka-Ushigahuchi, Tsukuba, Ibaraki 305-0062, Japan,⁵ Nara Institute of Science and Technology (NAIST), 8916-5, Takayama-cho, Ikoma, Nara 630-0101, Japan,⁶ Laboratory for Genome Exploration Research Group (Institute of Physical and Chemical Research, RIKEN), Genomic Sciences Center (GSC), RIKEN Yokohama Institute, 1-7-22, Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan,⁷ and Genome Science Laboratory, RIKEN Wako Main Campus, 2-1, Hirosawa, Wako, Saitama 351-0198, Japan⁸

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Abstract

We used an 8987-EST collection to construct a cDNA microarray system with various genomics information (full-length cDNA, expression profile, high accuracy genome sequence, phenotype, genetic map, and physical map) in rice. This array was used as a probe to hybridize target RNAs prepared from normally grown callus of rice and from callus treated for 6 hr or 3 days with the hormones abscisic acid (ABA) or gibberellin (GA). We identified 509 clones, including many clones that had never been annotated as ABA- or GA-responsive. These genes included not only ABA- or GA-responsive genes but also genes responsive to other physiological conditions such as pathogen infection, heat shock, and metal ion stress. Comparison of ABA- and GA-responsive genes revealed antagonistic regulation for these genes by both hormones except for one defense-related gene, thionin. The gene for thionin was up-regulated by both hormone treatments for 3 days. The upstream regions of all the genes that were regulated by both hormones had *cis*-elements for ABA and GA response. We performed a clustering analysis of genes regulated by both hormones and various expression profiles that showed three notable clusters (seed tissues, low temperature and sugar starvation, and thionin-gene related). A comparison of the *cis*-elements for hormone response genes between rice and *Arabidopsis thaliana*, we identified *cis*-elements for dehydration-stress response or for expression of amylase gene as *Arabidopsis* gene-specific or rice gene-specific, respectively.

Key words: Plant functional genomics; Rice; cDNA microarray; Germination/Dormancy; Full-length cDNA

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* To whom correspondence should be addressed. Tel. & Fax. +81-29-838-7007, E-mail: skikuchi@nias.affrc.go.jp

† Abbreviations: ABA; abscisic acid, GA; gibberellin, RED; Rice Expression Database, KOMÉ; Knowledge-based Oryza Molecular biological Encyclopedia

1. Introduction

The interaction between phytohormones, particularly between gibberellin (GA) and abscisic acid (ABA), is an important factor controlling the transition from embryogenesis to germination in seeds. The effects of these hormones are antagonistic, in that ABA promotes seed dor-

mancy whereas GA promotes seed germination. Seed germination and seed dormancy are thus antithetical phenomena, much like the stopping and restarting of growth. The mechanism of seed dormancy is composed of a system of repression of gene expression, and the genes expressed mainly in seeds and flowers are commonly regulated by ABA and GA.^{1,2} During germination of cereal grains, the embryo secretes GA to the aleurone layer, where it promotes the expression of several genes encoding hydrolytic enzymes.³ Expression of genes for these enzymes is repressed by ABA during seed development, in dormant seeds, and in seedlings under unfavorable conditions. Cereals are excellent plants in which to explore the molecular mechanisms involved in hormonally regulated gene expression, particularly the antagonism between GA and ABA.³ Reports of such explorations¹⁻³ have suggested the presence of cross-talk, but have investigated only part of the relationship between dormancy and germination in plants. One recent study showed that water stress markedly increased ABA accumulation in rice grains and substantially decreased grain GA content.⁴ The results suggests that water stress also alters the hormonal balance in rice grains during grain filling.

Over the past 10 year, the Rice Genome Project in Japan has generated useful tools for plant research, such as a over 28,000 full-length cDNA,⁵ a highly accurate genome sequence,^{6,7} a high-density linkage map,⁸ YAC-, PAC-, and BAC-based physical maps,⁹ about 50,000 transposon insertion lines,¹⁰ and an enormous collection of ESTs from large-scale cDNA sequencing using cDNA libraries derived from various tissues such as rice callus, root, shoot, leaf, and panicle.¹¹ Also, draft sequences of the *Oryza sativa* L. ssp. *indica*¹² and *japonica*¹³ genomes by the whole-genome shotgun sequencing method have been published by Beijing Genome Institute and the Syngenta. The enormous collection of approximately 110K ESTs includes the approximately 60,000 ESTs in the Rice Genome Project that have been analyzed thus far; from these 60K ESTs, more than 9000 partial cDNA sequences corresponding to unique genes have been identified.¹⁴ On the basis of the results of large-scale cDNA analysis, microarrays can be used to monitor gene expression profiles and to initiate functional analysis of the rice genome.¹⁵ The use of plant microarrays has become common recently in rice,^{15,16} barley,¹⁷ *Arabidopsis*,¹⁸ maize,¹⁹ and zinnia;²⁰ it has now become a standard investigative approach in plants and other organisms.^{21,22} We used these cDNAs from the RGP as probes to hybridize target RNAs prepared from normally grown callus and callus treated with hormones (ABA and GA). This report is the first step in the elucidation of the mechanisms of interaction between seed germination and dormancy.

2. Materials and Methods

2.1. Microarray construction

All cDNA clones were generated by large-scale cDNA analysis as part of the Rice Genome Project.¹⁴ Details of all cDNA clones on microarray are described in Supporting text on the *DNA Research* web site, http://www.dnares.kazusa.or.jp/10/6/03/supporting_text/support.html. λZAPII vectors were used for cDNA library construction. Inserted cDNA clones were amplified by PCR using M13 primers (TAKARA, Shiga, Japan); M4 (5'-GTTTTCCAGTCACGAC) and RV (5'-CAGGAAACAGCTATGAC). Plasmid template (1–2 ng) was added to 50 μl of PCR mixture containing 0.2 mM of each nucleotide, 0.4 μM of each primer, 2.5 mM of Mg²⁺, and 1.25 units of Taq DNA polymerase (Perkin Elmer, Tsukuba, Japan). Inserts were amplified by PCR with 30 cycles of 94°C for 1 min, 60°C for 2 min, 72°C for 2 min; an initial denaturation at 94°C for 1 min; and final extension at 72°C for 10 min. PCR products were purified on a QIAquick 96-column (QIAGEN, Tsukuba, Japan) and mixed with water and reagent D (Amersham Pharmacia, Tokyo, Japan) for microarray fabrication. In general, the total quantity of each PCR product was greater than 1 μg. The average size of full inserts in the cDNA clone was about 1000 bp. Electropherograms of amplified cDNAs can be viewed at http://cdna01.dna.affrc.go.jp/RMOS/eidou_pict/eidou_pict.html.

A total of 8987 DNAs were printed on an aluminum-coated, DMSO-optimized glass slide (Type 7, 2.5 cm × 7.5 cm) in duplicate using an Array Spotter Generation III (Amersham Pharmacia) with 280 μm spacing between the center of each element. The spotter was maintained at 55% humidity. After spotting, the slides were dried for about 1 hr under the same conditions and exposed to UV for cross-linking.

2.2. Plant material and RNA preparation

The callus used for total RNA extraction was derived from the scutellum of the *japonica* rice variety Nipponbare and cultivated in Murashige and Skoog medium²³ containing 10 μM of 2,4-dichlorophenoxyacetic acid. Such callus maintains the ability to develop roots and leaves. After the calli had been cultured in the medium for 30 days, they were transferred to a medium containing the plant hormones ABA or GA and cultured for 6 hr or 3 days. The concentration of each plant hormone was adjusted to 50 μM. After culturing, we used an RNeasy Plant Mini Kit (QIAGEN) to extract total RNA from the hormone-treated calli and from the controls. Messenger RNA was isolated with an Oligotex-dt30 (Super) mRNA purification kit (TaKaRa, Shiga, Japan).

2.3. Microarray experiments

See supporting text, which is published as supporting information of details of microarray experiments including labeled target preparation, microarray hybridization, data quantification and processing, data on expression profiling in rice callus treated with ABA and GA on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html.

2.4. Quantitative real-time PCR

Two micrograms of messenger RNA from each callus was denatured at 65°C for 5 min and then transferred to a bath at 37°C and incubated for 5 min. At the same time, the first-strand reaction mix containing murine reverse transcriptase and d(T)18 primer (Amersham Pharmacia) was held at 37°C for 5 min. The RNA solution was transferred to the first-strand reaction mix for synthesis of cDNA and incubated at 37°C for 60 min. Specific primers for real-time PCR were designed to work under the same experimental conditions (95°C for 10 min followed by 45 cycles of 95°C for 1 min and 60°C for 30 sec), generating products of about 200–250 bp at the 3'-untranslated region (3'UTR) of each cDNA clone. Genes encoding heat shock protein (AU062924) and poly-ubiquitin (AU108666, D22109) were tested as references for the hormonally treated and untreated samples. We chose poly-ubiquitin (AU108666) for the standard lines because it was amplified by PCR faster than the other 2 genes. Quantitative RT PCR was performed with iCycler (BioRad, Tsukuba, Japan) and Syber Green reagent (QIAGEN). For each reaction, standard lines for both the treated and untreated samples were made by 6 fivefold serial dilutions. The relative amounts of the target gene were calculated by comparison with standard lines of poly-ubiquitin (AU108666).

2.5. Determination of gene positions on rice chromosome and cis-element search

Our microarray was constructed from full-inserted rice ESTs collected from the rice EST project. These sequences of EST clones were searched for in the rice full-length cDNA database, the Knowledge-based Oryza Molecular biological Encyclopedia (KOME; <http://cdna01.dna.affrc.go.jp/cDNA/>),⁵ which includes a collection of 28,469 unique full-length cDNAs. Full-length cDNAs corresponding to the EST clones on the microarray were obtained using the BLAST program (> e-10). Each of the EST clones on the microarray has the end sequence of the 3' or 5' terminus of the gene inserted in the cloning vector, with an accession number. We used the end sequence of only the 3' terminus to obtain full-length cDNAs corresponding to EST clones with the end sequences of both the 3' and 5' termini. Full-length cDNAs corresponding to EST clones with the end sequence of only the 3' or the 5' terminus were ob-

tained by using the appropriate terminus sequence. After we had obtained full-length cDNAs corresponding to the ESTs, we determined the positions of these full-length cDNAs on the rice chromosome. All the mapping data of the 28,469 full-length cDNAs are available through KOME. The full-length cDNAs obtained were assigned to the rice (*japonica* cultivar) chromosome by using the BLAST program (identities: over 95; score: over 100) and the SIM4 program (for determination of position only).

After we had mapped the full-length cDNAs on the rice genome, we performed a *cis*-element search. From the 5'-end sequences of the corresponding full-length cDNAs, the promoter sequences were obtained by comparison with the rice genomic sequences. We selected 1000 bp of genomic sequences upstream from the 5' terminus of each full-length cDNA clone by using genome sequences from the results of TIGR Rice Genome Project (<http://www.tigr.org/tdb/e2k1/osa1/BACmapping/description.shtml>) including the information of INtegrated rice genome Explorer (INE: <http://rgp.dna.affrc.go.jp/giot/INE.html>)²⁴ and searched against about total 300 *cis*-elements known in plants using the PLACE *cis*-element database (<http://www.dna.affrc.go.jp/htdocs/PLACE/>).²⁵

2.6. Cis-element search for genes of *Arabidopsis thaliana* and specification of cis-element for rice and *Arabidopsis thaliana*

Arabidopsis thaliana (*Arabidopsis*) genes corresponding to the rice full-length cDNA obtained were searched by means of the KOME database. *Arabidopsis* full-length cDNA corresponding to *Arabidopsis* genes obtained were searched using the data from RIKEN *Arabidopsis* Genome Encyclopedia (RARGE: <http://rarge.gsc.riken.go.jp/>). From the 5'-end sequences of the full-length cDNA of *Arabidopsis*, the promoter sequences were obtained by comparison with the *Arabidopsis* genomic sequences using RARGE. We compared number of *cis*-elements between genes that response to both hormones in rice and genes of *Arabidopsis* corresponding to both hormone-responsive genes of rice, and the types of *cis*-elements of each species were characterized. For each of plant genes, we divided the total number of each type of *cis*-element in each species by the number of genes in each species. We then compared the numbers of each type of *cis*-element per gene between the two kinds of species.

3. Results

3.1. ABA and GA responsive genes in rice

All the genes selected based on changes in expression were contained in the area of median \pm 2SD over in a normal distribution. The 96 and 94 genes se-

lected with altered expression levels were identified by ABA treatment for 6 hr or 3 days, respectively. As well as experiments of ABA, the 232 and 87 genes selected with altered expression levels were identified by GA treatment for 6 hr or 3 days. Detailed results of these clone contents are described in the Supporting Text, Supplemental Tables 1, 2, 3, and 4 on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html. All of the expression profiles, including signal intensity, filtering results, and expression ratio of all clones are available as '1000_21_C0301_T0301,' '1000_2_C0201_T0201,' '1000_22_C0301_T0301,' and '1000_1_C0201_T0201' at Rice Expression Database (RED) in NIAS (<http://red.dna.affrc.go.jp/RED/>).

3.2. Responsive genes in callus treated with both GA and ABA

To elucidate the interaction between ABA and GA, we selected 19 genes (6-hr and 3-day treatments, 4 and 15, respectively) regulated by both hormone treatments from the results of 6-hr and 3-day treatments (Supplemental Table 5 on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html). Quantitative RT-PCR was used to confirm the changes in expression ratios of these genes and revealed a more than 1.5-fold change in the gene expression level. The results for standard lines of the control gene, poly-ubiquitin (AU108666), in the untreated samples were highly correlated with results in the treated sample ($r = 0.999520$).

Four genes (AU063650, D41439, C20180, AU075491) were up-regulated under the 6-hr treatment by both ABA and GA, as determined by microarray and quantitative RT-PCR. Under the 3-day treatment, 13 of 15 genes were identified as indicative of an antagonistic response between the two hormones (ABA up; GA down). Two genes were up-regulated by both hormones. Under the 6-hr treatment, the Osem gene (C20180),²⁶ the genes for LPT III lipid transfer protein (D41439)²⁷ and LMW heat-shock protein (AU063650),²⁸ and a gene with unknown function (AU075491) showed a non-antagonistic response to ABA (up) and GA (up). No genes having an antagonistic response between the two hormones (ABA down; GA up) were identified in our experiments. We speculated that the genes which were down-regulated by ABA and up-regulated by GA might not have altered gene expression levels under our experimental conditions or we may need to construct a larger-scale microarray for more genome wide expression analysis. The genes for aldose-reductase-related protein (AU101007)²⁹ and embryo globulin 1 (AU063570)² showed antagonistic regulation by both hormones under 3d-treatment (ABA up; GA down). Genes for the LEA family members, RAB24 protein (C28576), water-stress-regulated gene (AU174486),³⁰ the Osem gene (C20180),²⁶ group 3

LEA protein (AU174470),³¹ and hydrophobic LEA protein (C28696), were also antagonistically regulated by the hormones (ABA up; GA down). The Osem gene was up-regulated by both hormones under 6-hr treatment and antagonistically regulated under 3-day treatment. The functions of genes for thionin (D39662, D41338),³² glucose dehydrogenase (C19333),³³ and myo inositol phosphate synthase (AU174807)³⁴ were newly added as responsive to both ABA and GA. Four genes with no functional annotation (AU166971, C29020, C19796, C20014) were assigned functions as antagonistically responsive genes to ABA (up) and GA (down). Four 6-hr treatment-response genes and the 3-day treatment-response gene for thionin were up-regulated by both hormone treatments. Little is known about the genes up-regulated by both hormones.

3.3. Cis-element search for gene cross-talk between ABA and GA responses

We obtained 325 full-length cDNAs from all of 509 EST clones in Supplemental Tables 1, 2, 3, and 4 on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html, from our collection of approximately 30K full-length cDNAs. We carried out digital mapping using the method described in Materials and Methods for these 325 full-length cDNAs. We assigned these full-length cDNAs on the rice genome constructed by BAC contigs and searched *cis*-elements up to 1000 bp upstream of the cDNA from the 5' terminus of the rice genomic sequence. The *cis*-elements of 17 types of genes regulated by both hormones were investigated using the PLACE database. Supplemental Table 6 on our web site (http://cdna01.dna.affrc.go.jp/RMOS/publication_en.html) shows all the *cis*-elements (146 types) that exist in the upstream regions of these 17 genes. Table 1 summarizes the results in Supplemental Table 6 of a *cis*-element search of 17 types of genes regulated by both hormones. These 17 genes were matched to full-length cDNA at over 94% identity (Supplemental Table 6). They do not perfectly match each full-length cDNA because of factors affecting the accuracy of the EST sequence data, such as the inclusion of the "N" sequence. These 17 full-length cDNAs were assigned to a single locus of the rice genome with the BLAST program. All these mapping data are available on KOME (<http://cdna01.dna.affrc.go.jp/cDNA/>).

The results showed that 5 genes reported to have antagonistic responses to both hormones—those for hydrophobic LEA protein, RAB24 protein, group 3 LEA protein,³¹ aldose reductase,²⁹ and Osem²⁶ that were up-regulated by ABA and down-regulated by GA in our experiments—have not only an ABA-responsive element that acts as a receptor for GA as a negative regulator and for ABA as a positive regulator, but also GA-, water stress response-, and storage protein-responsive

Table 1. Summized *cis*-element search in 5' upstream regions.

Stage	Clone name	Accession	Putative gene identification	EL-cDNA	DOFCOREZM	DPBECORE	EBXKSNAPA	LTRECOREATCOR15	MYCORE	MYCATBED2	RAVJAAT	RYBPEATRNNAPA	SEHAMOTIFGMS	WROXATNORI
6 h, 3 d	EC1638	AU090658	Ocean gene (<i>Oryza sativa</i>)	001-119-E02	10	4	28	4	4	6	2	12	8	4
6 h	EH0778	AU031091	Unknown	001-024-C06	15	1	18	2	1	1	4			4
6 h	CH3428	AU093157	LMW heat shock protein (<i>Oryza sativa</i>)	006-203-F10	1		10	5	2	1	1			1
6 h	ST3943	AU174307	Lipid transfer protein (<i>Oryza sativa</i>)	006-205-A04	7	3	18	5	6	1	2	3	2	1
3 d	CH1555	AU166971	Unknown	J033118115	13		6		2		1			6
3 d	CH1659	AU161332	RAB24 protein (<i>Oryza sativa</i>)	J033115022	12		6	1	1		2	1	2	5
3 d	CH2085	AU100990	Hydrophobic LEA protein (<i>Oryza sativa</i>)	001-118-R01	10	3	16		3	1	1	3		3
3 d	CH2836	AU101008	Aldose reductase-related protein (<i>Avena fatua</i>)	J013074115	12	5	12		3		3			3
3 d	CH2882	AU163383	Embryo globulin 1 (<i>Hordeum vulgare</i>)	001-119-A01	8	2	6		4		5	1	2	2
3 d	CH3201	AU165265	Unknown	001-117-H11	16	2	6	1			2	2	1	1
3 d	EA0201	AU174471	Group 3 LEA Protein (<i>Oryza sativa</i>)	001-124-D08	12	6	16		1	2	4			1
3 d	EA0326	AU174487	Water stress-regulated gene (<i>Oryza sativa</i>)	J033070H10	10	4	12	1	3	2	1	1	1	1
3 d	EC0269	AU172698	Glucose and ribitol dehydrogenase (<i>Hordeum vulgare</i>)	002-169-E03	4	1	12	2	3	2	2			3
3 d	EC0946	AU172721	Unknown	001-118-E12	5		12	3	1	2	3	1	2	3
3 d	EC1379	AU090483	Unknown	001-016-C03	13	3	8			2	3	3	4	2
3 d	FE0401	AU174807	myo-inositol phosphate synthase (<i>Oryza sativa</i>)	J033131B11	9		6	1	1	1	2	1	3	2
3 d	ST3801	AU101091	Thionin (<i>Oryza sativa</i>)	001-107-G07	14	10	32	2	12	6	18	8	2	4

Stage: the treatment period of 6 hr or 3 days. Clone name: the original clone identification number of the sequence on Rice Microarray Opening Site (RMOS: <http://cdna01.dna.affrc.go.jp/RMOS/>). Putative gene identification: gene annotation obtained from homology search of the public database. The gene with the highest score was used as the putative gene identification for the sequence. FL cDNA: clone identifiers on full-length cDNA database, KOMÉ (<http://cdna01.dna.affrc.go.jp/cDNA/>). *cis*-elements: the number of *cis*-element within 1000 bp upstream of the 5' region of each gene. Details of all *cis*-element refer to PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE/>).

elements in their 5' upstream regions. These results also show clearly that our search results were correct. Other genes that were up-regulated by ABA and down-regulated by GA in our experiments, for myo-inositol phosphate synthase,³⁴ water stress regulated genes,³⁰ and glucose dehydrogenase³³ previously reported as responsive to ABA had not only ABA response elements that act as a receptor for GA as a negative regulator and for ABA as a positive regulator but also GA-, water stress response and possessed storage protein response elements in the 5' upstream regions.

The results of the *cis*-element search of genes up-regulated by both hormones (genes for heat shock protein, thionin, and Osem) showed that they had *cis*-elements for ABA response (EBOXBNNAPA, RAV1AAT),^{35,36} storage protein (EBOXBNNAPA, SEF4MOTIFGM7S),^{35,37} GA response (DOFCOREZM),³⁸ water stress response (MYBCORE),³⁹ low temperature response (LTRECOREATCOR15),⁴⁰ and drought response (MYCATRD22).⁴¹ In addition, the gene for the pathogen-related protein, thionin, had many more types of *cis*-element motifs than the other 18 ABA- and GA-responsive genes searched. The gene for thionin has *cis*-elements for genes responsive to ABA (68 positions), GA (14 positions), disease resistance (4 positions), drought stress (6 positions), water stress (12 positions), storage protein (34 positions), and low temperature (2 position) in Table 7.

The four genes with unknown function that were up-regulated by ABA and down-regulated by GA in our experiments had not only ABA response elements that act as a receptor for GA as a negative regulator and for ABA as a positive regulator but also GA-, and storage protein-responsive elements. The presence of these elements might support the finding that these unknown genes function as ABA- and GA-response and as storage-protein genes. The gene with unknown function (AU031091) that was up-regulated by both hormones showed that they had *cis*-elements for ABA-, GA-, and storage protein-responsive genes.

3.4. Cluster analysis of genes responsive to both hormones with regard to various expression profiles

To detect similar expression profiles and other profiles of interest, we used 16 genes including 3-day treatment-response genes, for which there was cross-talk between the expression profiles in ABA- and GA-treated callus, in a hierarchical cluster analysis of 43 experimental details of rice expression profiles available in the Rice Expression Database (RED: <http://red.dna.affrc.go.jp/RED/>).⁴² RED contains normalized expression data and basic graphical data derived from experiments on various RNAs hybridized to the Rice 8987 cDNA Array (over 600 experiments on genes in 26 physiological categories). A clustering anal-

ysis (Fig. 1) was performed on 16 genes and 43 experiments that were selected from approximately 600 expression profiles in RED. All expression patterns were obtained from four ratio data. The dendrogram showed three notable clusters (Clusters 1, 2, and 3 in Fig. 1).

Cluster 1 was composed of 5 genes (AU166971, C28576, C28696, AU174807, AU063570) from 6 experiments. These were newly detected as cross-talk genes responding to blast infection, heat shock, salt stress, and expression in endosperm tissue, seed pericarp, and seed coat of rice. Cluster 2 was composed of 10 genes from 3 experiments (C28576, AU174807, AU063570, C19333, AU174470, AU101007, C20419, AU174486, C20180, C29020). They had the same responses to ABA treatment, sugar starvation and low-temperature treatment. Cross-talk between ABA treatment and low temperature⁴³ or sugar starvation⁴⁴ has already been reported. These reported cross-talks show that the hierarchical clustering was performed correctly. The clustering results showed that the expression profile obtained with ABA treatment in our experiments in Cluster 2 was similar to the profile produced by sugar starvation and was similar to that seen for seed coat in Cluster 1 ($r = 0.854$). These similarities might suggest that ABA treatment induces conditions close to those produced by sugar starvation at the molecular level in seeds. Cluster 3 was composed of 9 experiments on 1 gene (D41338), that for thionin of rice. Thionin was up-regulated in all 9 experiments, including in N₂ starvation, glucose starvation, acid treatment, and brassinosteroid treatment. Although we were unable to compose a cluster with regard to the expression profile (experiments; 2122_1_C0001_T0001) of rice callus when the TEIL (Tobacco Ethylene INsensitive 3-like) gene was introduced (arrow 1 in Fig. 1), the expression profile was similar to that seen with GA treatment ($r = 0.855$, experiments; 1000_1_C0201_T0201). As expected, the expression pattern (arrow 2 in Fig. 1, experiments 2116_2_C0001_T0003) of rice seedlings treated with Uniconazole P (Sumitomo Chemical, Tokyo, Japan), an inhibitor of GA synthesis, was similar to that seen with ABA treatment ($r = 0.854$, experiments; 1000_2_C0201_T0201) and opposite to that in our GA experiments ($r = 0.256$). We also performed hierarchical clustering of the cross-talk genes that responded to the 6-hr treatment with ABA and GA. However, these genes did not noticeably cluster towards any particular expression profile in RED (data not shown).

3.5. Specification of *cis*-elements of rice and Arabidopsis genes

To characterize of mechanism for transcriptional regulation of the hormone-response system in rice, we analyzed *cis*-elements for genes of *Arabidopsis* corresponding to rice genes that respond to both hormones, and

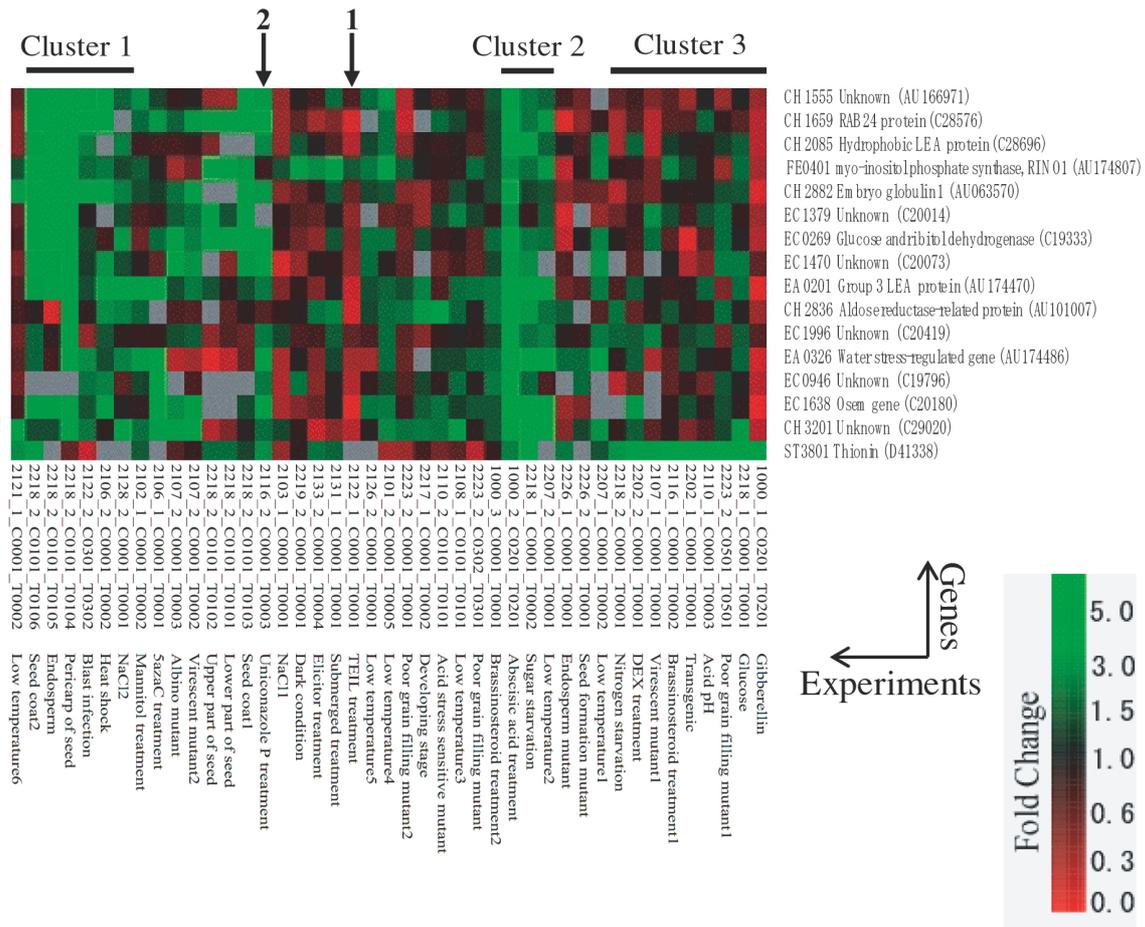


Figure 1. To detect cross-talk and specificity in our expression profile, we submitted 16 rice callus genes that showed cross-talk between ABA and GA treatments to a cluster analysis using UPGMA toward 43 rice expression profiles in the Rice Expression Database. The rows represent genes and the columns represent experiments. Details of the 43 experiments described at the end. Small labels below the cluster are RED experiment identifiers and experimental keywords. Small labels on the right side of the cluster indicate the EST clone name on our microarray, annotation and GenBank accession ID. The color scale indicates the degree of change (fold change) in expression. Details of 43 experiments; 1000_1_C0201_T0201: Rice callus treated with 50 μ M GA for 3 days, 2218_1_C0001_T0001: Rice callus treated with 90 mM glucose for 2 days, 2223_2_C0501_T0501: Superior spikelets of rice poor-grain-filling mutant at 13 days after flowering, 2110_1_C0001_T0003: Rice root treated with acidic condition (pH 5.5) for 1 day, 2202_1_C0001_T0001: 10-day-old seedling of transgenic rice (CDK activating kinase), 2116_1_C0001_T0002: Rice shoot treated with 1 μ M brassinolide for 6 hr, 2107_1_C0001_T0001: Leaves of weak virescent mutant of rice, 2202_2_C0001_T0001: 7-day-old seedling of rice treated with 1 μ M glucocorticoid (Dexamethasone: DEX) for 0 hr, 2218_2_C0001_T0001: Following nitrogen starvation, seedlings of 3-week-old rice treated with 3 μ M ammonium, 2207_1_C0001_T0002: Rice panicle treated with 12°C for 6 days, 2226_2_C0001_T0001: Panicle of seed-formation mutant of rice, 2226_1_C0001_T0001: Immature seed 14 days after pollination in embryo mutant of rice, 2207_2_C0001_T0001: Rice panicle treated with 12°C for 6 days, 2218_1_C0001_T0002: Rice callus treated with glucose (90 mM) for 2 days, 1000_2_C0201_T0201: Rice callus treated with 50 μ M ABA for 3 days, 1000_3_C0001_T0001: Rice callus treated with 50 μ M brassinolide for 3 days, 2223_2_C0302_T0301: Superior spikelets of rice poor-grain-filling mutant at 1 day after flowering, 2108_1_C0101_T0101: Wheat leaf treated with 4°C for 24 hr, 2110_2_C0101_T0101: Root of acid-stress-sensitive mutant of rice treated with acidic condition (pH 4.2) for 1 day, 2217_1_C0001_T0002: Rice leaf at heading stage 5, 2223_2_C0001_T0001: Inferior spikelets of rice poor-grain-filling mutant at 1 day after flowering, 2101_2_C0001_T0005: 3-week-old whole plants of rice treated with 4°C for 5 hr, 2126_2_C0001_T0001: Spikelets of early microspore stage of rice treated with 12°C for 1 day, 2122_1_C0001_T0001: Rice callus of transgenic rice (Tobacco Ethylene Insensitive 3-Like gene), 2131_1_C0001_T0001: Rice shoot under submerged condition for 48 hr, 2133_2_C0001_T0004: Suspension-cultured cell treated with blast-fungal-elicitor for 8 hr after treatment with MAPKK inhibitor, 2219_2_C0001_T0001: Leaf blade of rice grown under dark conditions for 40 hr, 2103_1_C0001_T0001: Rice leaf 24 days after treatment with 50 mM NaCl, 2116_2_C0001_T0003: Rice leaf treated with 1 μ M uniconazole P for 24 hr, 2218_2_C0101_T0103: Seed coat of rice seed 3 days and 6 days after pollination, 2218_2_C0101_T0101: Lower part of rice seed 3 days and 6 days after pollination, 2218_2_C0101_T0102: Upper part of rice seed of 3 days and 6 days after pollination, 2107_2_C0001_T0002: Leaves of virescent mutant of rice, 2107_2_C0001_T0003: Leaves of albino mutant of rice, 2106_1_C0001_T0001: Oc suspension cultured cell treated with 5-azacytidine for 3 days, 2102_1_C0001_T0002: Rice leaves treated with 0.1 M mannitol for 2 days, 2128_2_C0001_T0001: Rice shoot treated with 150 mM NaCl for 12 hr, 2106_2_C0001_T0002: Suspension cultured cell treated with 42°C for 2 hr, 2122_2_C0301_T0302: Rice leaf treated with blast fungus for 66 hr, 2218_2_C0101_T0104: Pericarp of rice seed of 10 days and 14 days after pollination, 2218_2_C0101_T0105: Endosperm of rice seed of 10 days and 14 days after pollination, 2218_2_C0101_T0106: Seed coat of rice seed of 10 days and 14 days after pollination, 2121_1_C0001_T0002: Suspension cultured cell of brome grass treated with 4°C for 6 days.

the types of *cis*-element of each species were compared. The numbers of all types of *cis*-elements of genes responsive to both hormones in each species are shown in Supplemental Table 6 (Rice) and Table 7 (*Arabidopsis*) on our web site, http://cdna01.dna.affrc.go.jp/RMOS/publication_en.html. The 14 genes are shown in the “clone name” column of Supplemental Table 7. The results of comparison between the 14 genes responding to both hormones in rice and the 14 genes of *Arabidopsis* corresponding to these genes in rice are summarized in Table 2. In all detected *cis*-elements, we selected *cis*-elements for the determination of specific elements in each species that were present in at least two genes in either species, and specific elements were more than \pm twofold in the ratio of rice:*Arabidopsis*. Also, we selected *cis*-elements for determination of common elements in both species that were present in at least 1 gene in both species. Five kinds of *cis*-element for dehydration-stress response (ACGTATERD1, MYB1AT, DRECRCOREAT, ABRELATERD1, MYCCONSENSUSAT) were specified as elements in *Arabidopsis* (italic character of “*cis*-element” column in Table 2). These five kinds of elements were not present in rice. The six kinds of *cis*-element for light response were specified as elements in *Arabidopsis* rather than in rice. *Arabidopsis* has a twice as many differences as found in the number of these six kinds of *cis*-elements of rice. The results might suggest differences in the mechanism of light response between the two species. In rice, elements for stress response, MYB2AT (water stress), MYCATRD22 (dehydration stress) and LTREATLTI78 (low temperature), were rich in the upstream regions of genes than *Arabidopsis*. The *cis*-elements for expression of amylase gene (CGACGOSAMY3) was found in rice. The number of *cis*-elements for protein storage was remarkably rich in both species (2SSEEDPROT-BANAPA, AACACOREOSGLUB1, CANBNNAPA and SEF3MOTIFGM in *Arabidopsis*, ACGTOSGLUB1 and RYREPEATLEGUMINBOX in rice). *Arabidopsis* has a greater variety elements for protein storage than does rice. The *cis*-element of ABREOSRAB21 that was founded as an ABA-responsive element of wheat *Em* and rice *rab21* genes was specified as elements in rice genes (see “rice specific” of “Specific or common” column in Table 2).

4. Discussion

We performed microarray experiments using callus tissue treated with phytohormones. Genes for embryogenesis, germination, seed dormancy, and grain filling were included among those with altered expression levels in calli treated with these hormones. We suggest that treatment of callus with ABA or GA demonstrates the dormancy and germination of seed. The fact that the experiments identified many already known ABA- and GA-responsive

genes in other tissues confirms that callus tissue can be used to elucidate the mechanisms of germination and dormancy.

The results of these experiments (GA 6-hr treatment, GA 3-day, ABA 6-hr, ABA 3-day) included genes responsive to various physiological events in addition to the ABA or GA response. In particular, many PR protein genes were responsive to ABA and GA treatment for 6-hr and 3-day periods. In the ABA response of callus, we detected PR protein genes such as those for pathogen-related thaumatin-like protein (6-hr treatment), chitinase IIb (6-hr), endochitinase (6-hr), class III chitinase (6-hr), thionin (3-day), and PBZ-1 (both). In the GA response of callus, we also detected genes such as those for pathogen-related thaumatin-like protein (6-hr), class III chitinase (6-hr), phenylalanine ammonia-lyase (3-day), and thionin (3-day). These results suggest that the ABA and GA response pathways have crosstalk with the pathogen-related pathway in rice callus. Jasmonic acid and salicylic acid reportedly function in defense against pathogen infection in plants.^{45,46} However, ABA and GA have not been reported to be related to pathogen infection.

Metallothionein was down-regulated by 6-hr and 3-day GA treatments. This result suggests that the rice cell may have to down-regulate metallothionein during GA treatment for activation of metal-sensitive protease, a gene which is modulated by the presence of metal cations during seed germination.⁴⁷ However, under ABA treatment the expression ratio of metallothionein did not change with 3-day treatment, although it was down-regulated by 6-hr treatment (Supplemental Table 1 on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html). We can guess that the rice cell begins to accumulate products of the gene with ABA accumulation, as reported for developing pollen of wheat.⁴⁸ In addition, the results of a *cis*-element search showing that the upstream sequence of this gene has ABA (EBOXBNNAPA)³⁵ and GA (DOFCOREZM)³⁸ response elements (data not shown) support the hypothesis that the gene is closely related not only to metal stress response but also to ABA and GA response. The results of a phenotype search using the Tos17 mutant panel database (Tos17; <http://tos.nias.affrc.go.jp>) showed that breaking the gene for metallothionein (AU055773) by Tos17 insertional mutagenesis gave a dwarf phenotype, which is a result of changes in GA metabolism (result number ND9040-0-104-1A in the Tos17 database). Full-length cDNA (AK058313) corresponding to (identity = 98%) the gene for metallothionein (AU055773) was a perfect match (identity = 100%) to chromosome 1 (BAC clone: AP003197, 74,385-75,589 bp) of the rice genome (*japonica* cultivar). To search the mutant line in the Tos17 database, users are required to register at the Tos17 web site. The Tos17 database is password pro-

Table 2. Specification of *cis*-elements of rice and *Arabidopsis* genes.

Specific or common	Rice	<i>cis</i> - element	<i>Arabidopsis</i>	Rice/ <i>Arabidopsis</i>	Category
Rice specific	42	CGACGOSAMY3	19	2.21	Amylase
Rice specific	19	MYBPZM	8	2.38	Development (pigmentation in floral organ)
Rice specific	10	HEXAMERATH4	5	2.00	Histone H4 promoter;
Rice specific	8	ABREOSRAB21	1	8.00	Hormone: ABA
Rice specific	22	RYREPEATLEGUMINBOX	11	2.00	Protein storage
Rice specific	13	ACGTOSGLUB1	1	13.00	Protein storage
Rice specific	23	MYCATRD22	10	2.30	Stress: Dehydration stress
Rice specific	7	LTREATLTI78	1	7.00	Stress: Low temperature stress
Rice specific	8	MYB2AT	2	4.00	Stress: Water stress
Common	31	WBOXATNPR1	22	1.41	Defense
Common	46	RAVIAAT	59	0.78	Hormone: ABA
Common	41	DPBFSCOREDCDC3	51	0.80	Hormone: ABA
Common	26	ACGTABREMOTIFA2OSEM	27	0.96	Hormone: ABA
Common	135	DOFCOREZM	186	0.73	Hormone: GA
Common	83	GT1CONSENSUS	142	0.58	Primary metabolism: Light
Common	93	GATABOX	137	0.68	Primary metabolism: Light
Common	31	IBOXCORE	50	0.62	Primary metabolism: Light
Common	22	INRNTPSADB	27	0.81	Primary metabolism: Light
Common	16	CACGTGMOTIF	26	0.62	Primary metabolism: Light
Common	15	POLASIG2	14	1.07	Primary metabolism: PolyA
Common	26	TAAAGSTKST1	42	0.62	Primary metabolism: Tissue (guard cell)
Common	116	GTGANTG10	119	0.97	Primary metabolism: Tissue (late pollen)
Common	54	POLLENILELAT52	79	0.68	Primary metabolism: Tissue (pollen)
Common	56	ROOTMOTIFTAPOX1	103	0.54	Primary metabolism: Tissue (root)
Common	109	CAATBOX1	173	0.63	Protein storage
Common	188	EBOXBNNAPA	160	1.18	Protein storage
Common	26	SEF4MOTIFGM7S	24	1.08	Protein storage
Common	33	RYREPEATBNNAPA	18	1.83	Protein storage
Common	27	CCAATBOX1	31	0.87	Stress: Heat shock
Common	21	LTRECOREATCOR15	18	1.17	Stress: Low temperature stress
Common	45	MYBCORE	23	1.96	Stress: Water stress
Common	19	MYBST1	19	1.00	Unclassified
Common	14	ASF1MOTIFCAMV	17	0.82	Unclassified
<i>Arabidopsis</i> specific	8	SEBFCONSSTPR10A	21	0.38	Defense
<i>Arabidopsis</i> specific	8	ARFAT	17	0.47	Hormone: Auxin
<i>Arabidopsis</i> specific	3	ERELEE4	8	0.38	Hormone: Ethylene
<i>Arabidopsis</i> specific	4	QARBNEXTA	10	0.40	Primary metabolism: Extensin
<i>Arabidopsis</i> specific	16	TATABOX5	35	0.46	Primary metabolism: Light
<i>Arabidopsis</i> specific	7	-10PEHVPSBD	17	0.41	Primary metabolism: Light
<i>Arabidopsis</i> specific	4	TATABOX4	13	0.31	Primary metabolism: Light
<i>Arabidopsis</i> specific	5	GT1CORE	11	0.45	Primary metabolism: Light
<i>Arabidopsis</i> specific	4	TATABOX3	9	0.44	Primary metabolism: Light
<i>Arabidopsis</i> specific	2	LRENPCABE	7	0.29	Primary metabolism: Light
<i>Arabidopsis</i> specific	0	<i>CGCARGAT</i>	46	n.d.	Primary metabolism: MADS-domain
<i>Arabidopsis</i> specific	11	POLASIG1	44	0.25	Primary metabolism: PolyA
<i>Arabidopsis</i> specific	11	POLASIG3	26	0.42	Primary metabolism: PolyA
<i>Arabidopsis</i> specific	3	MARTBOX	36	0.08	Primary metabolism: Scaffold
<i>Arabidopsis</i> specific	6	CANBNNAPA	17	0.35	Protein storage
<i>Arabidopsis</i> specific	4	AACACOREOSGLUB1	11	0.36	Protein storage
<i>Arabidopsis</i> specific	1	2SSEEDPROTBANAPA	9	0.11	Protein storage
<i>Arabidopsis</i> specific	3	SEF3MOTIFGM	8	0.38	Protein storage
<i>Arabidopsis</i> specific	0	<i>MYCONSENSUSAT</i>	160	n.d.	Stress: Dehydration stress
<i>Arabidopsis</i> specific	0	<i>ACGTATERD1</i>	152	n.d.	Stress: Dehydration stress
<i>Arabidopsis</i> specific	0	<i>ABRELATERD1</i>	57	n.d.	Stress: Dehydration stress
<i>Arabidopsis</i> specific	0	<i>MYBIAT</i>	43	n.d.	Stress: Dehydration stress
<i>Arabidopsis</i> specific	0	<i>DRECRTCOREAT</i>	13	n.d.	Stress: Dehydration stress
<i>Arabidopsis</i> specific	0	<i>ACGTABOX</i>	12	n.d.	Stress: Sugar repression

The 'Specific or common' column shows the *cis*-element of Rice specific, *Arabidopsis* specific or common between both plants. Number of Rice: The total number of *cis*-element of 14 crosstalk genes between ABA and GA treatments within 1000 bp upstream of the 5' region of each gene in rice. *cis*-element: The name of *cis*-elements in plant. Details of all *cis*-element refer to PLACE database (<http://www.dna.affrc.go.jp/htdocs/PLACE/>). An italic character shows the *cis*-element that exists only in *Arabidopsis*. Number of *Arabidopsis*: The number of *cis*-element for 14 genes of *Arabidopsis* corresponding to genes for both hormone response of rice. The data were obtained from RARGE (<http://rarge.gsc.riken.go.jp/>.) Rice/*Arabidopsis*: We calculated the ratio dividing the number of *cis*-element in rice by the number of *cis*-element in *Arabidopsis*. The 'n.d.' shows 'not determined.' Category: *cis*-element function was classified on the basis of the annotation of the element entry in PLACE database.

tected. However, every user can receive passwords to access the database once registered. Registration and

password protection facilitates efficient user support and feedback management, while maintaining a high level of

security.

Many heat shock proteins were induced by the ABA and GA treatments. In addition, alpha-amylase was not expressed in GA treatment for 6-hr. It has been reported that the synthesis of alpha-amylase, one of the key enzymes in seed germination, is suppressed during germination, but heat-shock proteins are induced.^{49,50} Also alpha-amylase is expressed after the expression of *GAmyb* in GA treatment.⁵¹ In our results, expression of the gene for alpha-amylase did not change with GA treatment for 6-hr and the genes for heat shock protein (AU162323, AU173046, D24882, C26730, AU069978, D40879, AU063648, AU063650, C93575) were up-regulated by the treatments (Supplemental Table 3 on the *DNA Research* web site, http://www.dna-res.kazusa.or.jp/10/6/03/supporting_text/support.html).

During germination of cereal seeds, alpha-amylase plays a critical role in providing the growing seedling with metabolizable carbohydrates. In rice, at least 10 genes encode for alpha-amylase isoforms; 2 of them are strongly under the control of the sugar level, namely *RAmy3D* and *RAmy3E*,^{52,53} although the GA-inducible *RAmy1A* gene is also modulated by sugars.⁵⁴ The alpha-amylase genes display tissue-specific expression in which genes *RAmy3B*, *RAmy3C*, and *RAmy3E* are preferentially expressed in the aleurone layer, genes *RAmy1A*, *RAmy1B*, and *RAmy3D* are expressed in both the embryo and aleurone, and genes *RAmy3A* and *RAmy2A* are not expressed in either tissue.⁵⁵ The full-length cDNA of AK073487 and AK103413 corresponding to the clones for the *Ramy3D* and *Ramy3E* genes that were spotted on our microarray were assigned to BAC clone AP004162 of chromosome 8 (3D: 83,215–85,200 bp; 3E: 77,092–78,997 bp). However, the clone for *Ramy1A* did not exist on our microarray. In microarray experiments using RNA of rice callus after GA treatment for 12 hr, the expression of the alpha-amylase 3D and 3E genes was found to be up-regulated, but the expression of heat-shock protein was unchanged (data not shown). These results show that alpha-amylase 3D and 3E genes with the Dof motif for response to GA were up-regulated temporarily in response to the GA treatment for 12 hr. We suggest that alpha-amylase is precisely and temporarily up-regulated by GA during germination after the expression of *GAmyb*.

The 3'-end sequences (AU174487 and AU172698) of two genes in the results of our ABA treatment for 3 days match perfectly those of a water stress-regulated gene and a gene for glucose dehydrogenase. Full-length cDNA (AK073837 and AK110652) corresponding to these were assigned to a single loci on rice chromosomes 1 and 5. The up-regulation of these genes in the experiments contrasted with other reports.^{30,33} We suggest that this difference occurred because of differences in the concentrations of hormone and treatment times. In one study,³⁰ water stress regulated gene was not modulated by 10 μ M ABA treatment for 1 hr in seedlings. In our expression

profile, this gene was up-regulated by 50 μ M ABA for 6 hr or 3 days and was down-regulated by 50 μ M GA for 3 days. The gene for glucose dehydrogenase, whose expression ratio was reportedly unchanged by treatment with 0.1 μ M ABA in the barley embryo,³³ was modulated under our conditions. The 5' upstream sequences of both genes included several *cis*-elements for the ABA response, a finding that supports the results of our microarray analysis (Table 1).

The number of response genes (only 4) with cross-talk expression between ABA and GA treatments for 6 hr is smaller than the 16 with cross-talk expression between ABA and GA treatments for 3 days. These results suggest that these genes expressed under 6-hr treatment play a role in signal transduction rather than in directly promoting germination and dormancy. In contrast, because the cross-talk genes expressed under 3-day treatment responded antagonistically to the two hormones, as shown in our results and various reports,^{1–3} we suggest that cross-talk genes expressed as a late response are directly concerned with germination and dormancy.

In our clustering, we newly detected not only similarity among seed tissues and callus under hormone treatment, but also a new function of thionin. We suggest that the up-regulation of the gene for thionin in over 10 types of physiological responses demonstrates that this protein functions under various conditions and is not just related to the pathogen response.

The upstream sequence of cross-talk genes that were up-regulated by ABA and down-regulated by GA in our experiments includes not only ABA-response elements that act as a receptor for GA as a negative regulator and for ABA as a positive regulator but also other *cis*-elements, such as those for GA-, drought stress-, water stress, low temperature stress-, and pathogen-response. Also, the upstream sequence of cross-talk genes that were up-regulated by both hormones includes not only ABA- and GA-response elements but also other *cis*-elements, such as those for drought stress-, water stress-, low temperature stress-, and pathogen-response. The existence of cross-talk among ABA, GA, and pathogen-response pathways in our expression profiles may be supported by the existence of WBOXATNPR1 elements, which were detected in the 12 cross-talk genes (Table 1) expressed under 6-hr or 3-day treatment with both hormones, along with the existence of many PR genes such as thionin and class III chitinase.

Full-length cDNA (AK062831) corresponding to clones of the thionin gene that were spotted on our microarray was assigned to BAC clone AP005932 of chromosome 8 (45,993–47,064 bp). The upstream region of the thionin gene on the rice genome has many kinds of *cis*-elements, such as those related to drought stress and water stress, low temperature stress, ABA response, GA response, protein storage, and pathogen response. The results of our *cis*-element search suggest that thionin

is an assistant gene rather than a gene directly linked with the ABA and GA response. They also suggest that the thionin gene functions in defense against various stresses, including germination and dormancy, rather than in disease resistance alone. However, other stress-response or pathogen-related genes (class III chitinase, metallothionein-like protein, and LMW heat-shock protein) do not have a variety of *cis*-elements, and the variety of factors to which they respond may be smaller than for thionin. We had assumed that genes directly linked to physiological phenomena would have very specific types and small numbers of *cis*-elements. From the results of the *cis*-element search, it is possible that the number and type of *cis*-elements may have a close relationship to the gene expression volume, intensity, and timing.

In a comparison of *cis*-elements between genes for two hormone response in rice and genes of *Arabidopsis* corresponding to genes for two hormone responses in rice, three kinds of ABA-responsive elements (RAV1AAT, DPBFCOREDCDC3, ACGTABRE-MOTIFA2OSEM) and one GA-responsive *cis*-elements were found to be conserved between rice and *Arabidopsis* (see “Common” of “Specific or common” column in Table 2). We suggest that both plants might respond to ABA or GA using conservative elements for ABA or GA response (“Common” of “Specific or common” column in Table 2). *Cis*-elements for stress response (heat shock, low temperature) were found to be conserved between rice and *Arabidopsis* (see “Common” of “Specific or common” column in Table 2). We suggested that these two species might utilized common *cis*-elements for heat shock- and low temperature-stress responses. Rice has specific *cis*-elements for dehydration stress (MYCATRD22), water stress (MYB2AT) and low temperature (LTREATLTI78), in addition to common *cis*-elements for stress response. Although the *cis*-element, MYBCORE, for water stress response includes both species as common *cis*-elements in Table 2, the number of elements for rice was 1.8-fold larger than for *Arabidopsis*. The results suggest that rice might have a different pathway for the water stress response from that of *Arabidopsis*. Also, we speculate that *Arabidopsis* has a unique system for the response to dehydration stress, because *cis*-elements for the dehydration-stress response were specified as elements in only *Arabidopsis*. The difference in the number of *cis*-elements for amylase gene between the two plants might suggest that the regulatory mechanism for the gene expression in the rice (crop) plant is more efficient than that of *Arabidopsis*. These specificities of each species might be derived from differences in the growth environments (water- and dehydration-environments) and of their physical organization, especially seed tissue.

The analysis of these data was performed by using rice functional genomics tools on National Institute of Agrobiological Sciences (http://www.nias.affrc.go.jp/index_e.html). We plan to perform more comprehensive

expression profiling to elucidate the mechanism of germination and dormancy by using a large-scale oligonucleotide array (approximately 22K transcriptional units) and time-course experiments.

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References

- Jacobsen, S. E. and Olszewski, N. E. 1996, Gibberellins regulate the abundance of RNAs with sequence similarity to proteinase inhibitors, dioxygenases and dehydrogenases, *Planta*, **198**, 78–86.
- Heck, G. R., Chamberlain, A. K., and Ho, T. H. 1993, Barley embryo globulin 1 gene, *Beg1*: characterization of cDNA, chromosome mapping and regulation of expression, *Mol. Gene Genet.*, **239**, 209–218.
- Lovegrove, A. and Hooley, R. 2000, Gibberellin and abscisic acid signalling in aleurone, *Trends Plant Sci.*, **5**, 102–110.
- Yang, J., Zhang, J., Zhu, Q., and Wanf, W. 2001, Hormonal changes in the grains of rice subjected to water stress during grain filling, *Plant Physiol.*, **127**, 315–323.
- Kikuchi, S., Satoh, K., Nagata, T. et al. 2003, Collection, mapping, and annotation of over 28,000 cDNA clones from *japonica* rice, *Science*, **301**, 376–379.
- Sasaki, T., Matsumoto, T., Yamamoto, K. et al. 2002, The genome sequence and structure of rice chromosome 1, *Nature*, **420**, 312–316.
- Feng, Q., Zhang, Y., Hao, P. et al. 2002, Sequence and analysis of rice chromosome 4, *Nature*, **420**, 316–320.
- Harushima, Y., Yano, M., Shomura, A. et al. 1998, A high-density rice genetic linkage map with 2275 markers using a single F2 population, *Genetics*, **148**, 479–494.
- Wu, J., Maehara, T., Shimokawa, T. et al. 2002, A comprehensive rice transcript map containing 6591 expressed sequence tag sites, *Plant Cell*, **14**, 525–535.
- Miyao, A., Tanaka, K., Murata, K. et al. 2003, Target site specificity of the Tos17 retrotransposon shows a preference for insertion within genes and against insertion in retrotransposon-rich regions of the genome, *Plant Cell*, **15**, 1771–1780.
- Sasaki, T., Song, J., Koga-Ban, Y. et al. 1994, Toward cataloging all rice genes: large-scale sequencing of randomly chosen rice cDNAs from a callus cDNA library, *Plant J.*, **6**, 615–624.
- Yu, J., Hu, S., Wang, J. et al. 2002, A draft sequence

- of the rice genome (*Oryza sativa* L. ssp. *indica*), *Science*, **296**, 79–92.
13. Goff, S. A., Ricke, D., Lan, T. H. et al. 2002, A draft sequence of the rice genome (*Oryza sativa* L. ssp. *japonica*), *Science*, **296**, 92–100.
 14. Yamamoto, K. and Sasaki, T. 1997, Large-scale EST sequencing in rice, *Plant Mol. Biol.*, **35**, 135–144.
 15. Yazaki, J., Kishimoto, N., Nakamura, K. et al. 2000, Embarking on rice functional genomics via cDNA microarray: use of 3 UTR probes for specific gene expression analysis, *DNA Res.*, **7**, 367–370.
 16. Kawasaki, S., Borchert, C., Deyholos, M. et al. 2001, Gene expression profiles during the initial phase of salt stress in rice, *Plant Cell*, **13**, 889–906.
 17. Negishi, T., Nakanishi, H., Yazaki, J. et al. 2002, cDNA microarray analysis of gene expression during Fe-deficiency stress in barley suggests that polar transport of vesicles is implicated in phytosiderophore secretion in Fe-deficient barley roots, *Plant J.*, **30**, 83–94.
 18. Seki, M., Ishida, J., Narusaka, M. et al. 2002, Monitoring the expression pattern of around 7,000 *Arabidopsis* genes under ABA treatments using a full-length cDNA microarray, *Funct. Integr. Genomics*, **2**, 282–291.
 19. Lee, J., Williams, M., Tingey, S., and Rafalski, J. 2002, DNA array profiling of gene expression changes during maize embryo development, *Funct. Integr. Genomics*, **2**, 13–27.
 20. Demura, T., Tashiro, G., Horiguchi, G. et al. 2002, Visualization by comprehensive microarray analysis of gene expression programs during transdifferentiation of mesophyll cells into xylem cells, *Proc. Natl. Acad. Sci. U.S.A.*, **99**, 15794–15799.
 21. Miki, R., Kadota, K., Bono, H. et al. 2001, Delineating developmental and metabolic pathways in vivo by expression profiling using the RIKEN set of 18,816 full-length enriched mouse cDNA arrays, *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 2199–2204.
 22. Oshima, T., Aiba, H., Masuda, Y. et al. 2002, Transcriptome analysis of all two-component regulatory system mutants of *Escherichia coli*, *Mol. Microbiol.*, **46**, 281–291.
 23. Murashige, T. and Skoog, F. 1962, A revised medium for rapid growth and bio assays with tobacco tissue cultures, *Physiol. Plant.*, **15**, 473–497.
 24. Sakata, K., Antonio, B. A., Mukai, Y. et al. 2000, INE: a rice genome database with an integrated map view, *Nucl. Acids. Res.*, **28**, 97–101.
 25. Higo, K., Ugawa, Y., Iwamoto, M., and Higo, H. 1998, PLACE: a database of plant *cis*-acting regulatory DNA elements, *Nucl. Acids. Res.*, **26**, 358–359.
 26. Hattori, T., Terada, T., and Hamasuna, S. 1995, Regulation of the *Osem* gene by abscisic acid and the transcriptional activator *VP1*: analysis of *cis*-acting promoter elements required for regulation by abscisic acid and *VP1*, *Plant J.*, **7**, 913–925.
 27. Vignols, F., Wigger, M., Garcia-Garrido, J. M., Grellet, F., Kader, J. C., and Delseny, M. 1997, Rice lipid transfer protein (LTP) genes belong to a complex multigene family and are differentially regulated, *Gene*, **195**, 177–186.
 28. Tseng, T., Yeh, K., Yeh, C., Chang, F., Chen, Y., and Lin, C. Y. 1992, Two rice (*Oryza sativa*) Full-Length cDNA clones encoding low-molecular-weight heat-shock proteins, *Plant Mol. Biol.*, **18**, 963–965.
 29. Bartels, D., Engelhardt, K., Roncarati, R., Schneider, K., Rotter, M., and Salamini, F. 1991, An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein, *EMBO J.*, **10**, 1037–1043.
 30. Takahashi, R., Joshee, N., and Kitagawa, Y. 1994, Induction of chilling resistance by water stress, and cDNA sequence analysis and expression of water stress-regulated genes in rice, *Plant Mol. Biol.*, **26**, 339–352.
 31. Moons, A., De Keyser, A., and Van Montagu, M. 1997, A group 3 LEA cDNA of rice, responsive to abscisic acid, but not to jasmonic acid, shows variety-specific differences in salt stress response, *Gene*, **191**, 197–204.
 32. Iwai, T., Kaku, H., Honkura, R. et al. 2002, Enhanced resistance to seed-transmitted bacterial diseases in transgenic rice plants overproducing an oat cell-wall-bound thionin, *Mol. Plant Microbe Interact.*, **15**, 515–521.
 33. Alexander, R., Alamillo, J. M., Salamin, F., and Bartel, D. 1994, A novel embryo-specific barley cDNA clone encodes a protein with homologies to bacterial glucose and ribitol dehydrogenase, *Planta*, **192**, 519–525.
 34. Yoshida, K. T., Fujiwara, T., and Naito, S. 2002, The synergistic effects of sugar and abscisic acid on myo-inositol-1-phosphate synthase expression, *Physiol. Plant.*, **114**, 581–587.
 35. Stalberg, K., Ellenstom, M., Ezcurra, I., Abolov, S., and Rask, L. 1996, Disruption of an overlapping E-box/ABRE motif abolished high transcription of the *napA* storage-protein promoter in transgenic *Brassica napus* seeds, *Planta*, **199**, 515–519.
 36. Kagaya, Y., Ohmiya, K., and Hattori, T. 1999, RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants, *Nucl. Acids. Res.*, **27**, 470–478.
 37. Allen, R. D., Bernier, F., Lessard, P. A., and Beachy, R. N. 1989, Nuclear factors interact with a soybean beta-conglycinin enhancer, *Plant Cell*, **1**, 623–631.
 38. Washio, K. 2001, Identification of Dof proteins with implication in the gibberellin-regulated expression of a peptidase gene following the germination of rice grains, *Biochim. Biophys. Acta.*, **1520**, 54–62.
 39. Urao, T., Yamaguchi-Shinozaki, K., Urao, S., and Shinozaki, K. 1993, An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence, *Plant Cell*, **5**, 1529–1539.
 40. Baker, S., Wilhelm, K., and Thomashow, M. 1994, The 5'-region of *Arabidopsis thaliana* *cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression, *Plant Mol. Biol.*, **24**, 701–713.
 41. Abe, H., Yamaguchi-Shinozaki, K., Urao, T., Iwasaki, T., Hosokawa, D., and Shinozaki, K. 1997, Role of *Arabidopsis* MYC and MYB homologs in drought- and abscisic acid-regulated gene expression, *Plant Cell*, **9**, 1859–1868.
 42. Yazaki, J., Kishimoto, N., Ishikawa, M., and Kikuchi, S. 2002, Rice Expression Database: the gateway to rice

- functional genomics, *Trends Plant Sci.*, **7**, 563–564.
43. Yamaguchi-Shinozaki, K. and Shinozaki, K. 1994, A novel *cis*-acting element in an *Arabidopsis* gene is involved in responsiveness to drought, low-temperature, or high-salt stress, *Plant Cell*, **6**, 251–264.
 44. Finkelstein, R. R. and Lynch, T. J. 2000, Abscisic acid inhibition of radicle emergence but not seedling growth is suppressed by sugars, *Plant Physiol.*, **122**, 1179–1186.
 45. Creelman, R. A., Tierney, M. L., and Mullet, J. E. 1992, Jasmonic acid/methyl jasmonate accumulate in wounded soybean hypocotyls and modulate wound gene expression, *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 4938–4941.
 46. Seskar, M., Shulaev, V., and Raskin, I. 1998, Endogenous methyl salicylate in pathogen-inoculated tobacco plants, *Plant Physiol.*, **116**, 387–392.
 47. Fontanini, D. and Jones, B. 2001, Study of metallopeptidase isozymes from malted barley (*Hordeum vulgare* cv. Morex), *J. Agric. Food Chem.*, **49**, 4903–4911.
 48. Reynolds, T. L. and Crawford, L. R. 1996, Changes in abundance of an abscisic acid-responsive, early cysteine-labeled metallothionein transcript during pollen embryogenesis in bread wheat, *Plant Mol. Biol.*, **32**, 823–829.
 49. Grindstaff, K. K., Fielding, L. A., and Brodl, M. R. 1996, Effect of gibberellin and heat shock on the lipid composition of endoplasmic reticulum in barley aleurone layers, *Plant Physiol.*, **110**, 571–581.
 50. Belanger, F., Brodl, M. R., and Ho, T. D. 2003, Heat shock causes destabilization of specific mRNAs and destruction of endoplasmic reticulum in barley aleurone cells, *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 1354–1358.
 51. Gubler F, J. JV. 2003, Gibberellin-responsive elements in the promoter of a barley high-pI alpha-amylase gene, *Proc. Natl. Acad. Sci. U.S.A.*, **4**, 1435–1441.
 52. Yu, S. M., Kuo, Y. H., Sheu, G., Sheu, Y. J., and Liu, L. F. 1991, Metabolic derepression of alpha-amylase gene expression in suspension-cultured cells of rice, *J. Biol. Chem.*, **266**, 21131–21137.
 53. Yu, S.-M., Lee, Yi-Ching, Fang, S.-C., Chan, M.-T., Hwa, S. F., and Liu, L.-F. 1996, Sugars act as signal molecules and osmotica to regulate the expression of alpha-amylase genes and metabolic activities in germinating cereal grains, *Plant Mol. Biol.*, **30**, 1277–1289.
 54. Morita, A., Umemura, T., Kuroyanagi, M., Futsuhara, Y., Perata, P., and Yamaguchi, J. 1998, Functional dissection of a sugar-repressed alpha-amylase gene (*RAmy1A*) promoter in rice embryos, *FEBS Lett.*, **423**, 81–85.
 55. Karrer, E. E., Litts, J., and Rodriguez, R. L. 1991, Differential expression of alpha-amylase genes in germinating rice and barley seeds, *Plant Mol. Biol.*, **16**, 797–805.