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Differential Analysis of Protein Expression in RNA-Binding-Protein Transgenic and Parental Rice Seeds Cultivated under Salt Stress

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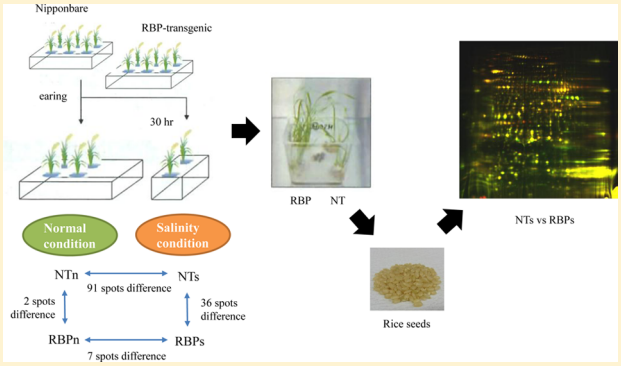
Supporting Information

ABSTRACT: Transgenic plants tolerant to various environmental stresses are being developed to ensure a consistent food supply. We used a transgenic rice cultivar with high saline tolerance by introducing an RNA-binding protein (RBP) from the ice plant (*Mesembryanthemum crystallinum*); differences in salt-soluble protein expression between nontransgenic (NT) and RBP rice seeds were analyzed by 2D difference gel electrophoresis (2D-DIGE), a gel-based proteomic method. To identify RBP-related changes in protein expression under salt stress, NT and RBP rice were cultured with or without 200 mM sodium chloride. Only two protein spots differed between NT and RBP rice seeds cultured under normal conditions, one of which was identified as a putative abscisic acid-induced protein. In NT rice seeds, 91 spots significantly differed between normal and salt-stress conditions. Two allergenic proteins of NT rice seeds, RAG1 and RAG2, were induced by high salt. In contrast, RBP rice seeds yielded seven spots and no allergen spots with significant differences in protein expression between normal and salt-stress conditions. Therefore, expression of fewer proteins was altered in RBP rice seeds by high salt than those in NT rice seeds.



KEYWORDS: *proteomics, 2D-DIGE, salt stress, rice allergens, RNA binding protein, transgenic rice*

INTRODUCTION

Now genetically modified (GM) foods have been developed, which enhances their resistance to insects or herbicides, their growth, preferable nutrients, and so on. In addition, stress-tolerance gene-transgenic plants are under-developed to make the plants tolerant to stresses such as cold, heat, and salt.[1] Because the introduction of stress-tolerance gene is suggested to affect expressions of multiple genes, unintended effects on protein expression levels may appear in transgenic plants compared with that in nontransgenic (NT) plants. In such cases, proteomic approach of safety assessment for transgenic plants seems to be necessary, in particular to know their allergenicity.

Rice is a grain that is cultured and consumed worldwide, and many transgenic rice lines have been developed to improve productivity and add nutrients, such as Fe-fortified rice,[2] beta-carotene (provitamin A) rice (Golden rice),[3] and high-level tryptophan rice.[4] Consumers are concerned that transgenic rice may be more allergenic. Rice allergy is not common, but there are several reports of immediate hypersensitivity reactions after rice ingestion, leading to rhinoconjunctivitis,[5] bronchial asthma,[6] and atopic dermatitis.[7] Major rice allergens of 14−16 kDa, RAs, were identified from a rice salt-soluble fraction as members of the alpha-amylase/trypsin inhibitor-like protein family with high sequential homology each other, which includes RA17 (or RAG1), RA14 (or RAG2), and RA5.[8−11] Glyoxalase I[12] and 56 kDa glycoprotein[13] are also rice allergens, and globulins that bind to rice-allergic-patients serum IgE are also possible rice allergens.[14] Many plant allergens are reported to be categorized into pathogenesis-related (PR) proteins that are induced through the action of defense signaling.[15] The structure similarity of alpha-amylase and trypsin inhibitor family protein and PR-5 protein, zeamatin, has also been reported.[16] Therefore, expression change of allergenic proteins after stress tolerance-gene transfection seems to be important.

A consistent food supply would be particularly helped by the production of stress-resistant rice.[17] Such strains can be generated by introducing transcription factors that regulate the expression of stress-response proteins or genes isolated from stress-tolerant plants. Transfer of transcription factors or stress-tolerance gene may enhance stress tolerance but may also trigger unidentified mechanisms and endogenous proteins that are not part of the stress response. Proteomic analysis may be used to

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identify unintended changes in protein expression, particularly of harmful proteins such as allergens. In fact, we and another group have reported comprehensive analyses of differences in protein expression between transgenic and NT lines using gel-based proteomics.[14,18,19] NT host plant lines are generally used as comparators in the analysis of allergens in transgenic plants.[20] We have recently demonstrated that protein expression in NT lines varies by cultivar and growth condition.[21,22] Such variations in protein expression have been seen in other plants, such as soybean,[23] and these variations should be considered when assessing differences between stress-tolerant transgenic plants and NT lines.[24] To assess the effect on allergen expression, it is also worth knowing the condition-dependent variations in allergen expression in NT lines.[25]

In this study, we used a transgenic rice line expressing RNA-binding protein (RBP) from the ice plant (*Mesembryanthemum crystallinum*), which is one of the stress-tolerant lines, to assess unintended protein expression including allergen expression in comparison with the NT line. Ice plants can survive in high-saline soil, and our group has revealed that RBP contributes salt stress in ice plants.[26] RBP-transgenic rice acquired the ability to live under saline conditions, in which NT rice cannot survive, but the mode of action of RBP to live in high salt is unknown. To clarify changes in the expression of endogenous unintended proteins in NT and RBP-transgenic rice seeds, we cultured both rice cultivars in normal water medium and 200 mM NaCl medium, at which concentration the NT rice did not survive. Other studies on salt stress-responsive proteins used rice tissues such as roots and shoots[27,28] but not rice seeds. We then intended to identify which salt-soluble proteins of the rice seed were altered by exposure to salt stress by using 2D-DIGE to compare protein expression in NT and RBP rice seeds in the presence and absence of salt stress; differentially expressed proteins were identified by MALDI-TOF MS/MS.

EXPERIMENTAL SECTION

Rice Plant Materials and Protein Extraction

Ice plant RBP cDNA was obtained by bacterial functional screening method.[29] RBP cDNA driven by cauliflower mosaic virus 35S promoter including omega sequence of tobacco mosaic virus was constructed in the binary vector, pAB7113,[30] and the constructed vector was introduced into *Rhizobium radiobacter (Agrobacterium tumefaciens)* EHA105, followed by transfection to *Oryza sativa* cv. Nipponbare to establish RBP-transgenic rice lines.[31] NT and RBP-transgenic rice were cultivated in normal water medium or 200 mM NaCl for 30 h after heading. Rice mature seeds were grouped as follows ($n = 4$ per group): NT rice cultured in normal medium (NTn), NT rice cultured in saline medium (NTs), RBP rice cultured in normal medium (RBPn), and RBP rice cultured in saline medium (RBPs). Equal amounts of protein from two transgenic lines were mixed and used for 2D-DIGE analysis.

Proteins were extracted from rice seeds with 1 M NaCl, as described,[21,32] and extracts were stored at −80 °C until use. The protein concentration of the rice seed extracts was determined with a 2D-Quant Kit (GE Healthcare UK, Little Chalfont, U.K.), then purified with a 2D Clean-Up Kit (GE Healthcare).

2D-DIGE

2D-DIGE analysis was performed as described[21] with slight modification. In brief, equal quantities of salt-soluble proteins from all four groups were mixed and used as an internal standard. The internal standard was labeled with Cy2, and proteins from each rice group were labeled with Cy3 or Cy5, according to the manufacturer's protocol. Cy-labeled proteins (25 μg from each sample) were mixed and applied to an Immobiline Drystrip (pH 3−10 NL, 13 cm, GE Healthcare), and 1D isoelectric focusing (IEF) was performed at 20 °C under the following conditions: 500 V for 4 h, 1000 V for 1 h, and 8000 V for 4 h. After reduction and alkylation, the proteins were separated by 2D SDS-PAGE on 10−20% acrylamide gels (DRC, Tokyo, Japan) at 200 V for 3 h. Fluorescence images were acquired with a Typhoon 9400 variable image analyzer (GE Healthcare). Fluorochromes were detected as follows: Cy2 with a 488 nm bandpass (520BP40) filter, Cy3 with a 532 nm bandpass (580BP30) filter, and Cy5 with a 633 nm bandpass (670BP30) filter. The spots were detected and matched using Decyder software version 7 (GE Healthcare).

The fluorescence intensity of each protein spot was normalized to that of the internal standard. We calculated normalized values for protein spots from RBP and NT rice, with or without salt stress. Spot expression differences between NTn versus RBPn, NTn versus NTs, RBPn versus RBPs, and NTs versus RBPs were tested by Student's *t*-test with Bonferroni correction, and $p < 0.05$ was considered to indicate a significant difference. The "ratio" denotes the mean value of a relative normalized protein spot between groups, and two-fold differences were considered to represent altered expression.

In-Gel Digestion and Protein Identification by MALDI-TOF MS/MS

Spots of interest were excised from 2D gels in which 100 μg protein was separated, destained, dried, and in-gel digested for 2 h at 37 °C in trypsin solution (30 μg/mL Trypsin Gold-Mass Spec grade (Promega, Madison, WI) and ProteaseMax (Promega)). Digested peptides were mixed with α-cyano-4-hydroxycinnamic acid (α-CHCA, Sigma Aldrich), and MS spectra and MS/MS fragment ion masses were determined with a 4800 MALDI-TOF/TOF analyzer (Applied Biosystems, CA). All mass spectra were acquired in positive ion reflector mode with 2500 shots per spot and externally mass calibrated with a Peptide Mass Protein MALDI-MS Calibration Kit (Sigma-Aldrich Japan). The mass range from 700 to 4000 Da and the 10 most intense ion peaks from the MS run were further submitted to fragmentation using MS/MS mode operated with 1 kV collision energy. The collision-induced dissociation was performed using helium as the collision gas. The signal-to-noise criterion was set to 25 or greater. The monoisotopic masses were processed for identification. The peak list files were generated from the raw mass spectrum data using the "peak to mascot" script of the 4000 Series Explorer Software according to the settings: mass range from 60 to precursor −20 Da, peak density of 10 peaks per 200 Da, signal-to-noise of 5, minimal area value of 20, and maximal 200 peaks per precursor. The peptide sequence tag from all product ions was submitted to a computer database search analysis with MS/MS ion search mode of Mascot (Matrix Science, Boston, MA). The NCBInr database was selected to search using Mascot, and the taxonomy of the database category was set to *Oryza sativa* (updated on Aug 3, 2009) which contained 132 827 sequences. Carbamidomethyl cysteine and oxidated methionine were selected as fixed and variable modifications of fragmented peptides, respectively. Trypsin was selected as a specific enzyme, and the number of allowable missed cleavages in tryptic digestion was set at 1. Mass error tolerance of a precursor ion was set to ±1.2 and ±0.6 Da for the product ions. Decoy database and false discovery rate were

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Table 1. List of Identified Protein Spots with Differing Expression in NT and RBP Rice

	fold change[a]				
master no.	RBPn/NTn	NTs/NTn	RBPs/RBPn	RBPs/NTs	protein name
22		0.36		2.6	70 kDa heat shock protein
27		0.29		2.0	heat shock protein 101
28		0.29		2.2	heat shock protein 101
30		0.28		2.1	heat shock protein 101
31		0.43			putative 2-oxoglutarate dehydrogenase; E1 subunit
42		0.29		2.3	elongation factor 2
43		0.42			elongation factor 2
45		0.22		2.6	elongation factor 2
47		0.20		2.9	elongation factor 2
48		0.37		2.9	putative seed maturation protein
49		0.34		3.1	putative seed maturation protein
52		0.40		2.2	putative aminopeptidase N
53		0.33		2.2	putative seed maturation protein
60		0.33			alpha 1; 4-glucan phosphorylase H isozyme
61		0.31		2.2	alpha 1; 4-glucan phosphorylase H isozyme
83		0.28		2.1	heat shock protein 90
127		0.26	0.48		malic enzyme
129		0.33			malic enzyme
166		0.48			phosphoglucose isomerase (Pgi-a)
180		4.8	2.8	0.48	glanule-bound starch synthase I
184		7.1	3.5	0.45	granule-bound starch synthase I
185		6.2	2.6	0.45	granule-bound starch synthase I
186		9.9	3.8	0.42	granule-bound starch synthase I
222		0.48			Cupin family protein; expressed
233		0.42			wheat adenosylhomocysteinase-like protein
248		0.45			19 kDa globulin precursor
315		2.0		0.42	late embryogenesis abundant protein; expressed
320		2.2		0.40	late embryogenesis abundant protein; expressed
332		0.42			alcohol dehydrogenase 1
354		0.42			aldolase C-1
377		0.40			glyceraldehyde-3-phosphate dehydrogenase
378		0.38			glyceraldehyde-3-phosphate dehydrogenase
379		0.38			glyceraldehyde-3-phosphate dehydrogenase
380		0.43			putative glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
381		0.38			putative glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
382		0.33			putative glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)
444	2.3	4.0	2.1		putative abscisic acid-induced protein
468		2.1			glutelin
601		0.48			1-cys peroxiredoxin-A
614		2.0			19 kDa globulin precursor
618		2.3			gamma interferon inducible lysosomal thiol reductase family protein; expressed
639		2.4			19 kDa globulin precursor
698		2.3		0.5	cold shock domain protein 2
699		2.3			19 kDa globulin precursor
754		2.8			putative Bowman Birk trypsin inhibitor
759		2.6			putative Bowman Birk trypsin inhibitor
790		2.3			seed allergenic protein RAG2
811		2.0			seed allergenic protein RAG1
838		2.1			putative globulin (with alternative splicing)
849		2.2			19 kDa globulin precursor
870		2.1			putative globulin (with alternative splicing)/ trypsin amylase inhibitor-like protein
	1/2	51/91	6/7	20/36	number of identified spots/number of total differential spots[b]

[a]The fold change of the normalized fluorescence intensity is shown. [b]Fold change of unidentified spots is shown in Supplemental Table 2 in the Supporting Information.

used. Only significant hits, as defined by the Mascot probability analysis ($p < 0.05$) and ion score >40, were accepted.

Immunoblot Using RAG2-Specific Rabbit IgG

The salt-soluble proteins (4 μg) of NT and RBP rice were separated by SDS-PAGE in a 10−20% acrylamide gel (D.R.C.,

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Tokyo, Japan) and the gel was stained with Quick-CBB (Wako Pure Chemical Industries, Osaka, Japan). For 1D-immunoblot analysis, the separated proteins were transferred to a 0.2 μm BA83 Protran nitrocellulose membrane (GE Healthcare). The membrane was incubated with 0.5% (w/v) casein-PBS blocking buffer for 2 h at room temperature and then incubated with rabbit anti-rice RAG2 protein antibody (diluted 1:1000 with 0.1% casein−PBS) for 1 h at room temperature. After washing three times with 0.05% Tween-20/PBS, the membranes were incubated with horseradish-peroxidase-linked anti-rabbit IgG (1:2000 diluted with 0.1% casein−PBS; GE Healthcare) for 1 h at room temperature. After three more washes with 0.05% Tween-20/PBS, the color reaction was developed with Konica Immunostain (Konica Minolta, Tokyo, Japan) according to the manufacturer's protocol. The band intensity of each sample was measured using Scion Image software, and the significance in differences of the intensity was calculated by Student's *t*-test with Bonferroni correlation.

RESULTS

Preparation of the Seeds of RBP-Transgenic and NT Rice with or without Salinity

Northern blotting confirmed transcription of the RBP gene at 665 bp even under normal conditions (unpublished data). To compare protein expression in NT and RBP rice with and without salt stress, we cultured both rice cultivars in normal water medium and in 200 mM NaCl medium for 30 h after heading. The NT rice became wilted in saline medium; in contrast, the RBP rice grew normally in saline and normal medium. The salt-soluble fractions of the seed extracts were used to compare protein expression by 2D-DIGE.

Protein Expression in NTn and RBPn Rice Seeds under Normal Conditions

First, we compared differences in protein expression between NT and RBP rice seeds cultivated in normal medium (NTn and RBPn). Supplementary Figure 1 in the Supporting Information shows a representative merged image of NTn (green) and RBPn rice (red). The total number of spots was ~600 using DeCyder software. Only two spots exhibited a two-fold difference between NTn and RBPn rice; these are circled in Supplementary Figure 1 in the Supporting Information and listed in Table 1. Spot 444 was excised from the gel, trypsin-digested, and identified by MALDI-TOF MS/MS as putative abscisic acid (ABA)-induced protein. Spot 709 (Supplemental Table 2 in the Supporting Information) could not be identified because of a low concentration of protein in the spot.

Protein Expression in NT Rice Seeds under Normal and Saline Conditions

We evaluated changes in protein expression in NT rice cultured with or without 200 mM NaCl. Expression of 91 protein spots differed over two-fold in NTs versus NTn (Figure 1). Fifty of these spots were identified by MALDI-TOF MS/MS homology search. Allergenic proteins RAG1 (spot 811), RAG2 (spot 790), 19 kDa globulin precursor, and IgE-binding proteins (spots 248, 614, 639, 699, 849) were induced in NTs (Table 1). Among the other nonallergen proteins, granule-bound starch synthase I, a putative Bowman Birk trypsin inhibitor, was induced in NTs. In contrast, saline-inhibited proteins were identified as heat shock proteins, elongation factor, putative seed maturation protein, and malic enzyme.

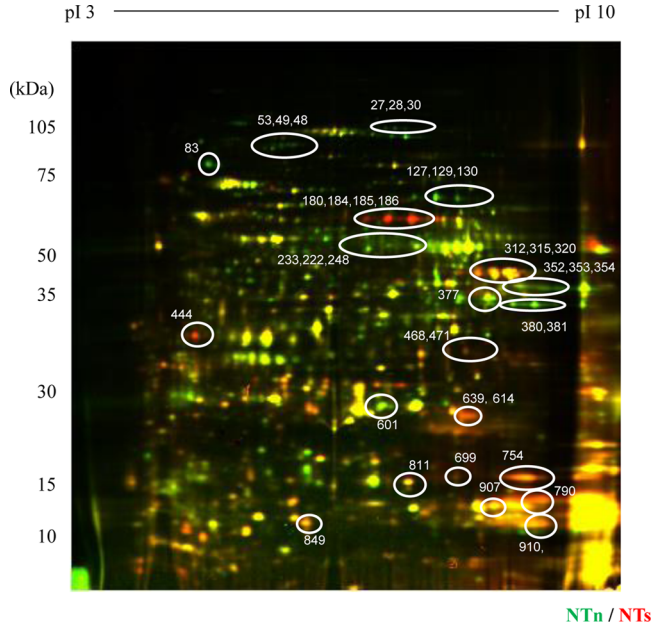

Figure 1. Representative 2D-DIGE merged image of protein expression in NTn and NTs. The gel was scanned using a Typhoon 9400 variable image analyzer to generate Cy3 (green, NTn) and Cy5 (red, NTs) images. The circled spots significantly differed (ratio >2 or <0.5) between NTn and NTs by Student's *t*-test ($p < 0.05$). The numbers beside the circles correspond to spot numbers in Table 1 and Supplemental Table 2 in the Supporting Information.

Protein Expression in RBP-Transgenic Rice Seeds under Normal and Saline Conditions

Changes in protein expression between control RBPn and stressed RBPs were determined. Only seven spots increased/decreased by >2-fold between RBPn and RBPs (Figure 2). These proteins also drastically differed between NTn and NTs, but the magnitude of the fold change was smaller than NT rice (Table 1). These differential proteins did not include allergenic proteins RAG1, RAG2, and 19 kDa globulin precursor proteins. In addition, malic enzyme (spot 127), granule-bound starch synthase I (spots 180, 184, 185, 186), and putative ABA-induced protein (spot 444) showed slight changes between RBPn and RBPs, in contrast with the drastic changes observed between NTs and NTn.

Protein Expression in NT and RBP-Transgenic Rice Seeds under Saline Conditions

To evaluate differences in protein expression due to RBP gene transfer, we compared NTs and RBPs. Thirty-six protein spots differed by >2-fold between NTs and RBPs (Figure 3). Expression of these proteins also differed significantly in salt-stressed NT rice; however, their expression was unchanged between RBPs and all rice cultured under normal conditions, such as heat shock proteins (spots 27, 28, 30), elongation factor (spots 42, 45, 47), putative seed maturation protein (spots 48, 49, 53), α-1,4-glucan phosphorylase H isozyme, cold shock domain protein (spot 698), and late embryo abundant protein; expressed (spots 315, 320). These protein expressions might be controlled by different pathway of ABA signaling in RBPs rice. The expression of granule-bound starch synthase I (spots 180, 184, 185, 186) increased six-fold in NTs versus NTn, and increased three-fold in RBPs versus RBPn, indicating a significant difference between RBPs and NTs.

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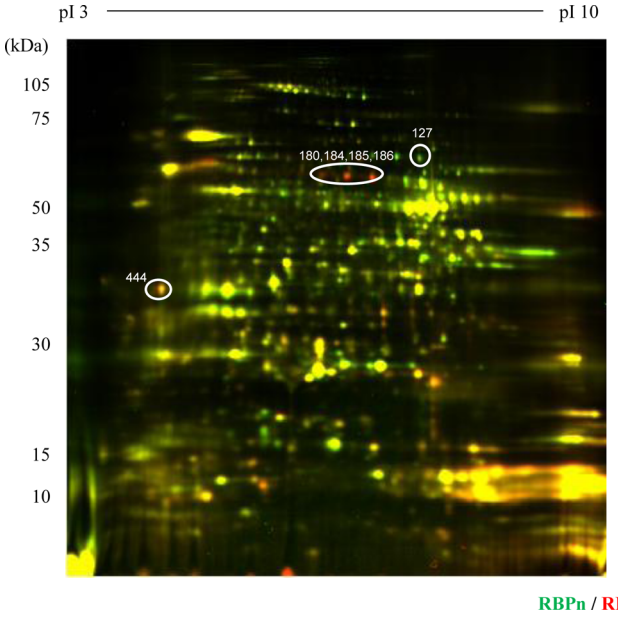

Figure 2. Representative 2D-DIGE merged image of protein expression in RBPn and RBPs. The gel was scanned at two separate wavelengths using a Typhoon 9400 variable image analyzer to generate Cy3 (green, RBPn) and Cy5 (red, RBPs) images. The circled spots significantly differed (ratio >2 or <0.5) between RBPn and RBPs by Student's *t*-test ($p < 0.05$). The numbers beside the circles correspond to spot numbers in Table 1 and Supplemental Table 2 in the Supporting Information.

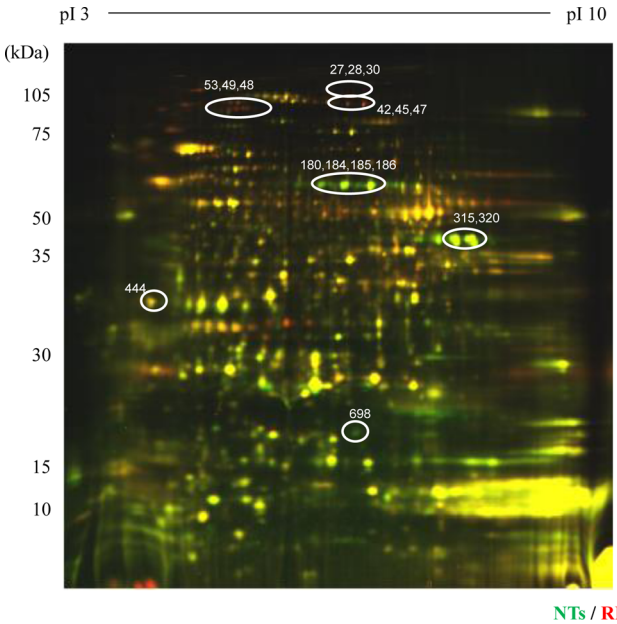

Figure 3. Representative 2D-DIGE merged image of protein expression in NTs and RBPs. Representative fluorescence images of NTs (Cy3, green) and RBPs (Cy5, red) extracts are shown. The circled spots significantly differed (ratio >2 or <0.5) between NTs and RBPs by Student's *t*-test ($p < 0.05$). The numbers beside the circles correspond to spot numbers in Table 1 and Supplemental Table 2 in the Supporting Information.

Expression of Total RAs Proteins in NT and RBP Rice

The differential analysis of protein expression in NTn and NTs revealed induction of allergenic proteins RAG1 and RAG2, members of the RA (alpha-amylase/trypsin inhibitor-like) protein family, under salt stress. RAs appear as multiple spots in 2D gels.[7] We performed immunoblotting using rabbit anti-RAG2 antibody, which also detects other RAs with high sequence homology, to quantify total RA expression. The RAs were slightly increased in NTs versus NTn, but the differences did not reach two-fold (Figure 4A). Measurement of the intensity of the RAs band revealed expression almost 1.1 times that of NTn and showed no significant differences among groups ($p > 0.05$, Figure 4B).

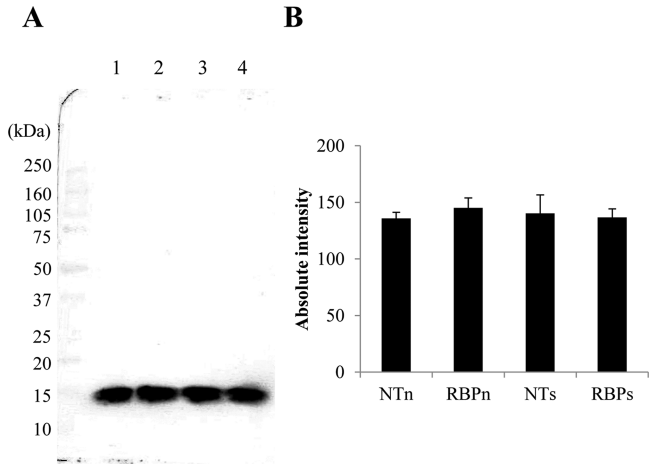

Figure 4. Immunoblotting with RAG2-specific rabbit antibody. The labeled proteins were detected (A) Lane 1, NTn; Lane 2, RBPn; Lane 3, NTs; Lane 4, RBPs. Band intensities are shown in panel B. Data show mean ± SEM. All seeds and conditions showed a 1.1-fold increase over NTn. Intergroup differences were statistically analyzed by ANOVA, followed by the Bonferroni method.

DISCUSSION

Transgenic plants with improved tolerance to environmental stress are beneficial to the food supply. They are typically generated by introducing genes or transcriptional factors isolated from plants tolerant to cold,[33] drought,[34] or high salinity.[35] Stress-tolerance genes and the transcriptional factors that regulate stress-response genes have not been thoroughly characterized.[17] The risk associated with transgenic plants includes the possibility of inducing expression of harmful molecules such as allergenic proteins. In this study, we used a transgenic rice line expressing RBP from the ice plant, which contributes salt stress in ice plants.[26] As RBPs have generally been reported to have a crucial role in post-transcriptional regulation in gene expression, RBP from ice plant also seems to have some unique RNA-binding activity and crucial role in post-transcriptional regulation of certain proteins, but such target proteins of the RBP that give rice the ability to live under high salt condition are not known. We therefore evaluated differences in endogenous protein expression, including allergenic proteins, in NT and RBP rice seeds.

Comparison of NT and RBP rice cultured under normal conditions revealed differential expression of only two proteins (Supplementary Figure 1 in the Supporting Information). The number seems to be too small compared with other rice tissues. One reason for this is that our differential analysis was based on reproducible spots whose appearance in different gels was >80%. The growth conditions may also play a role. Moreover, because rice seed is abundant in proteins such as globulin, it may not be possible to identify trace amounts of proteins. We then compared protein expression in RBP and NT rice under saline conditions.

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To determine whether the differences were salt- or gene-induced, we also compared the RBP rice to NT rice cultured under normal and saline conditions. Over 28 proteins exhibited altered expression in NTs versus NTn (Figure 1), including stress-response proteins such as heat shock protein 101 and growth-related proteins such as granule-bound starch synthase I (Table 1). Notably, expression of a part of the spots of allergens RAG1 and RAG2 was partially increased in NTs rice. We previously reported the variations in protein expressions of rice allergenic proteins (RAs) including RAG1 and RAG2 between rice species such as sp. *japonica* and *india*.[21,22] In this manuscript, we first clarified the increase in expressions of RAG1 and RAG2 in NT rice cultured under salt stress condition by using a 2D-DIGE method (Figure 1). The expression levels of RAs in RBP rice cultured with high salinity were similar to those cultured under normal condition (Figure 2). These results suggest RA expression varies between rice species and growth conditions; however, total RA expression did not differ between NT rice under normal and saline conditions, as indicated by immunoblotting with RAG2-specific rabbit antibodies (Figure 4). These results indicate that a part of RA spots in NTs was increased in their expression in comparison with those in NTn, but the differences in total RAs expressions between NTn and NTs were not significant. In RBP rice seed cultured under normal conditions, expression of RAG1 and RAG2 allergens seems to be as high as that in NT rice seeds grown in high salt. Therefore, RAG1/2 allergens seem to be increased in NT but not RBP rice seed under high salt conditions. Further studies on the variations of RA expression in rice seeds under other environmental stresses and on the function of RA in the response to environmental stress are needed. As for the total allergenicity of RBP rice, RBP seeds contain the same level of allergens as NT rice seeds do. Therefore, consumer costs in increased allergens seemed to be negligible.

In contrast with NT rice, protein expression differed only slightly between salt-stressed RBP rice and RBP rice cultured under normal conditions (Figure 2). The three proteins that differed between RBPn and RBPs also differed between NTn and NTs, but the magnitude of the change in RBP rice was smaller. The mechanism of salt tolerance in RBP-transgenic rice remains unknown, but our results suggest the RBP-transgenic rice may maintain levels of protein expression as they are under normal conditions.

Finally, we compared the protein expression in NTs and RBPs to evaluate the effect of the RBP gene. Salt-stress-responsive proteins differed significantly in NT rice but changed only slightly or not at all in RBP rice (Figure 3). A few other proteins also varied following gene transfer, but they were not identified because of their low abundance.

These results suggest that differences between NTn and RBPn were few and minor, but the differences were striking during culture under high salinity. Because stress-tolerant transgenic plants may be viable under conditions in which control plants cannot grow, this study suggests it is important to prepare the proper controls to assess the safety of transgenic plants. In this study, changes in protein expression in RBP rice were smaller than those in NT rice under salt stress versus normal conditions. In normal culture, expression of ABA-induced protein was greater in RBP rice than in NT rice. This protein was increased in both rice lines cultured in high salinity; however, the magnitude of the change was greater in NT rice than in RBP rice. ABA pretreatment of rice enhances salt tolerance, mediated by various metabolic enzyme.[36] The mechanism by which ABA-induced protein mediates salt tolerance in RBP rice requires further study. Furthermore, like heat-shock proteins, expression levels of some proteins were decreased under high salinity condition of NT rice but not in RBP rice, indicating the existence of a different pathway of ABA signaling in RBP rice. It seems to be important to know the mechanism by which a different pathway of ABA-signaling is activated in RBP rice.

We used salt-soluble proteins to evaluate differences in protein expression between NT and RBP rice seeds to focus on changes in allergenic protein expression. Therefore, the difference in salt-insoluble proteins responsive to high salinity in NT and RBP rice seeds might have been missed in this study. To reveal the mechanism of salt-tolerance in rice, a differential analysis using whole proteins from rice seeds and other tissues might be necessary.

In conclusion, we used 2D-DIGE analysis to evaluate protein expression in NT and RBP rice cultured under normal or high saline conditions. The following results were obtained: (1) many proteins, including allergenic proteins, exhibited altered expression in NT rice cultured in saline versus normal medium; (2) only a few proteins exhibited expression differences in RBP rice under saline and normal conditions, and the expression of allergenic proteins remained unchanged; (3) the magnitude of the change in RBP protein expression was smaller than that of NT rice; and (4) comprehensive analysis of protein expression in stress-tolerance-gene-transfected rice seems to be useful tool to know the expression change of stress-responding proteins and also predict stress-responding pathways.

■ ASSOCIATED CONTENT

Supporting Information

Merge image of 2D-DIGE analysis of proteins in the NT rice and the RBP rice cultured under normal condition. Annotated MS/MS spectra of spot 53 and spot 222. Identification of the protein spots by MALDI-TOF MS/MS. List of unidentified protein spots with differing expression in NT and RBP rice. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CBB, Coomassie brilliant blue; IEF, isoelectric focusing; IgE, immunoglobulin E; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; PBS, phosphate-buffered saline, pH 7.2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2D-DIGE, two-dimensional fluorescence difference gel electrophoresis

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