JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Metabolic Profiling of Transgenic Potato Tubers Expressing *Arabidopsis* Dehydration Response Element-Binding Protein 1A (DREB1A)

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Supporting Information

ABSTRACT: Untargeted metabolome analyses play a critical role in understanding possible metabolic fluctuations of crops under varying environmental conditions. This study reports metabolic profiles of transgenic potato tubers expressing the *Arabidopsis DREB1A* transcription factor gene, which induces expression of genes involved in environmental stress tolerance. A combination of targeted and untargeted metabolomics demonstrated considerable metabolome differences between the transgenic lines and nontransgenic parent cultivars. In the transgenic lines, stimulation of stress responses was suggested by elevated levels of the glutathione metabolite, γ-aminobutyric acid (GABA), and by the accumulation of β-cyanoalanine, a byproduct of ethylene biosynthesis. These results suggest that the *Arabidopsis DREB1A* expression might directly or indirectly enhance endogenous potato stress tolerance systems. The results indicate that transgenesis events could alter the metabolic compositions in food crops, and therefore metabolomics analysis could be a most valuable tool to monitor such changes.

KEYWORDS: *transgenic crops, metabolomics, β-cyanoalanine, stress responses*

INTRODUCTION

Crop domestication involved the attempt to confer beneficial traits, such as yields, stress tolerance, shapes, and colors, to various plant species through selective breeding. During the past three decades, the development of genetic engineering techniques enabled breeders to transfer beneficial traits across species barriers directly into target crops. The first successes in plant genetic engineering were the introduction of herbicide tolerance and insect resistance into crops.[1,2] In response to global warming and climate change, massive changes in cultivation practices will be necessary to sustain crop productivity under environmental stresses such as recurrent drought and changes in the physical properties of soil, as well as biotic stresses from as yet unknown pests and pathogens.

It is expected that stress tolerance can be conferred on plants via the manipulation of endogenous defense systems involving the biosynthesis of secondary metabolites that function as antimicrobial compounds and insect deterrents and by the manipulation of structural elements that are involved in plant architecture.[3] One of the most promising means of engineering endogenous stress responses is the manipulation of transcription factor genes that epistatically regulate the metabolic activities that produce defense metabolites.[4] However, the production capacities of such defense metabolites in wild progenitors might become inactive during the domestication process.[5] It is therefore possible that the reinforcement of endogenous stress tolerance may result in the activation of unknown and/or latent stress responses, leading to unexpected metabolic changes and accumulation of undesirable metabolites. Thus, precautionary research is required to ensure the safety of genetically engineered crops.[6,7]

Implementation of metabolomics is a key to clarify biochemical constituents of crops. We performed our metabolite profiling study using a combination of untargeted metabolomics and quantitative analyses of targeted metabolites. Untargeted metabolomics is expected to provide global metabolome information together with specific metabolites for which levels fluctuate under certain conditions. The targeted analyses are designed to quantify selected metabolites of specific importance, including bioactive compounds and the marker metabolites identified through the untargeted metabolomics.

In this study, we characterized transgenic potato tubers[8,9] expressing the *Arabidopsis DREB1A* transcription factor gene under the control of either the CaMV35S promoter (transgenic potato genotype, 35S-3) or the *Arabidopsis rd29a* promoter (transgenic potato genotypes, D163 and D164). In *Arabidopsis*, DRE-binding protein/C-repeat binding factor (DREB/CBF) proteins bind the dehydration-responsive element/C-repeat

Received: September 22, 2012
Revised: November 26, 2012
Accepted: January 3, 2013
Published: January 3, 2013

(DRE/CRT) *cis*-acting elements and regulate the expression of both dehydration- and low-temperature-induced genes.[10] DREB proteins are classified into two subclasses: DREB1/CBF and DREB2. These genes act in response to low temperature and dehydration, respectively, and a number of transgenic studies have been directed toward engineering stress tolerance through the transgenic manipulation of *DREB* expression.[9,11−15] The *rd29a* gene is induced by desiccation, cold, high-salt conditions, and abscisic acid in *Arabidopsis*.[16] The transgenic approach using a combination of the *Arabidopsis rd29A* promoter and *DREB1A* successfully improved environmental stress tolerance in different plant species.[11,17]

The tuber metabolome profiles from transgenic and nontransgenic plants demonstrated considerable differences in their metabolic status, although no significant differences were found in the contents of the toxic compounds such as glycoalkaloids, α-solanine, and α-chaconine. The metabolite levels involved in the metabolism of glutathione and γ-aminobutyric acid (GABA) were clearly elevated in the transgenic lines, suggesting the possible stimulation of stress responses by the expression of *DREB1A*. In addition, β-cyanoalanine (β-CA) was detected in both transgenic and nontransgenic samples, and the accumulation levels were higher in the transgenic samples. β-CA is known to be a biosynthesis byproduct of ethylene (ET), which is a stress-related phytohormone.[18−20] It is therefore possible that *Arabidopsis DREB1A* expression might have directly or indirectly influenced endogenous stress responses, including ET biosynthesis, in the potato, resulting in the accumulation of β-CA. The current results indicate that variable environmental conditions could affect metabolic profiles of crops, irrespective of transgenic and nontransgenic backgrounds; the effect of transgenic manipulation on metabolic profiles should be closely monitored using holistic metabolic analyses.

MATERIALS AND METHODS

Metabolic Profiling. Tubers were harvested from three lines of transgenic *Solanum tuberosum* cv. Desiree containing the *Arabidopsis DREB1A* gene under the control of either the *rd29A* promoter (*rd29A::DREB1A*) or the cauliflower mosaic virus 35S promoter (*35S::DREB1A*).[8,9] Plants were grown in a special netted house (semicontainment green house) that was operated without controlling the temperature and humidity. Cut tissues from a surface layer (2 mm thick) of tubers were immediately frozen in liquid N_2 and stored at −80 °C until use. A total of 12 samples (three replicates from each) were obtained from nontransgenic Desiree (NT), the *DREB1A* transgenic lines *rd29A::DREB1A* (D163 and D164), and a *35S::DREB1A* (35S-3) line. Metabolic profiling was carried out according to the method described previously[21] using the global unbiased metabolic profiling platform composed of three separate mass spectrometric platforms, as described below.[22] For sample extraction, 20 mg of each sample was thawed on ice and extracted using the automated MicroLab STAR system (Hamilton Co., Reno, NV, USA) in 400 μL of methanol containing recovery standards. The extracts were analyzed on an untargeted metabolomics platform that consisted of three independent instrumentation methods: ultrahigh-performance liquid chromatography−tandem mass spectrometry (UHLC-MS/MS^2) optimized for basic species, UHLC-MS/MS^2 optimized for acidic species, and gas chromatography−mass spectrometry (GC-MS). Ion peaks were matched to standard compounds stored in a reference library, and their relative levels were quantified.[22]

The UHPLC-MS analysis was performed using a Waters Acquity UHPLC (Waters Corp., Milford, MA, USA) coupled to an LTQ mass spectrometer (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) with an electrospray ionization source. Two separate UHPLC-MS injections were performed on each sample: one optimized for positive ions and one for negative ions. Derivatized samples for GC-MS were analyzed using a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole MS unit operating at unit mass resolving power. Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (m/z), preferred adducts, and in-source fragments, as well as their associated MS/MS^2 spectra. Comparison of experimental samples to process blanks (water only) and solvent blanks allowed the removal of artifactual peaks.[21,22]

Statistical analysis was performed using JMP (SAS, http://www.jmp.com) and R (http://cran.r-project.org/) to assess the differences in metabolite accumulation between the distinct lines. A log transformation was applied to the observed relative concentrations for each biochemical metabolite because the variance generally increases as a function of each biochemical metabolite's average response. A summary of the metabolites that achieved statistical significance ($p < 0.05$) is shown in Supplementary Table 1 (Supporting Information). A Welch two-sample t test was used to identify metabolites that differed between the nontransgenic (NT) samples and those from the three promoter constructs (35S-3, D163, and D164), according to the principle described by Oliver et al.[21] The false-positive rate associated with multiple comparisons was calculated using the false-discovery rate (FDR) method of Storey and Tibshirani;[23] q values for all tests are included in Supplementary Table 1 (Supporting Information). The q values for the vast majority of significant tests ($P < 0.05$) fell below the 10% FDR ($q < 0.10$). In the biochemical pathway analysis, all tests with significance of $P < 0.05$ were considered without restriction by q value.[21]

Determination of Glycoalkaloids and β-CA. Potato tuber tissues were extracted using hot methanol/water (70:30, v/v), and glycoalkaloids were analyzed using a liquid chromatograph (LC)−linear ion trap/time-of-flight mass spectrometer (TOF-MS; Nano Frontier LD, Hitachi High-Technologies Corp., Tokyo, Japan). Metabolites were separated using a Cadenza CD-C18 column (column temperature of 40 °C, 2 × 150 mm; Imtakt Corp., Kyoto, Japan) in a linear gradient elution using solvent A [H_2O containing 0.1% (v/v) formic acid] and solvent B [acetonitrile containing 0.1% (v/v) formic acid]. The elution was kept at an initial A:B ratio of 95:5 from 0 to 5 min and then linearly shifted to A:B = 5:95 from 5 to 50 min. Two major solanidine glycosides, α-solanine $[M + H]^+$ (m/z 868.6) and α-chaconine $[M + H]^+$ (m/z 852.6) with different sugar modifications, were distinguished by their different elution times of 27.9 and 28.3 min, respectively, in the ion selective mode of mass chromatograms (the lower detection limit was 4 ng for α-solanine). For the determination of glycoalkaloids, a calibration curve ($R^2 = 0.988$) was obtained using α-solanine (Sigma-Aldrich, St. Louis, MO, USA) as the reference standard (between 4 ng and 40 μg). The fragment ion (m/z 398.32) ascribed to solanidine (the alkaloid aglycone) in our LC-MS analysis was detected only at the elution times corresponding to α-solanine and α-chanonine (Supplementary Figure 1, Supporting Information), indicating the absence of differentially modified glycoalkaloids and hydrolysis of the glycoside moieties of the glycoalkaloids.

For β-CA determination, tuber tissues were frozen in liquid N_2 and homogenized with 80% (v/v) methanol, and ribitol (Sigma-Aldrich) was added to the homogenized samples. After centrifugation, the supernatant was filtered through a DISMIC-13P filter (Advantec, Tokyo, Japan) and subjected to methyloximation derivatization (at 30 °C for 90 min in anhydrous pyridine) and derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich). The derivatized samples were analyzed by GC-MS using a Saturn 2200 GC-MS system (Varian, Palo Alto, CA, USA) with a CP-SIL5 CB low bleed/MS column (15 m × 0.25 mm; Varian). The port temperature for split injections (a split ratio of 50%) was 270 °C, and helium was used as the carrier gas at a flow rate of 1.5 mL/min. The temperature program was started at 80 °C with a linear rise from 80 to 330 °C (8 °C/min) and maintained for the final 6 min at 330 °C. The peak areas of metabolites were automatically calculated using the 2200 Work-

station (Varian), and β-CA was determined in comparison to standard β-CA (Sigma-Aldrich).

γ-Glutamyl-β-CA was prepared as described by Watanabe et al.[24] Briefly, a reaction mixture (100 μL) containing 1 unit of γ-glutamyl transpeptidase (Sigma-Aldrich), 10 mM Tris-HCl (pH 8.5), 10 mM β-CA, and 10 mM glutamic acid was incubated for 1 h at 37 °C. The reaction was stopped by adding 100 μL of ethanol, and the formation of the reaction product, γ-glutamyl-β-CA $[M - H]^-$ (m/z 242.2), was confirmed using the LC-ESI-TOF-MS (Nano Frontier LD, Hitachi High-Technologies Corp.) under the same conditions as used for the glycoalkaloid determination.

Semiquantitative RT-PCR Analysis. A detailed comparison of gene expression levels was carried out using semiquantitative RT-PCR for genes involved in ET biosynthesis and β-CA metabolism. These genes include β-CA synthase, Cys synthase, 1-aminocyclopropane-1-carboxylate (ACC) synthase (ACS), ACC oxidase (ACO), γ-glutamyl transpeptidase (GGT), and nitrilase (NIT).

Gene-specific primers for γ-glutamyl transpeptidase (*St-GGT1*) and nitrilase (*St-NIT4*) were designed by referring to genomic sequences deposited in The Solanaceae Genomics Resource (http://solanaceae.plantbiology.msu.edu/). For *GGT1* transcript amplification, we identified a putative *GGT1* sequence (PGSC0003DMT400077372) in the potato, using the *Arabidopsis* GGT1 amino acid sequence at the Potato Genome Sequencing Consortium Data Release. This gene is named *St-GGT1*. The PCR amplification was designed to amplify the fragment encompassing the region from 693 to 1322 of the predicted open reading frame, which corresponds to exon2 thru exon4 of the predicted *St-GGT1* coding sequence. Another putative *GGT* sequence (*St-GGT2*), PGSC0003DMT400077372, was identified in the potato. These two *GGT* sequences are 65% identical. A potato nitrilase (*St-NIT4*) candidate (PSGC0003DMT400067695) was identified at the Potato Genome Sequencing Consortium Data Release, referring to the amino acid sequence of *Arabidopsis NIT4B* (At5g22300) involved in β-CA metabolism in *Arabidopsis*.[25]

In the potato, at least three *ACS* genes are expressed: *St-ACS1A*, *St-ACS1B*, and *St-ACS2*.[26] In this study, we used a primer set to simultaneously analyze both *St-ACS1A* and *St-ACS1B* and a separate set for *St-ACS2*.[26] Potato *ACO* gene (*St-ACO1*, *St-ACO2*, and *St-ACO3*) transcript levels were analyzed according to the sequences reported by Zanetti et al.,[27] whereas only the *St-ACO3* transcript accumulation was detectable in the samples used in this study. β-CAS (*StCAS1*, Genbank ID AB027000) and CS (*StCS1*, Genbank ID AB029511; *StCS2*, Genbank ID AB029512) gene transcripts were analyzed using the primers designed from the deposited sequences (Supplementary Table 2, Supporting Information). The primer sets for the analyses of *actin* (Genbank ID U60488) and *DREB1A* (Genbank ID AB007787) genes are listed in Supplementary Table 2 (Supporting Information).

Total RNA was isolated using Plant RNA Isolation Reagent (Life Technologies, Tokyo, Japan), and genomic DNA contamination was eliminated using an RNase-free DNaseI Set (Takara Bio, Kyoto, Japan). First-strand cDNA was synthesized in a 10 μL reaction mixture containing 250 ng of total RNA using an oligo$(dT)_{16}$ as the reverse primer. The reverse transcription reactions using AMV Reverse Transcriptase XL (Takara Bio) were carried out at 45 °C for 10 min and 90 °C for 5 min and then chilled to 5 °C for 5 min. PCR was carried out using 0.2 μL of the reverse transcription products as the template in a 10 μL of reaction mixture containing 1 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.025 unit/μL PrimeSTAR HS DNA polymerase (Takara Bio), and 0.2 μM concentrations of primers. The PCR programs were prepared for each primer set (Supplementary Table 2, Supporting Information) with a PCR cycle at 94 °C for 10 s, a specifically determined annealing temperature for 30 s (Supplementary Table 2, Supporting Information), and a temperature of 72 °C for 30 min, followed by an extension period of 30 s at 72 °C. Aliquots of RT-PCR reactions were collected at five-cycle intervals to monitor the linear amplification phase. The potato actin gene was amplified using a specific primer set (Supplementary Table 2, Supporting Information) as the internal reference under the same PCR conditions for the genes of interest. RT-PCR products were resolved by 2% (w/v) agarose gel electrophoresis and visualized by staining with SYBR Green I (Lonza, Rockland, ME, USA).

RESULTS

Metabolome Comparison. Untargeted metabolomics approaches offer advantages to characterize unintended metabolic differences among transgenic and nontransgenic crops.[28,29] We detected a total of 165 metabolite candidates from the potato samples (Supplementary Table 1, Supporting Information). Among them, 113 metabolites match named structures of authentic standard compounds, and 52 represent currently unknown structural identities. The metabolites were mapped onto general biochemical pathways, as illustrated in the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/) and the Plant Metabolic Network (http://www.plantcyc.org/). Many of the significant changes and trends observed are common to the three transgenic lines (35S-3, D163, and D164), although there may have been some differences in the magnitude of the responses (Supplementary Table 1, Supporting Information). There were some compounds that seem to be specifically different in the D163 or D164 lines (which are very similar in genotypic sense) or in 35S-3.

Several measurements indicated the presence of stress responses in the transgenic samples. The accumulation levels of some metabolites, such as those involved in the GABA shunt or glutathione pathways, seem to point to the activation of oxidative stress responses in the transgenic samples.[21] Thus, the increases in GABA and 2-pyrrolidone levels, as well as Gln levels, suggested that the GABA shunt in the transgenic lines (Figure 1) was stimulated. In addition, marked accumulation of γ-glutamyl amino acids (Figure 2) suggests the activation of the

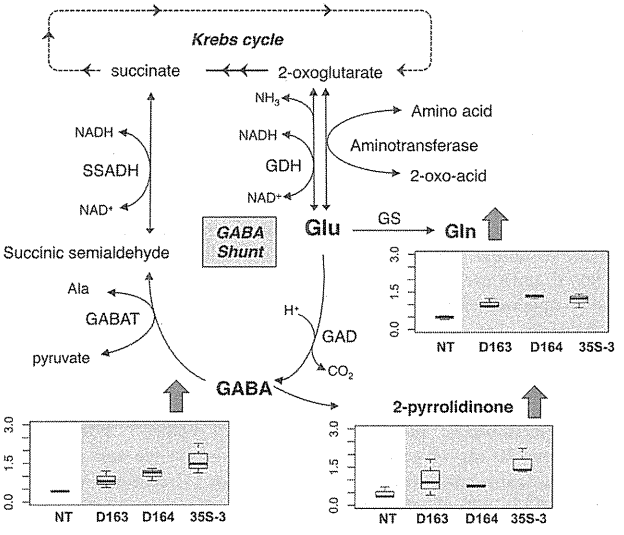

Figure 1. Relative comparison of metabolite levels in the glutamate/glutamine pathway. Metabolite levels in the transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers are shown by the box plots. The box shows first quartile, median, and third quartile values. The whiskers represent upper and lower data. The y-axis is the median scaled value. Results from the transgenic lines are shown in the shaded area of the panels. GS, glutamine synthetase; GABAT, γ-aminobutyrate aminotransferase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; SSAD, succinic semialdehyde dehydrogenase.

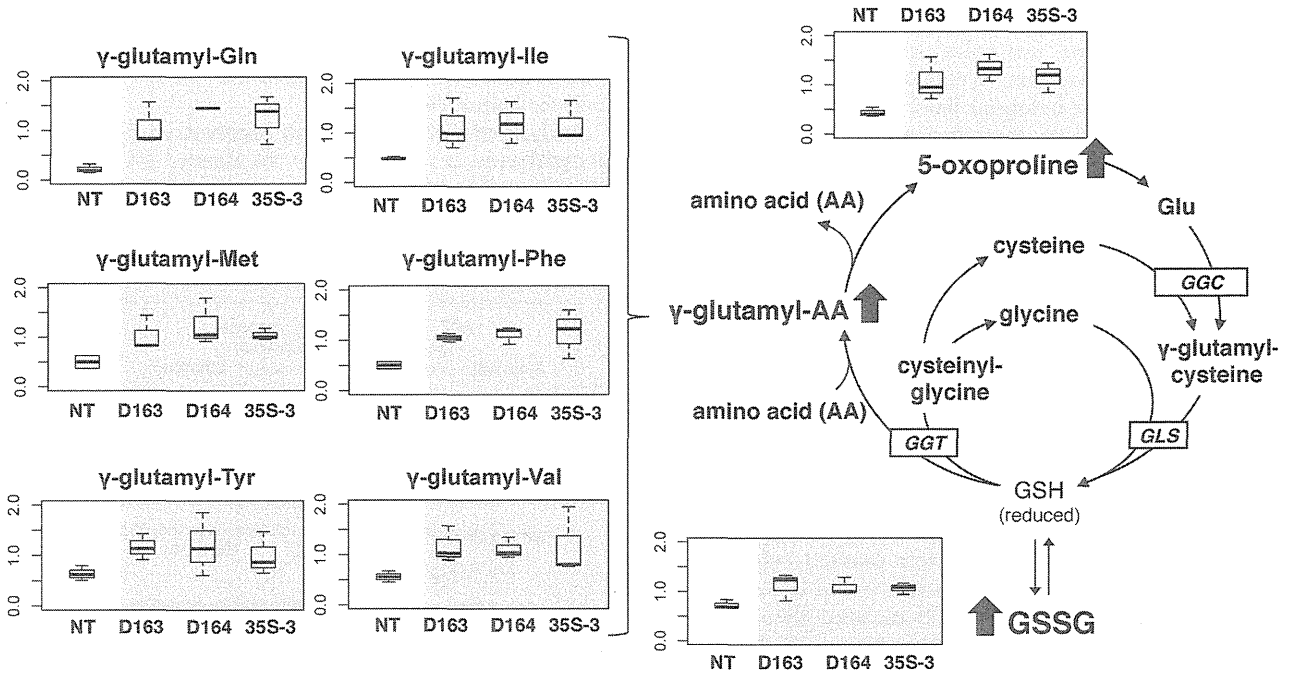

Figure 2. Relative comparison of the GSH pathway metabolite levels in the transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers. The levels of oxidized glutathione (GSSG), 5-oxoproline, γ-glutamylphenylalanine, γ-glutamylisoleucine, γ-glutamyltyrosine, γ-glutamylmethioine, γ-glutamylglutamine, and γ-glutamylvaline are illustrated by the box plots. The box shows first quartile, median, and third quartile values. The whiskers represent upper and lower data. The *y*-axis is the median scaled value. Results from the transgenic lines are shown in the shaded area of the panels. GGC, GGT, and GLS represent γ-glutamyl cyclotransferase, γ-glutamyl transpeptidase, and glutathione synthase, respectively.

GSH recycling pathway in the transgenic lines. The increased accumulation of oxidized glutathione (GSSG) and γ-glutamyl amino acids was prominent in the transgenic potato tubers. 5-Oxoproline levels were also elevated in the transgenic lines (Supplementary Table 1, Supporting Information). Ascorbate, an important antioxidant, seemed to be dramatically elevated in the D163 and 35S-3 tubers (Supplementary Table 1, Supporting Information). However, it is not clear whether the *DREB1A* transgene expression led to oxidative damage or if these metabolic responses are part of a wider oxidative stress-protective mechanism in potato. A stress response was also suggested by the higher levels of β-CA accumulation in the transgenic plants, especially in the 35S-3 line [Supplementary Table 1 (Supporting Information) and Figure 3]. β-CA is produced as a byproduct of the ET biosynthesis.[30] ET is a phytohormone that plays crucial roles in a variety of physiological processes throughout plant development and in response to biotic and abiotic stresses.[31−36]

The sugar metabolism might also have been affected in the transgenic lines: the levels of sucrose, glucose-1-phosphate, glucose-6-phosphate, and fructose-6-phosphate were higher in the transgenic lines than in the controls. It is possible that the transport of carbon assimilation products might have been stimulated in the transgenic plants (Supplementary Table 1, Supporting Information). Glu biosynthesis, which is key to the acquisition of nitrogen (through the GS/GOGAT cycle), is influenced by the plants' carbon status. High levels of sucrose, relative to amino acid pools, are known to induce Glu synthase at the transcriptional level.[37]

Ethanolamine levels were enhanced in the transgenic lines (Supplementary Table 1, Supporting Information). It has been reported that ethanolamine is involved in the biosynthesis of

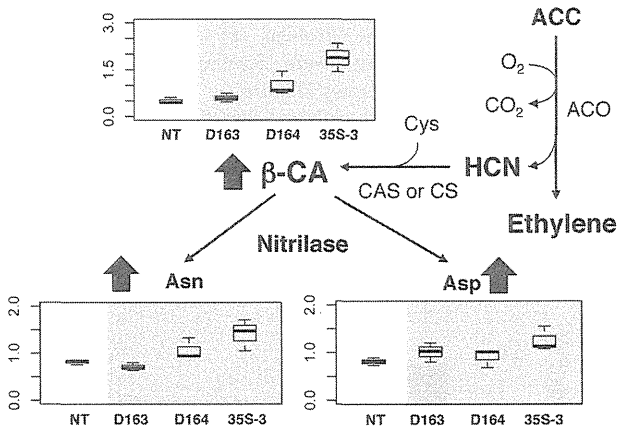

Figure 3. Relative comparison of the metabolite levels involved in the ET biosynthesis pathway. The transgenic (D163, D164, and 35S-3) and nontransgenic (NT) potato tubers were subjected to the metabolome profiling assay, and the results are shown by the box plots. Results from the transgenic lines are shown in the shaded area of the panels. 1-Aminocyclopropanecarboxylic acid (ACC) is the substrate of the ACC oxidase (ACO) reaction to produce ET, releasing HCN as a byproduct. Either β-cyanoalanine synthase (CAS) or cysteine synthase (CS) is responsible for the production of β-cyanoalanine (β-CA) from Cys and HCN, and nitrilases metabolize β-CA to Asp or Asn.

glycine betaine as an osmoprotectant in plants in response to high salinity.[38] However, our targeted analysis did not detect a significant increase in the glycine betaine levels in the transgenic lines (data not shown). The purine nucleosides, adenosine and guanosine, were elevated in all transgenic lines,

possibly reflecting an increase in the activity of purine salvage pathways. Also, some metabolite levels (e.g., methionine sulfoxide, quinate, tyramine, kynurenate, and dehydroascorbate) decreased in the transgenic lines, but the responses were not consistent among the three transgenic lines (Supplementary Table 1, Supporting Information).

Targeted Analysis for Toxic Metabolites. Potato tissues are known to accumulate the toxic glycoalkaloids α-solanine and α-chanonine, which represent 95% of total glycoalkaloids in tubers.[39] The glycoalkaloid contents of potato tubers vary depending on environmental conditions during cultivation, the harvesting process, and the storage period. In most cases, the glycoalkaloid content remains within the range of 7−187 μg/100 g FW.[40] The levels of α-solanine and α-chanonine in NT were 14.6 ± 5.81 and 33.5 ± 9.78 μg/g FW, respectively. The levels of these glycoalkaloids in the D163 and D164 lines were comparable to those in NT, whereas in the 35S-3 line these levels were significantly lower than those in the NT. The fragment ion (m/z 398.32) ascribed to solanidine (the alkaloid aglycone) in our LC-MS analysis was detected only at the elution times corresponding to α-solanine and α-chanonine, indicating the absence of differentially modified glycoalkaloids (Supplementary Figure 1, Supporting Information). These results demonstrate that the expression of the *DREB1A* transcription factor in the potato did not affect the glycoalkaloid levels.

The neural toxicity of β-CA is well-known, particularly in the legume family plants.[41] In a study in which β-CA was intraperitoneal or orally administered to chicks and rats, the neurotoxic effects of β-CA were ascribed to its action toward the *N*-methyl-D-aspartate class of glutamate receptors.[42] Because the metabolome profiles are shown by relative comparison, β-CA contents were determined by a targeted analysis of the sampled tissues (Figure 4). The β-CA levels in the transgenic potato tubers were significantly higher than those in the NT tubers (1.80 ± 0.32 μg/g FW). In the transgenic lines, 35S-3, D163, and D164 had β-CA levels of 5.88 ± 0.86, 3.66 ± 0.50, and 3.38 ± 1.4 μg/g FW, respectively.

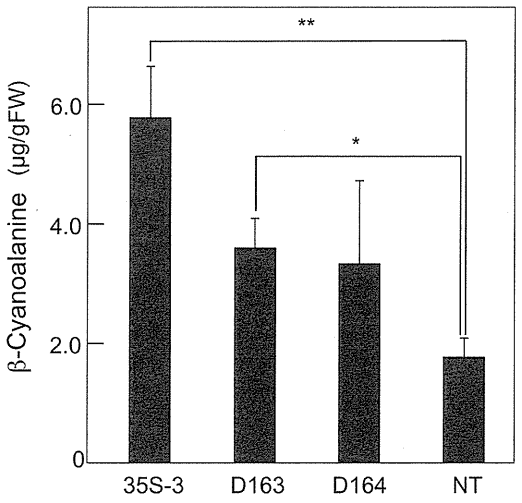

Figure 4. β-CA contents of transgenic (35S-3, D163, and D164) and nontransgenic (NT) potato tuber extracts. Error bars indicate the mean ± SD of three separate extracts. Asterisks indicate significant differences between NT and transgenic potatoes using a two-tailed Student's t test (*, $P < 0.05$; **, $P < 0.01$).

The toxic level of β-CA, given by the lethal dose at which 50% mortality (LD_{50}) is observed, is 134 mg/kg;[43] however, β-CA levels in potato tubers in our studies were far lower than toxic levels for human consumption.

Analysis of Gene Expression Involved in ET and β-CA Biosyntheses. It is possible that the elevated β-CA levels are caused by enhanced ET biosynthesis in the *DREB1A* transgenic potatoes. To test this hypothesis, we compared the transcript levels of the genes involved in ET biosynthesis in potato tubers (Figures 5 and 6). The initial step of ET biosynthesis is

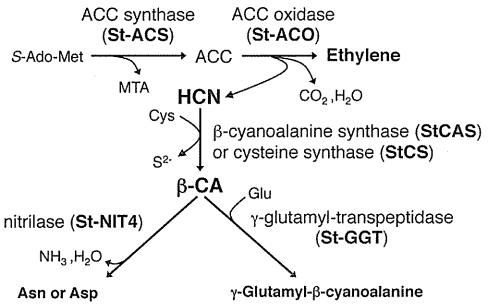

Figure 5. Schematic representation of the genes involved in β-CA production in potato. Transcript levels of ACC synthase gene (*St-ACS*) and ACC oxidase gene (*St-ACO*) were analyzed according to the sequence information reported by Destéfano-Beltrán et al.[26] and Zanetti et al.,[27] respectively. β-Cyanoalanine synthase (*StCAS*) and cysteine synthase (*StCS*) gene transcripts were analyzed according to the deposited sequences.[47] The genes responsible for the metabolic degradation of β-CA, γ-glutamyl transferase (*St-GGT1*, *St-GGT3*) and nitrilase (*St-NIT4*), were analyzed with reference to genomic sequences deposited in The Solanaceae Genomics Resource (http://solanaceae.plantbiology.msu.edu/).

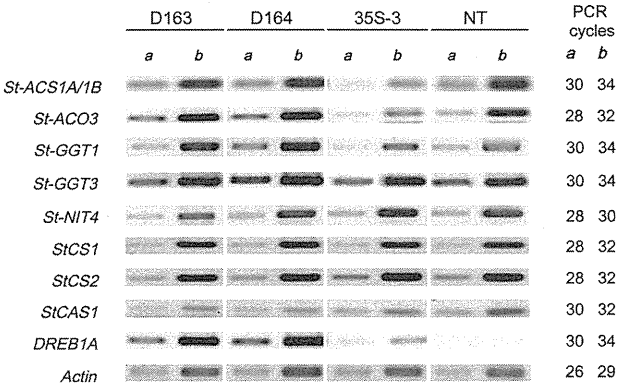

Figure 6. Transcript levels of genes related to ET and β-CA synthesis in the tubers from the *DREB1A* transgenic (35S-3, D163, and D164) and nontransgenic (NT) lines. Semiquantitative RT-PCR was performed using a gene-specific primer set for each gene listed in Supplemental Table 2 (Supporting Information) for *St-GGT1* (potato γ-glutamyl transferase-1), *St-GGT3* (potato γ-glutamyl transferase-3), *St-NIT4* (potato nitrilase-4), *StCS1* (potato cysteine synthase-1), *StCS2* (potato cysteine synthase-2), *StCAS1* (potato β-cyanoalanine synthase-1), *St-ACO3* (potato ACC oxidase-3), *St-ACS1A/1B* (potato ACC synthase-1A/1B), *DREB1A*, and *actin*. Two different PCR reaction cycles (shown by ***a*** and ***b***) were used for the analysis of each gene transcript level. The numbers shown on the right side correspond to those PCR cycles yielding the amplification products shown in lanes ***a*** and ***b***, respectively.

dx.doi.org/10.1021/jf304071n | J. Agric. Food Chem. 2013, 61, 893−900

catalyzed by ACS, yielding ACC and 5′-methylthioadenosine from *S*-adenosylmethionine (*S*-Ado-Met). ACC is then oxidized by ACO to give the end product, ET, releasing cyanoformic acid, which is spontaneously converted into HCN and CO_2.[18−20] The HCN is captured by β-CAS and CS to produce β-CA.

In potato plants, at least three *ACS* genes are expressed: *St-ACS1A* and *St-ACS1B*, which share 98% identity in their primary structures and are ubiquitously expressed in the potatoes, and the *St-ACS2* gene transcript, which has been detected in potato leaves, leaf petioles, roots, and tubers.[26] *St-ACS1A* and *St-ACS1B* are strongly induced in hypocotyls by indole-3-acetic acid and in leaves by wounding.[26] In our study, the transcript levels of *St-ACS1A/B* were comparable among the D163, D164, and NT lines, whereas those in the 35S-3 line were clearly decreased (Figure 6). The *St-ACS2* transcript was not detected in the tuber samples in this study.

ACO is known to be induced by various stresses in plants.[31−36] We analyzed the transcript accumulations of the potato *ACO* genes (*St-ACO1*, *St-ACO2*, and *St-ACO3*). It has been reported that the expression levels of *St-ACO1* and *St-ACO2* were higher in leaves and lower in roots and tubers.[27] We found that *St-ACO3* transcript levels were higher in the D163 and D164 lines than in the NT and 35S-3 lines (Figure 6), whereas *St-ACO1* and *St-ACO2* transcripts were not detectable in this study (data not shown).

In potato, β-CAS (*StCAS1*) and cysteine synthase (*StCS1* and *StCS2*) are involved in β-CA production.[44] There were no clear differences in *StCS1/2* and *StCAS1* transcript levels between the transgenic lines and the NT line (Figure 6). It has been reported that levels of β-CAS activity and the resulting protein accumulation were elevated by ET without corresponding transcript levels, suggesting that ET induces β-CAS protein accumulation at the posttranscriptional level.[44]

Increases in Asp and Asn levels in the transgenic lines (Figure 3) may be related to β-CA catabolism.[25,45] β-CA degradation occurs through GGT and NIT4 activity in *Arabidopsis*, producing γ-glutamyl-β-CA and Asp/Asn, respectively.[24] In the potato, we identified a putative *GGT* sequence (*St-GGT1*), using the *Arabidopsis* GGT1 amino acid sequence in the Potato Genome Sequencing Consortium Data Release (http://potatogenomics.plantbiology.msu.edu/). A potato nitrilase candidate (*St-NIT4*) was also identified in the same database, referred to the amino acid sequence of *Arabidopsis* NIT4B (At5g22300).[25] Figure 6 shows the higher levels of *St-GGT1* and *St-NIT4* transcripts in the D163 and D164 lines than in the 35S-3 line, suggesting possible stimulation of β-CA degradation in the D163 and D164 lines. However, γ-glutamyl-β-CA accumulation was not detectable in any of the potato lines in this study (data not shown). In contrast, the γ-glutamyl amino acid levels were consistently higher in the transgenic lines than in the NT line [Supplementary Table 1 (Supporting Information) and Figure 2]. It remains unclear whether the higher *St-GGT1* transcript levels were responsible for the accumulation of γ-glutamyl amino acids in the transgenic lines. In the metabolic profiling study, the levels of both Asp and Asn were likely to be higher in the transgenic potato tubers (Supplementary Table 1, Supporting Information), which may be ascribed to the stimulated metabolism of β-CA to Asp and Asn.

DISCUSSION

Our metabolic profiling study suggests that endogenous potato stress responses might be stimulated by the transgenic expression of *Arabidopsis DREB1A*, regardless of the promoters used. Kasuga et al.[17] have reported that *DREB1A* expression caused growth retardation in a study that employed the constitutive promoter CaMV35S. Thus, the constitutive expression of *DREB1A*, which is stress-inducible[10] and induces expression of its target genes, might not be favored under normal growth conditions. *DREB1A* expression elicited by the stress-inducible *rd29a* promoter successfully increased stress tolerance in the potato.[8,9] It should be noted that the *DREB1A* transcript was not detected in these transgenic potatoes without the presence of stress factors.[8,9] The high levels of *DREB1A* transcripts detected in D163 and D164 (Figure 6) suggest that these plants might have behaved as if they experienced certain environmental stresses, including the up-regulation of endogenous defense-response genes, because the potato plants used in this study were not grown in environmentally controlled conditions. The presence of a unique transcript fragment (PUT-157a-Solanum_tuberosum-42922 in The Solanaceae Genomics Resource), of which the known primary structure is 57% identical to *DREB1A*, suggests that a functional stress tolerance mechanism in potatoes is similar to the DREB1A-inducible stress response. Thus, it is possible that the metabolic responses we observed might have mirrored the outcome of interactive endogenous stress responses that were intensified by the transgenic expression of *Arabidopsis DREB1A*. However, regardless of the promoters used, *DREB1A* transcript levels may be expected to coincide with changes in the metabolic profiles. However, the *DREB1A* transcript levels (Figure 6) did not match the accumulated levels of some metabolites (e.g., the β-CA level was higher in 35S-3 line than in the rd29a line), indicating that gene transcript levels were not linearly related to the extent of metabolic fluctuations.

The prominent accumulation of γ-glutamyl amino acids (Figure 2) suggested the activation of the GSH recycling pathway in the transgenic potatoes (Figures 1 and 2). Oliver et al.[21] have reported that the levels of γ-glutamyl amino acids were dramatically increased in desiccation-tolerant *Sporobolus stapfianus* during dehydration and that two different desiccation-tolerant species, the bryophyte *Tortula rulalis* and the lycophyte *Selaginella lepidophylla*, also showed elevated levels of γ-glutamyl amino acids during dehydration. Oliver et al.[21] also reported that *S. stapfianus* leaves exhibited significant accumulation of *GGT* transcripts during dehydration. They hypothesized that increased levels of γ-glutamyl amino acids might be involved in the desiccation tolerance by providing protection from stresses caused by reactive oxygen species.

Involvement of γ-glutamyl amino acids in oxidative stress tolerance has not been extensively studied in plants. In the γ-glutamyl cycle in animals, GGT transfers the γ-glutamyl moiety from GSH to water or an amino acid, yielding either Glu or a corresponding γ-glutamyl amino acid, which is then transported back into the cytoplasm.[46] γ-Glutamyl amino acids also serve as the substrates of γ-glutamyl cyclotransferase (GGC), which converts γ-glutamyl amino acids into 5-oxoproline, releasing the corresponding amino acids.[46] 5-Oxoproline is hydrolyzed to Glu by an ATP-dependent enzyme, 5-oxoprolinase.[46] In *Arabidopsis*, it has been reported that the apoplastic GGT1 and GGT2 control the degradation of oxidized GSH in the extracellular space, and the vacuolar enzyme GGT4 is

responsible for the degradation of stored GSH conjugates to release Glu as well as Cys−Gly conjugates.[47]

It is not known whether plant *GGT* genes are involved in γ-glutamyl amino acid production. In this study, we observed a significant increase in γ-glutamyl amino acids and 5-oxoproline in the transgenic potatoes. It has been suggested that GGC and 5-oxoprolinase are primarily involved in GSH recycling.[48] However, the *GGC* gene remains unidentified and uncharacterized in plants.[49] The identification and characterization of *GGC* in plants is essential to understand the stimulated increase in γ-glutamyl amino acids and the physiological roles of γ-glutamyl amino acids and GSH metabolism, particularly in stress tolerance.

Because potatoes are grown for human consumption, a large concern is the presence of unfavorable metabolites. In our study, we found that there was no increase in potato glycoalkaloid levels in the transgenic tuber samples. However, our untargeted metabolomics revealed elevated levels of β-CA in the transgenic potatoes, which was confirmed by the targeted analysis. The natural occurrence of β-CA and γ-glutamyl-CA, of which the toxicological effects are identical, were first characterized in *Vicia angustifolia* and *Vicia sativa*.[41,50] The toxicity levels[43] and species-dependent toxicity[51] of β-CA are known, and the β-CA levels that accumulated in the current analytical samples (both transgenic and nontransgenic materials) should not be harmful to humans.

Although the number of analyzed plants was limited, the current results indicate that further research and analyses are required to confirm whether or not these differences are indeed significant and if they reflect a clear change in the metabolic profile from nontransgenic to transgenic potato tubers. It is possible that latent metabolic activities in both transgenic and nontransgenic crops may be reactivated, as suggested by the stimulated stress responses. The global metabolome profiles, particularly the profiles of unfavorable metabolites, could not be reasonably explained by the gene transcript levels alone. Untargeted metabolomics together with targeted analyses play a critical role in the clarification of metabolic fluctuations in crops. Further routine metabolome profiling is necessary to ensure food safety. In addition, metabolome profiling studies with transgenic crops conferred stress tolerance shed light on plant stress responses under environmental stresses, generating novel hypotheses that could contribute to further development of stress tolerance technologies.

■ ASSOCIATED CONTENT

Supporting Information

Additional figure and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This study was partly supported by a grant from the Ministry of Health, Labor and Welfare of Japan.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

ACC, 1-aminocyclopropane-1-carboxylate; ACO, ACC oxidase; ACS, ACC synthase; CA, cyanoalanine; CAS, CA synthase; CN, cyanide; CS, cysteine synthase; ET, ethylene; GABA, γ-aminobutyric acid; GABAT, γ-aminobutyrate aminotransferase; GAD, glutamate decarboxylase; GDH, glutamate dehydrogenase; GGC, γ-glutamyl cyclotransferase; GGT, γ-glutamyl transpeptidase; GLS, glutathione synthase; GOGAT, glutamate synthase; GS, glutamine synthetase; HCN, hydrogen cyanide; NT, nontransgenic; SSAD, succinic semialdehyde dehydrogenase

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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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Journal of proteome research

Reprinted from
Volume 13, Number 2, Pages 489-495

Journal of proteome research

Article

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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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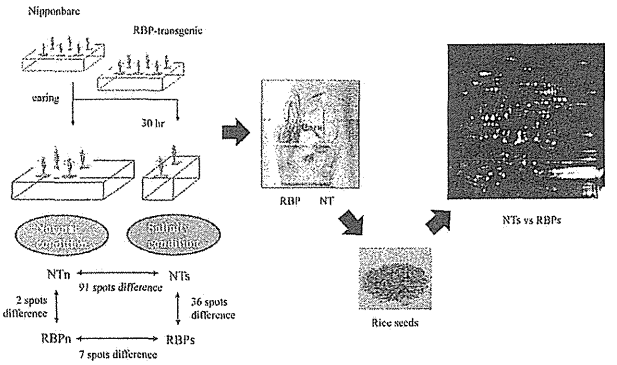
Supporting Information

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

Received: June 30, 2013
Published: January 13, 2014

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

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.,

dx.doi.org/10.1021/pr4006487 | J. Proteome Res. 2014, 13, 489–495

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.

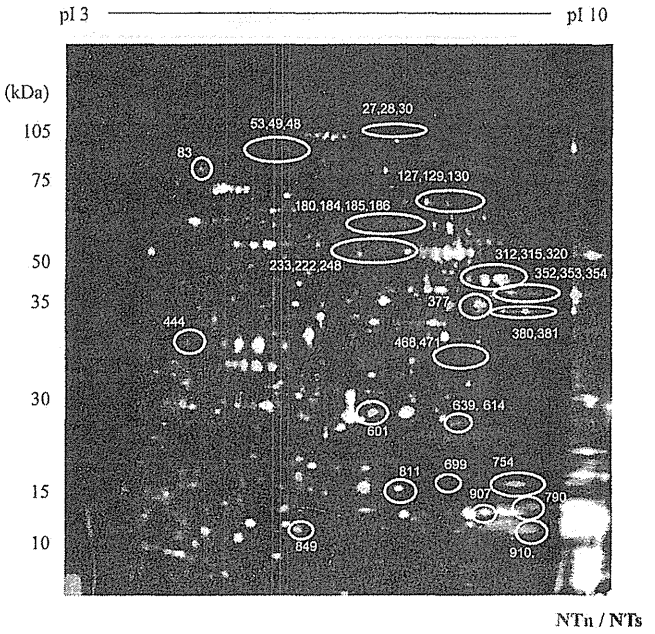


Figure 1. Representative 2D-DIGE merged image of protein expression in NTn and NTs. The gel was scanned using a Typhoon 9400 variable image analyzer to generate Cy3 (green, NTn) and Cy5 (red, NTs) images. The circled spots significantly differed (ratio >2 or <0.5) between NTn and NTs by Student's *t*-test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

dx.doi.org/10.1021/pr4006487 | J. Proteome Res. 2014, 13, 489−495

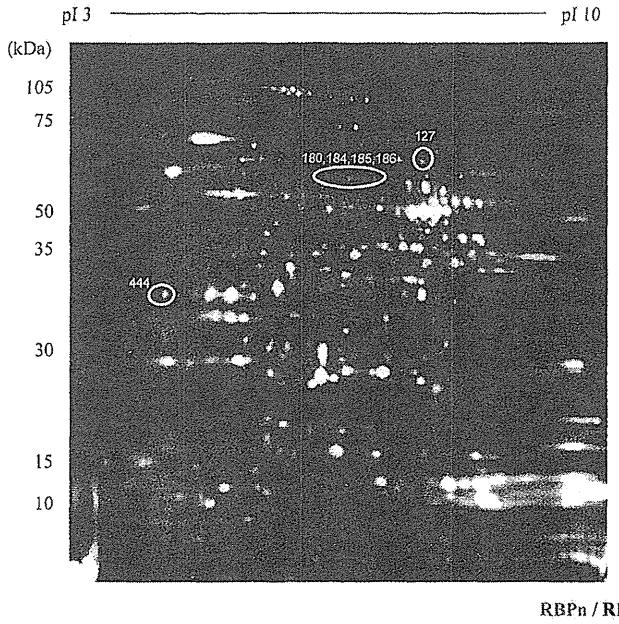


Figure 2. Representative 2D-DIGE merged image of protein expression in RBPn and RBPs. The gel was scanned at two separate wavelengths using a Typhoon 9400 variable image analyzer to generate Cy3 (green, RBPn) and Cy5 (red, RBPs) images. The circled spots significantly differed (ratio >2 or <0.5) between 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.

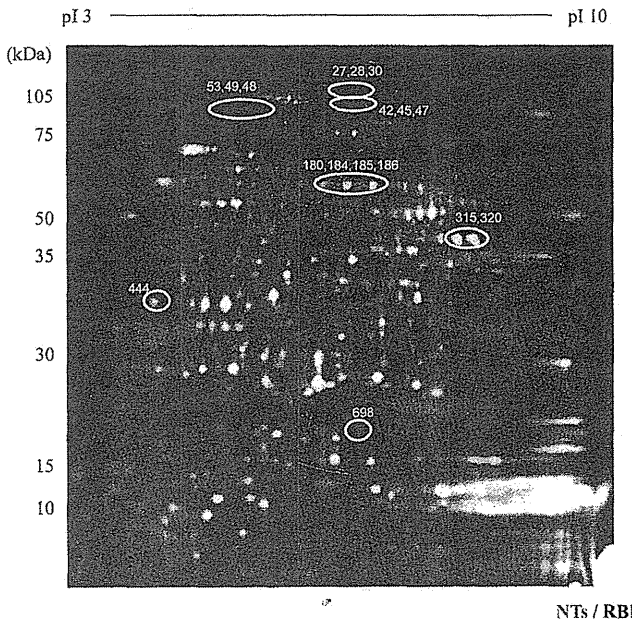


Figure 3. Representative 2D-DIGE merged image of protein expression in NTs and RBPs. Representative fluorescence images of NTs (Cy3, green) and RBPs (Cy5, red) extracts are shown. The circled spots significantly differed (ratio >2 or <0.5) between NTs and RBPs by Student's *t*-test ($p < 0.05$). The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

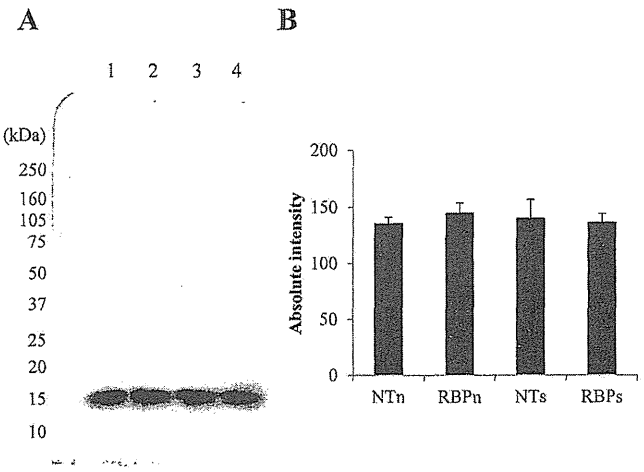


Figure 4. Immunoblotting with RAG2-specific rabbit antibody. The labeled proteins were detected (A) Lane 1, NTn; Lane 2, RBPn; Lane 3, NTs; Lane 4, RBPs. Band intensities are shown in panel B. Data show mean ± SEM. All seeds and conditions showed a 1.1-fold increase over NTn. Intergroup differences were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

dx.doi.org/10.1021/pr4006487 | J. Proteome Res. 2014, 13, 489−495

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.

■ ACKNOWLEDGMENTS

This study was supported by a grant from the Ministry of Health, Labour and Welfare.

■ 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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Identification and Detection of Genetically Modified Papaya Resistant to Papaya Ringspot Virus Strains in Thailand

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Received September 13, 2013; accepted October 18, 2013; advance publication released online October 29, 2013

Many lines of genetically modified (GM) papaya (*Carica papaya* Linnaeus) have been developed worldwide to resist infection from various strains of papaya ringspot virus (PRSV). We found an unidentified and unauthorized GM papaya in imported processed papaya food. Transgenic vector construct that provides resistance to the PRSV strains isolated in Thailand was detected. An original and specific real-time polymerase chain reaction method was generated to qualitatively detect the PRSV-Thailand-resistant GM papaya.

Key words genetically modified organism; papaya; polymerase chain reaction; genomic DNA

Papaya (*Carica papaya* Linnaeus) is a widely grown fruit crop in tropical and subtropical areas.[1] A major constraint on papaya production has been infection by papaya ringspot virus (PRSV), which reduces papaya yields.[2] In response to this problem, genetically modified (GM) papaya that resists PRSV infection has been developed. Since the first successful generation of GM papaya in 1991,[3,4] many GM papaya lines, carrying different transgenic vector constructs, have been planted in various places, such as Hawaii, Florida, China, Jamaica, Taiwan, Thailand, Australia, Malaysia, the Philippines and Vietnam.[1]

Since Japan announced a mandatory safety assessment of GM foods, and processed foods containing GM ingredients, on April 1, 2001, foods on the Japanese market have been monitored for contamination with unauthorized GM products. We have developed qualitative detection methods for various GM crops, such as potato,[5,6] maize,[7–10] rice,[11,12] and flax,[13] using polymerase chain reaction (PCR) methods. For papaya, GM papaya line 55-1, a commercialized PRSV-resistant GM papaya developed in Hawaii, was the first authorized GM fruit allowed in Japan after a safety assessment by Food Safety Commission of Japan in 2009.[14] Subsequently, the use of any other GM papaya lines in foods has been prohibited. In 2011, an unauthorized GM papaya line, PRSV-YK, which was developed to resist the YK strain of PRSV in Taiwan, was found in some processed papaya products (papaya leaf tea, pickles and jam) on the Japanese market. The transgenic construct for PRSV-YK is similar to that of the GM papaya line 16-0-1/17-0-5 developed in Taiwan.[15] To monitor foods containing GM papaya on the Japanese market, we developed a qualitative detection method for GM papaya line 55-1 and for PRSV-YK using real-time PCR.[16–18] In addition, a histochemical assay[19] was developed to identify GM papaya line 55-1. Unauthorized GM papaya from Thailand has been found in Europe since 2012. Consequently, some papayas were rejected at the borders of some European countries.[20] Japan imports many papayas from overseas, thus it is required to monitor commercially processed products that include papaya as a major ingredient for contamination with the unauthorized GM papaya lines. In the present study, the presence of a new unauthorized GM papaya, PRSV-SC, was found in a processed papaya commodity (dried papaya) in Japan, and a new detection method for detecting PRSV-SC was developed using real-time PCR.

MATERIALS AND METHODS

Papaya Samples Processed papaya product (dried papaya) was purchased over the Internet in Japan. Hawaiian non-GM papaya (Sunset) fruit was purchased from a Japanese trade agency *via* the Hawaii Papaya Industry Association through the Consumer Affairs Agency, Government of Japan.

Purification of DNA Dried papaya was ground using a mixing mill. DNA was extracted and purified from 2 g of the samples using an ion-exchange resin-type DNA extraction and purification kit (Genomic-tip; Qiagen, Hilden, Germany) according to a previous report.[16] The purified DNA was quantified by measuring UV absorption at 260 nm using an ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, U.S.A.).

PCR Each PCR reaction mixture (50 μL) contained 5 μL 10× cloned *Pfu* reaction buffer (Agilent Technologies, Santa Clara, CA, U.S.A.), 0.18 mM deoxyribonucleotide triphosphate (dNTP) (Agilent Technologies), 0.3 μM forward and reverse primers, 2.5 U *Pfu Turbo* DNA polymerase (Agilent Technologies) and 50 ng template DNA. The PCR conditions were 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. A final terminal elongation occurred at 72°C for 10 min. PCR was performed using the GeneAmp PCR System 9700 (Life Technologies, Carlsbad, CA, U.S.A.). To determine the nucleotide sequence of the transgenic vector construct harbored in the GM papaya, DNA fragments were amplified by PCR using the following primer set:

Forward primer: 5′-GAC ATC TCC ACT GAC GTA AGG G-3′ (p324)

Reverse primer: 5′-CTATCRCTC TCT CCA GTT TTT G-3′ (p323)

DNA Sequencing PCR-amplified DNA fragments were extracted from the agarose gels and purified using a QIAquick PCR purification kit (Qiagen). The fragments were directly sequenced from both strands using forward and reverse primers with an ABI PRISM 3700 DNA analyzer and BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies), according to the manufacturer's instructions. Nucleotide se-

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quences were analyzed using GENETYX software ver. 10.0.3 (GENETYX Corp., Tokyo, Japan).

Real-Time PCR Real-time PCR assays were performed using the ABI PRISM 7900 Sequence Detection System (Life Technologies). The 25 μL reaction mixture consisted of 2.5 μL sample DNA solution (25 ng), 12.5 μL Gene Expression Master Mix (Life Technologies), 0.8 μM forward and reverse primers, and 0.1 μM probe. The PCR conditions were 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. GM papaya was detected using the following primers and probe:

Forward primer: 5′-CAT TTC ATT TGG AGA GAA CAC G-3′ (SC-F)

Reverse primer: 5′-ACC AGC ATC CAC AGC TTC-3′ (SC-R)

Probe: 5′-FAM-ACT CTA GAG GAT CCA TGT CCA A-TAMRA-3′ (SC-P)

To detect the papaya endogenous internal control gene, *chymopapain* (*Chy*; GenBank accession no. AY803756), the fol-

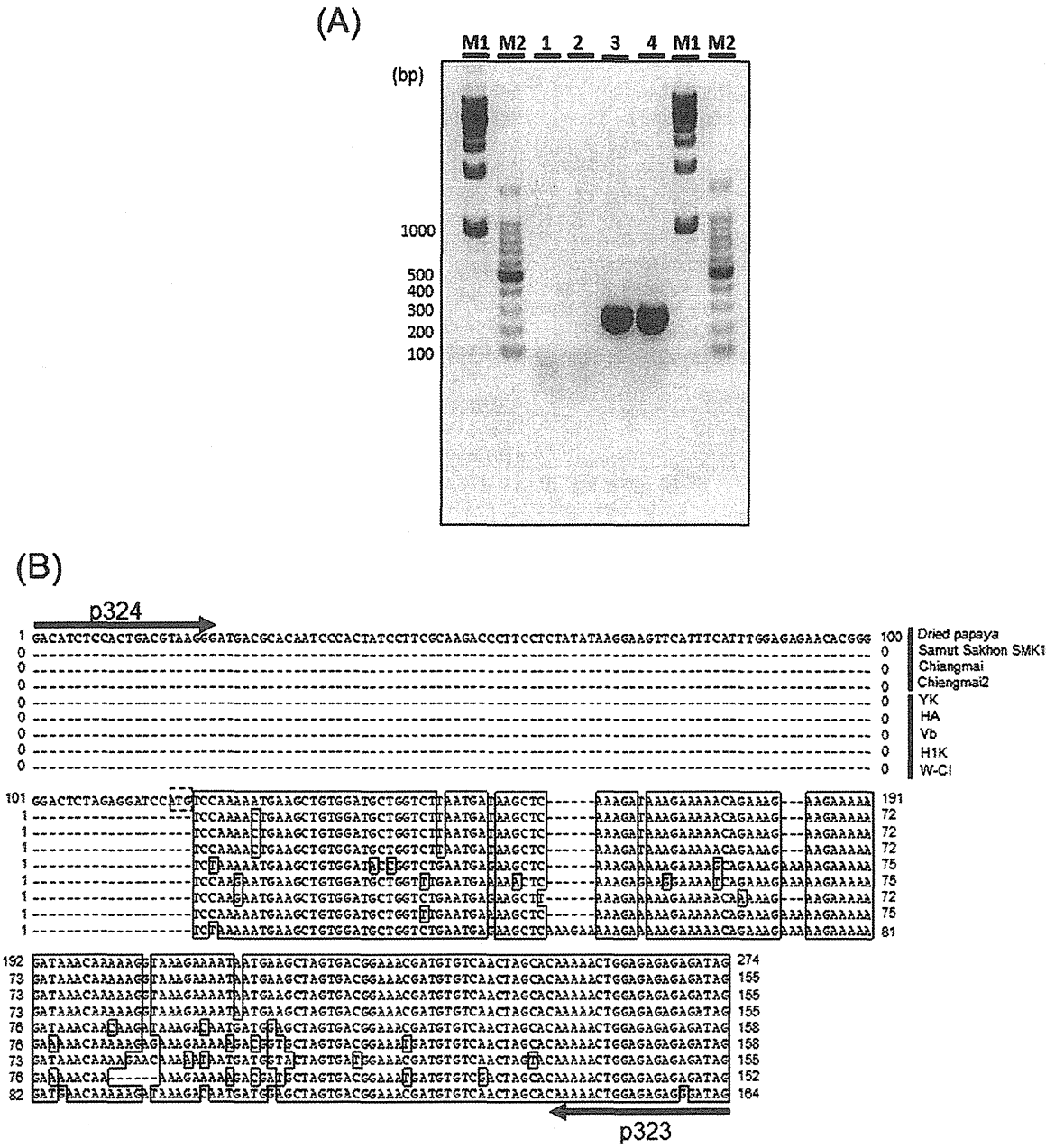

Fig. 1. PCR Targeting Construct-Specific Sequence of Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* LINNAEUS)

(A) DNA templates (lane 1, no DNA was added; lane 2, non-GM papaya (Sunset); lanes 3 & 4, dried papaya product) were used for the PCR test using the primer set, p324 and p323, that hybridize to the cauliflower mosaic virus (CaMV) 35S promoter and the PRSV's coat protein (CP) gene, respectively. The PCR-amplified products were run on a 2% (w/v) agarose gel. Lane M1, 1-kbp DNA ladder marker, lane M2, 100-bp DNA ladder marker (B) Nucleotide sequence alignment of PRSV CP cloned from various isolates and strains (Samut Sakhon SMK1 [Thailand]; Chiangmai [Thailand]; Chiengmai2 [Thailand]; YK [Taiwan]; HA [Hawaii]; Vb [China]; H1K [Florida]; W-CI [Taiwan]) and the PCR products obtained using dried papaya product. Homologous sequences are boxed. The start codon for the GM papaya is boxed with a dashed line. Numerals beside the sequences indicate the number of nucleotides from the 5′ terminus. Arrows indicate the position of the primers p324 and p323.

lowing primers and probe were used according to a previous report[17]:

Forward primer: 5′-CCA TGC GAT CCT CCC A-3′ (Q-Chy-1F2)

Reverse primer: 5′-CAT CGT AGC CAT TGT AAC ACT AGC TAA-3′ (Q-Chy-2R)

Probe: 5′-FAM-TTCCCTTCAT(BHQ1)CCA TTC CCA CTC TTG AGA-3′ (Q-Chy-P)

BHQ1 (black-hole quencher 1) was labeled in Q-Chy-P at the thymidine underlined in the nucleotide sequence.

All primers and probes were diluted with an appropriate volume of distilled water and stored at −20°C until used. Each real-time PCR reaction was tested in duplicate. Results were analyzed using SDS 2.1 sequence detection software (Life Technologies) for the ABI PRISM 7900 Sequence Detection System.

RESULTS AND DISCUSSION

Identification of Unauthorized GM Papaya To investigate the potential contamination of processed papaya products with a new unauthorized GM papaya, we used genomic DNA purified from the products as the PCR template. The primer set p323 and p324 was used as previously reported.[16] The forward primer (p324) was designed to hybridize to the sequence of the most common promoter, the cauliflower mosaic virus (CaMV) 35S promoter, which is used in the transformation of papaya to alter various papaya traits.[1] The reverse primer (p323) was designed to hybridize to the highly conserved sequence of the coat protein (CP) gene, which has been cloned from various isolates and strains of PRSV (GenBank accession nos. Samut Sakhon SMK1, DQ085864; Chiangmai, DQ085856; Chiengmai2, AY010720; YK, X97251; HA, S46722; Vb, AF243496.1; H1K, AF196839.1; W-CI, AY027810.2). When using DNA purified from a dried papaya product as the template, electrophoresis of the PCR products showed a single band of 200–300 bp (Fig. 1A). The DNA purified from the non-GM papaya (Sunset) as a control generated no PCR products of this length. A direct sequence analysis of the PCR product and a BLASTn analysis indicated that the 3′ end sequence was homologous to the CP gene in some Thai PRSV isolates (Samut Sakhon SMK1, Chiangmai, Chiengmai2), except that the fourth codon after the start codon had a single nucleotide polymorphism ("aat" in the dried papaya and "act" in Samut Sakhon SMK1, Chiangmai, and Chiengmai2) (Fig. 1B). The predicted amino acid se-

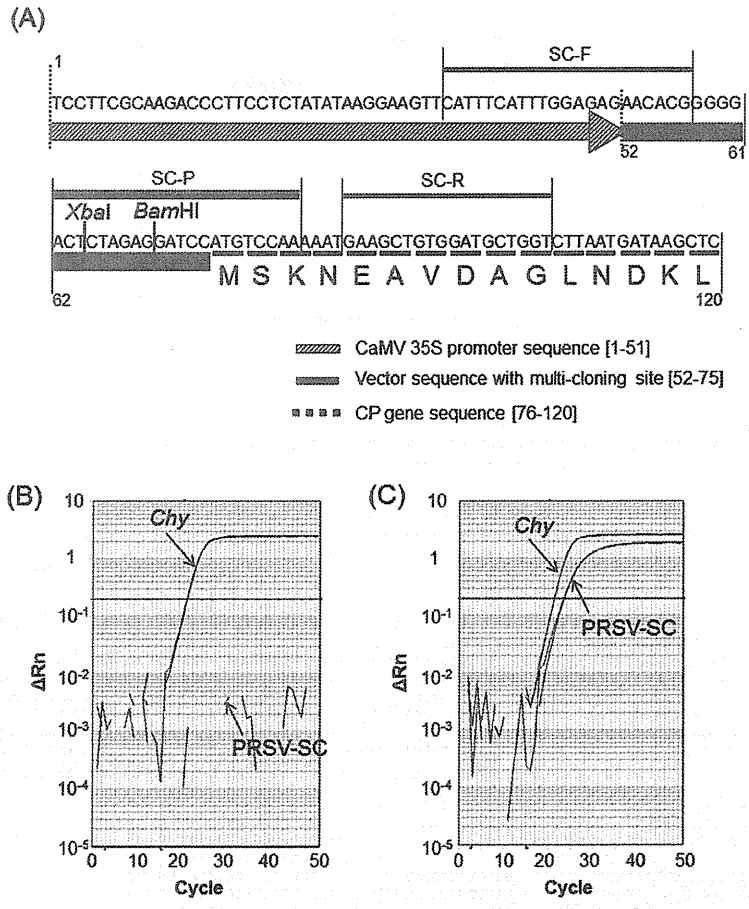

Fig. 2. Detection of a Papaya Ringspot Virus (PRSV)-Resistant Genetically Modified (GM) Papaya (*Carica papaya* Linnaeus) Line, PRSV-SC, Using Real-Time PCR

(A) A fragment of the transgenic vector construct sequence from the line PRSV-SC was obtained; restriction sites are marked by vertical arrows. The primers (SC-F and SC-R) and probe (SC-P) designed for detecting the construct-specific sequence of PRSV-SC are indicated by lines above the sequence. Numerals indicate the number of nucleotides from the 5′ terminus. DNA purified from non-GM papaya (Sunset) (B) and the PRSV-SC contaminated dried papaya product (C) were tested using real-time PCR for endogenous *Chy* detection with primer set (Q-Chy-1F2 and Q-Chy-2R) and probe (Q-Chy-P) and for PRSV-SC detection with SC-F, SC-R and SC-P. The threshold value was set at 0.2. Positive amplification curves are designated by arrows.

quences of the CP gene were identical to that of Thailand's PRSV isolates.[21] Furthermore, a multiple cloning site (containing restriction sites for *Xba*I and *Bam*HI) and the insertion of a start codon between the CaMV 35S promoter and the N-terminus of the CP gene were detected (Fig. 2A). The transgenic vector construct sequences of the CaMV 35S promoter and the CP gene in the GM papaya developed in Hawaii (line 55-1, GenBank accession no. FJ467933.1) and Taiwan (lines 16-0-1, 17-0-5)[15] showed no similarity (data not shown) to the sequence from the dried papaya product. In addition, our preliminary study using real-time PCR showed that the dried papaya product also contained high concentrations of two transgenic sequences, the *nopaline synthase* terminator, which is used in transgenic cassettes, and *neomycin phosphotransferase II*, which is used as a selectable marker (data not shown). These results suggest that the dried papaya product was contaminated with a new unauthorized GM papaya line, which we named PRSV-SC.

Designing a Novel Construct-Specific Detection Method for PRSV-SC DNA fragmentation occurs during the manufacturing of processed foods.[22,23] To qualitatively detect PRSV-SC in processed foods, specific primers and a probe for a real-time PCR assay were designed to produce a short amplicon (70 bp) based on the detected transgenic construct sequence. Also, to prevent false-negative results using real-time PCR, the PRSV-SC detection method was designed to generate a target amplicon shorter than the endogenous papaya *Chy* detection method (amplicon size 72 bp). The primers were designed to amplify the region between the transgenic vector backbone and the CP gene sequence. The probe (SC-P) was designed to target the site of the CP gene's start codon (Fig. 2A).

The real-time PCR assay for PRSV-SC detection confirmed that the dried papaya was positive for PRSV-SC, producing Ct values of 23.48 and 23.34 with a threshold value of 0.2 in a duplicate test (Fig. 2C). Endogenous *Chy* detection was positive for all samples, with Ct values of 21.27 and 21.28 for the dried papaya and 20.77 and 20.87 for the non-GM papaya (Sunset), each with a threshold value of 0.2 in a duplicate test (Figs. 2B, C). No amplification signals were obtained from the non-template control, from genomic DNA derived from 14 other crops (maize, rice, soybean, flax, canola, chickpea, wheat, sugarbeet, cottonseed, potato, papaya, tomato, eggplant, and green pepper), or from other GM papaya lines, such as 55–1 (Hawaii) and PRSV-YK (Taiwan), using the developed PRSV-SC detection method (data not shown). These results indicated that the method is specific for detecting PRSV-SC.

In the present study, as a result of monitoring processed foods for contamination with unauthorized GM papaya, we found a dried papaya product containing a transgenic vector construct for the expression of PRSV's CP gene, which was cloned from isolates in Thailand. A novel construct-specific real-time PCR detection method was developed for detecting PRSV-SC. Because the genetic background of PRSV-SC was unknown, it was not possible to estimate the content level of PRSV-SC in the papaya product. Further studies are underway to determine the PRSV-SC detection limits of the real-time PCR, and whether the qualitative PCR method using the p323/p324 primer set can function as an initial screening for the presence of GM papaya in foods.

Acknowledgment This study was partly supported by Grants from the Ministry of Health, Labour and Welfare of Japan.

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