

Inter-laboratory optimization of protein extraction, separation, and fluorescent detection of endogenous rice allergens

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Inter-laboratory optimization of protein extraction, separation, and fluorescent detection of endogenous rice allergens

Rie Satoh¹, Reiko Teshima², Kazumi Kitta¹, Gang-Hua Lang¹, Kathleen Schegg³, Kenneth Blumenthal⁴, Leslie Hicks⁵, Bénédicte Labory-Carcenac⁶, David Rouquié⁶ , Rod A. Herman⁷, Corinne Herouet-Guicheney⁸, Gregory S. Ladics⁹, Scott McClain¹⁰, Lars K. Poulsen¹¹, Laura Privalle¹², Jason M. Ward¹³, Nancy Doerrler¹⁴ and Jean-Baptiste Rasclé^{6,*} 

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In rice, several allergens have been identified such as the non-specific lipid transfer protein-1, the α -amylase/trypsin-inhibitors, the α -globulin, the 33 kDa glyoxalase I (Gly I), the 52–63 kDa globulin, and the granule-bound starch synthetase. The goal of the present study was to define optimal rice extraction and detection methods that would allow a sensitive and reproducible measure of several classes of known rice allergens. In a three-laboratory ring-trial experiment, several protein extraction methods were first compared and analyzed by 1D multiplexed SDS-PAGE. In a second phase, an inter-laboratory validation of 2D-DIGE analysis was conducted in five independent laboratories, focusing on three rice allergens (52 kDa globulin, 33 kDa glyoxalase I, and 14–16 kDa α -amylase/trypsin inhibitor family members). The results of the present study indicate that a combination of 1D multiplexed SDS-PAGE and 2D-DIGE methods would be recommended to quantify the various rice allergens.

Key words: rice grain; allergen; proteomics; multiplex immunodetection; two-dimensional difference gel electrophoresis (2D-DIGE)

Following the first commercial planting of a genetically modified (GM) crop in 1996, agricultural

biotechnology has been rapidly adopted in many countries.¹⁾ Many countries require that a comprehensive safety assessment to be conducted before a GM crop can be either imported or cultivated. General guidelines for the safety assessment were laid out by the *Codex Alimentarius* Commission in 2003.²⁾ Although the principles in these guidelines remain in use today, specific regulations vary by country or region, and requirements continue to evolve.^{2–4)} Accordingly, the safety assessment paradigms for existing commercial GM crops focus on the safety of transgenic protein(s), along with an evaluation to detect any possible unintended changes in the crop plant or its derivatives. Components of the safety assessment include agronomic evaluation, compositional analyses of the crop, bioinformatics tools for toxicity and allergenicity assessment, specific toxicity studies, and animal feeding trials with whole GM food/feed. The concept of substantial equivalence is the basis of GM crop safety assessment. Therefore, any potential change in the whole GM plant compared with that of its non-GM comparator(s) shall be investigated by analytical comparison.

Allergenicity assessment of GM crops is one of the pillars in the safety review process of these products. Allergenicity, as it pertains to food allergy, describes the immune-mediated adverse health effects (allergic reactions) that can be induced in sensitised subjects following dietary exposure to relevant allergens in food. Food

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allergy is an important health problem.⁵⁾ Allergic reactions can be triggered by a variety of environmental agents including foods. Almost all antigens involved in eliciting allergic responses to food are proteins. To address concerns for food allergic consumers and the need to prevent introduction of new allergenic foods into the supply chain, allergenicity risk assessment has been the focus of different guidance documents for the assessment of GM crops and novel foods. For GM food crops, the focus is to ensure that the genetic modification does not raise levels or change characteristics of endogenous compounds, such as endogenous allergens, that would adversely impact human and animal health. Endogenous allergen assessments, as recommended by the European Food Safety Authority (EFSA), are based on the possibility, as yet unobserved empirically, that the genetic modification might have induced potential unintended over-expression of endogenous allergen(s).⁶⁾ Several proteomics approaches have been developed in order to quantify the levels of clinically relevant endogenous allergens in GM crops.^{6,7)}

Although it is recognized that specific serum screening using serum from food allergic individuals is the most frequently used reagent platform for detection and definition of an allergenic protein in protein extracts from edible plant parts, there are several limitations associated with the use of human sera. Because of these limitations, namely access to qualified patients and viable volumes of sera, other serum-free technologies have been investigated in recent years. At present, novel analytical methods and molecular profiling techniques, not based on human sera, are becoming available and could be considered as complementary and/or alternative methods for the comparative assessment of endogenous allergen content between the GM crop and its non-GM comparator(s).^{6,7)}

The EFSA guidance has also recommended the inclusion of endogenous allergens in the comparative compositional analysis as additional parameters to be measured.⁴⁾ Various methods are available for qualitative and quantitative comparison of plant proteomes. To date, two-dimensional gel electrophoresis, combined with mass spectrometry or enzyme-linked immunosorbent assay remains the most widely used qualitative approach for comparing plant proteomes to identify differentially expressed proteins.⁸⁾ Herein, rice as a developed crop amenable to GM transformation technology, is considered for its allergen content and the quantified evaluation of those allergens from an analytical perspective.

Rice is the staple food of over half of the world's population and contains carbohydrate and abundant proteins as an energy source. It is known that allergenicity of rice is partly dependent on globulin and albumin fraction proteins.⁹⁾ Several rice proteins with molecular masses of 9, 14–16, 26, 33, 52, 60, and 63 kDa have also been reported to be recognized by serum IgE of patients showing hypersensitive reactions to rice ingestion. These rice allergens have been identified and include the non-specific lipid transfer protein-1 (nsLTP1),¹⁰⁾ the α -amylase/trypsin-inhibitors,¹¹⁾ the α -globulin (19 kDa globulin),^{12,13)} the 33 kDa glyoxalase I (Gly I),¹⁴⁾ the 52 kDa and 63 kDa globulin,¹⁵⁾ and the granule-bound starch synthetase (GBSSI).¹⁶⁾ In

2010, Teshima et al.¹⁷⁾ reported that a two-dimensional difference gel electrophoresis (2D-DIGE) method was useful for analyzing the natural variation in protein expression among different rice cultivars. In 2013, Lang et al.¹⁸⁾ investigated the influence of different extraction solutions on the rice allergen detection from salt-soluble, salt-insoluble, and total protein fractions. This study indicated that the type of protein extraction buffer has an influence on the efficiency and the quality of rice allergen detection, and should be taken into consideration when conducting such analyses.

The goal of the present study was to refine and validate the existing methodologies developed by Teshima et al.¹⁷⁾ and Lang et al.¹⁸⁾ that would allow a sensitive and reproducible detection of several classes of known rice allergens in several laboratories. To evaluate the possible influence of the extraction method on the measured content and ability to identify rice proteins, three different extraction buffers were first tested in three independent laboratories. The level of expression of five rice allergens were determined by one-dimensional (1D) SDS-PAGE followed by multiplex immunodetection as described in Lang et al.¹⁸⁾ An inter-laboratory validation of 2D-DIGE analysis was then conducted in five independent laboratories, as described in Teshima et al.,¹⁷⁾ to measure the content of three rice allergens.

Materials and methods

Rice material. Two varieties of Japonica rice (*Oryza sativa* cv. Nipponbare and Koshihikari), one Indica rice (Kasalath), and one Thai rice (Bleiyo) were obtained from the world core rice collection in the Gene Bank of the National Institute of Agrobiological Sciences (NIAS), Japan. In order to get a representative sample, approximately 100 grains per cultivar were ordered. For the optimization of rice protein extraction method, rice grains of Nipponbare and Bleiyo cultivars were used. For the 2D-DIGE experiments, Nipponbare, Koshihikari, Kasalath, and Bleiyo cultivars were used. The grains were processed into powder at room temperature with a multi-beads shocker (Yasui Kikai Coop., Osaka, Japan) and immediately dispatched frozen on dry ice to the participating laboratories (Table 1). This allowed every participant (i) to use a representative sample of each rice variety and (ii) to use the same rice powder starting material.

Optimization of the rice protein extraction method.

Rice proteins were extracted from 20 to 100 mg of rice powder of the Nipponbare and Bleiyo cultivars. The extractions were performed in three different extraction buffers:

- NaCl-based buffer (either 1 M NaCl in laboratories A and B, or 0.5 M NaCl in laboratory C, in 30 mM Tris-HCl pH 6.8) made in-house, and supplemented with protease inhibitors (cOmplete ULTRA Tablets Mini from Roche Cat. No. 05892970001 or Halt Protease Inhibitor Cocktail Cat. No. 87785, from Thermo Fisher Scientific). It's been previously shown that 0.5 M to 1 M NaCl is an optimal concentration range to extract salt-soluble proteins;^{15–19)}

Table 1. Participating laboratories.

Laboratory identification	Laboratory name
A—Optimization of the rice protein extraction method	
Laboratory A	Bayer S.A.S., Bayer CropScience, 355 rue Dostoïevski, 06903 Sophia Antipolis, France
Laboratory B	National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan
Laboratory C	National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
B—2D-DIGE experiments	
Laboratory 1	Proteomics & Mass Spectrometry Facility, Donald Danforth Plant Science Center, 975 North Warson Road St. Louis, Missouri 63132, USA
Laboratory 2	Department of Biochemistry, State University of New York at Buffalo, 3435 Main St., 140 Fander Hall Buffalo, NY 14214
Laboratory 3	Nevada Proteomics Center, University of Nevada Reno, 1664N. Virginia St. Main Stop 200, Reno, NV 89557
Laboratory 4	National Food Research Institute, National Agriculture and Food Research Organization, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan
Laboratory 5	National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

- P-PER buffer (Cat. No. 89803) obtained from Thermo Fisher Scientific, supplemented with protease inhibitors (cOMplete ULTRA Tablets Mini from Roche or Halt Protease Inhibitor Cocktail from Thermo Fisher Scientific) in accordance with the manufacturer's protocol;
- Buffer D (8 M Urea, 4% CHAPS, 60 mM DTT), made in-house or obtained from GE Healthcare Life Science.

The extracted protein concentrations were determined with a BCA assay kit (Cat. No. 23225, Thermo Fisher Scientific Inc., MA, USA), a 2-D Quant kit (Cat. No. 80648356, GE Healthcare UK Ltd., Little Chalfont, England), or a 660 nm Protein Assay Reagent Kit (Cat. No. 22660, Thermo Fisher Scientific Inc.).

The extracted rice proteins were resolved by SDS-PAGE by loading the maximal volume per lane (3 µg/lane in laboratories (A) and (C), and 2 µg/lane in laboratory (B)), using a 15% (w/v) separating gel with a 4% (w/v) stacking gel. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue R-250 or transferred to polyvinylidene difluoride (PVDF) membranes by electroblotting with a TransBlot SD, a TransBlot Turbo semi-dry transfer cell, or Wet/Tank Blotting Systems (Bio-Rad Laboratories). The multiplexed detection of the rice allergens was done by immunodetection as described in Lang *et al.*,¹⁸⁾ using a mixture of rabbit antisera against rice nsLTP1, α -amylase/trypsin inhibitor, α -globulin, 33 kDa Gly I, and GBSSI.

2D-DIGE. Salt-soluble protein extraction was performed as described in Teshima *et al.*¹⁷⁾ with 1 M NaCl extraction buffer supplemented with protease inhibitors. Twenty-five microgram salt-soluble protein samples were purified using a 2-D Clean-Up Kit (GE Healthcare), followed by fluorescent labeling. Each protein extract was labeled in duplicate with Cy3 or Cy5 as described in Table 2, according to manufacturer's protocol. In addition, a Cy2-labeled protein extract obtained using a pool of equal protein quantities of the Nipponbare, Koshihikari, Rexark, and Bleiyo cultivars, was used as an internal standard.

Equal quantities of the proteins labeled with three different Cy dyes were mixed and separated on a 2D gel, as described in Teshima *et al.*¹⁷⁾ Fluorescence intensity was measured with a Typhoon 9400 variable image analyzer (GE Healthcare). Filters of 520 nm bandpass (520BP40), 580 nm bandpass (580BP30), and 670 nm bandpass (670BP30) were used for detection of, respectively, Cy2, Cy3, and Cy5 fluorochromes. Spot matching and differential analyses were performed with the following image analysis programs:

- SameSpot (Nonlinear Dynamics) in laboratory 1;
- DeCyder image analysis software version 7.0 or version 6.5 (GE Healthcare) in laboratory 2, 3, 5;
- PDQuest (Bio-Rad Laboratories) in laboratory 4.

Statistical analysis. For each rice cultivar, four replicate gels were used in this study. The ratios of fluorescence intensity for each of the spots across each of the cultivars against the internal standard spots were calculated with the image analysis software. The mean of four replicate spot fluorescence intensities was calculated, and ratios of the mean value against the mean internal standard value were calculated. Tukey's honest significant difference (HSD) test was performed to calculate the *p*-value on the intensity of 15 allergen spots for each of all combinations of four cultivars for each laboratory.

Results and discussion

Selection of the optimal salt-soluble rice proteins extraction method

To evaluate the possible influence of the extraction method on the amount and composition of extracted rice proteins that would allow a sensitive and reproducible detection of several classes of known rice allergens, three independent laboratories were chosen for ring-trial comparison. One was located in Europe and two in Japan (Table 1(A)). Each laboratory extracted rice grain proteins using three different extraction buffers as described in the Materials and methods section. The extracted proteins were then separated on a 1D SDS-PAGE. The rice allergen detection was performed

Table 2. 2D-DIGE experimental design.

Fluorescent dye	Samples				
Cy3	Internal standard* (x2)	Nipponbare (x2)	Koshihikari (x2)	Rexark (x2)	Bleiy0 (x2)
Cy5	Internal standard* (x2)	Koshihikari (x2)	Nipponbare (x2)	Bleiy0 (x2)	Rexark (x2)
Cy2	Internal standard* (x2)	Internal standard* (x2)	Internal standard* (x2)	Internal standard* (x2)	Internal standard* (x2)

*The internal standard corresponds to a pool of equal protein quantities of the Nipponbare, Koshihikari, Rexark, and Bleiy0 cultivars.

by multiplex immunodetection as described in Lang et al.¹⁸⁾ This method allows for simultaneous detection of five major rice allergens, the GBSSI, the α -globulin, the 33 kDa Gly I, the 14–16 kDa α -amylase/trypsin inhibitor family members, as well as the nsLTP1.

Amounts of salt-soluble proteins extracted from rice grains

The evaluation of extraction buffers demonstrated that Buffer D had the greatest capacity to solubilize salt-soluble proteins with an average protein concentration of 1412 $\mu\text{g}/\text{mL}$. This was compared with 763 and 529 $\mu\text{g}/\text{mL}$ for total protein concentrations represented by the NaCl-based and the P-PER buffers, respectively (Table 3). In terms of total protein extracted on a mass per rice weight basis, Buffer D outperformed the other two methods, with an average 45 μg of protein per mg of rice grain, compared to 6–21 $\mu\text{g}/\text{mg}$ of rice grain for the NaCl-based and the P-PER buffers, respectively.

SDS-PAGE and multiplex immunodetection of rice allergens from salt-soluble proteins

The proteins amenable to extraction and solubilization from rice grain were separated by SDS-PAGE electrophoresis, followed by Coomassie Brilliant Blue

staining (Fig. 1(A)). A separate, multiplex immunodetection of five major rice allergens as described in Lang et al. 2013 (Fig. 1(B)) was also performed to allow specific visualization and identification of rice allergens.¹⁸⁾ The goal of both detection methods was to observe any differences in overall extraction (i.e. relative content and concentration) of rice proteins. In using immunodetection, the impact of extraction buffer could be specifically demonstrated for known rice allergens.

By evaluation of the Coomassie-stained SDS-PAGE experiments, the NaCl-based method provided the best overall representation of rice proteins (i.e. protein profile) compared with the other two buffers (Fig. 1(A)). A major difference in protein profile for Buffer D was that it under-represented most of the bands observed in the extracts of the other two buffers and over-represented one primary protein (center lanes, Fig. 1(A)). The main band observed with Buffer D located at around 30 kDa corresponds to the glutenin acidic subunit, as previously described in Lang et al.¹⁸⁾ This phenomenon is mainly explained by the presence of 8 M urea in the buffer.

In the multiplexed immunodetection of five rice allergens, the NaCl-based and the P-PER methods provided the clearest specific protein allergen profiles (Fig. 1(B)). Buffer D was also different in terms of relative band intensities; not only were there fewer bands, but those that were detected were lower in

Table 3. Summary of detection of five allergens by 1D-western blot analysis.

Rice varieties	Extraction method	Protein concentration ($\mu\text{g}/\text{mL}$)	Total protein quantity $\mu\text{g}/\text{mg}$ of rice sample	Allergen					
				GBSSI	Gly I	α -globulin	RAG2 family	nsLTP1	
<i>Laboratory A</i>									
Nipponbare	NaCl	976 \pm 308	9.76	N.D.	+	+	+	(smear)	+
	P-PER	861 \pm 54	43.05	N.D.	+	+	+	(smear)	+
	Buffer D	1290 \pm 524	51.6	N.D.	+	+	+	(smear)	+
Bleiy0	NaCl	431 \pm 16	4.31	N.D.	+	+	+	(smear)	+
	P-PER	877 \pm 35	43.85	N.D.	+	+	+	(smear)	+
	Buffer D	1661 \pm 759	83.05	N.D.	+	+	+	(smear)	+
<i>Laboratory B</i>									
Nipponbare	NaCl	950 \pm 30	38	+	+	+	+	(2 bands)	+
	P-PER	180 \pm 27	9	N.D.	+	+	+	(2 bands)	+
	Buffer D	1100 \pm 26	11	+	+	+	+	(2 bands)	+
Bleiy0	NaCl	900 \pm 36	36	N.D.	+	+	+	(3 bands)	+
	P-PER	170 \pm 58	8.5	N.D.	+	+	+	(3 bands)	+
	Buffer D	1110 \pm 135	11.1	N.D.	+	+	+	(3 bands)	+
<i>Laboratory C</i>									
Nipponbare	NaCl	653 \pm 6	6.5	+	+	+	+	(2 bands)	+
	P-PER	540 \pm 70	27	+	+	+	+	(2 bands)	+
	Buffer D	1300 \pm 66	52	+	+	+	+	(2 bands)	+
Bleiy0	NaCl	493 \pm 6	5	N.D.	\pm	\pm	+	(3 bands)	\pm
	P-PER	603 \pm 15	30	N.D.	\pm	\pm	+	(3 bands)	\pm
	Buffer D	1200 \pm 57	48	+	\pm	\pm	+	(3 bands)	\pm

Notes: N.D.: Not detected. 100 mg, 20 mg, or 25 mg of rice powder were suspended in 1 ml of NaCl-based buffer, P-PER buffer, and Buffer D, respectively. Rice proteins were then extracted according to "Materials and Methods."

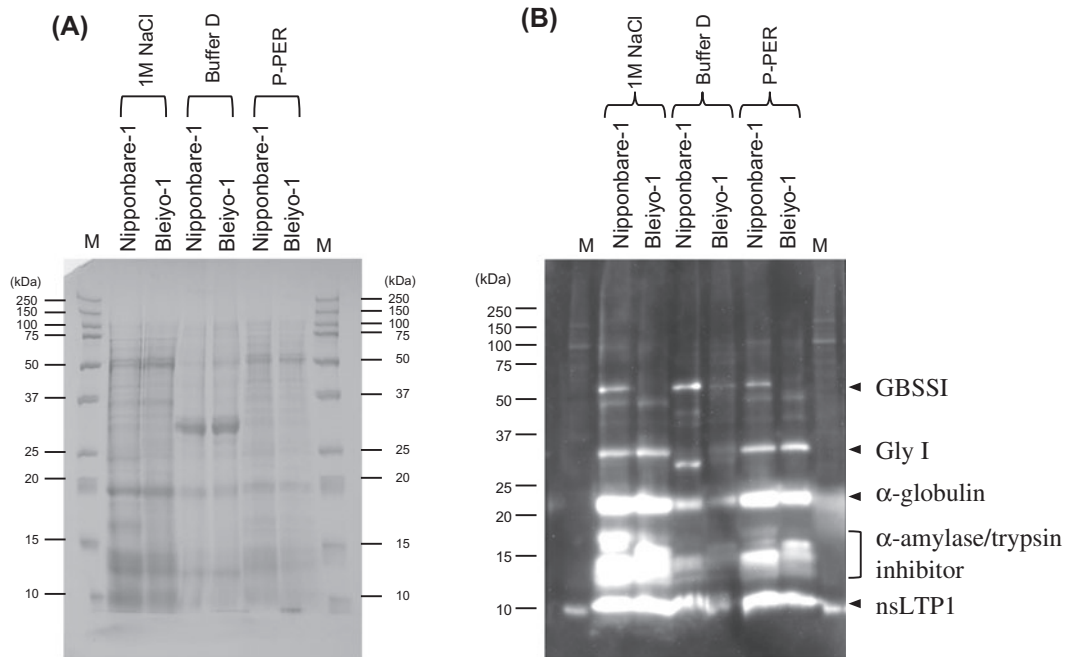


Fig. 1. SDS-PAGE analysis of rice proteins and multiplexed detection of rice allergens.

Notes: (A) representative coomassie brilliant blue staining of SDS-PAGE obtained in Laboratory C and (B) representative multiplexed detection of rice allergens obtained in Laboratory C

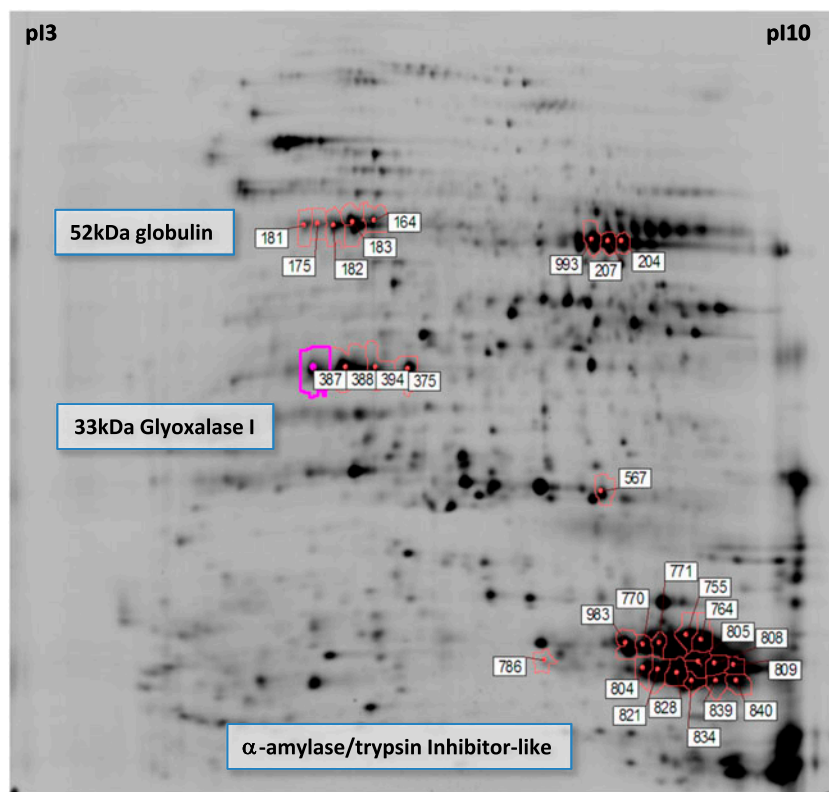


Fig. 2. Representative 2D-DIGE analysis of rice seed proteins.

Notes: Representative 2D-DIGE profile obtained in Laboratory 5 obtained on the Nipponbare variety. The mapping of the rice allergens spot was based on previous work by Teshima *et al.*¹⁷⁾ The salt-soluble proteins were labeled with Cy fluorescence dye, and separated by 2D-PAGE. Spots were identified using the DeCyder image analysis software ver. 7.0 or ver. 6.5 (Ge Healthcare). Fluorescence intensities of each spot from four gels were revised with the internal standard.

apparent concentration (center lanes, Fig. 1(B)). This was demonstrated most clearly for the α -globulin, 33 kDa Gly I, and the α -amylase/trypsin inhibitor family members. It's been previously reported that

the salt solubility of the GBSSI protein is very low,¹⁸⁾ and the extracted levels are usually close to the detection limit as observed in the present study.

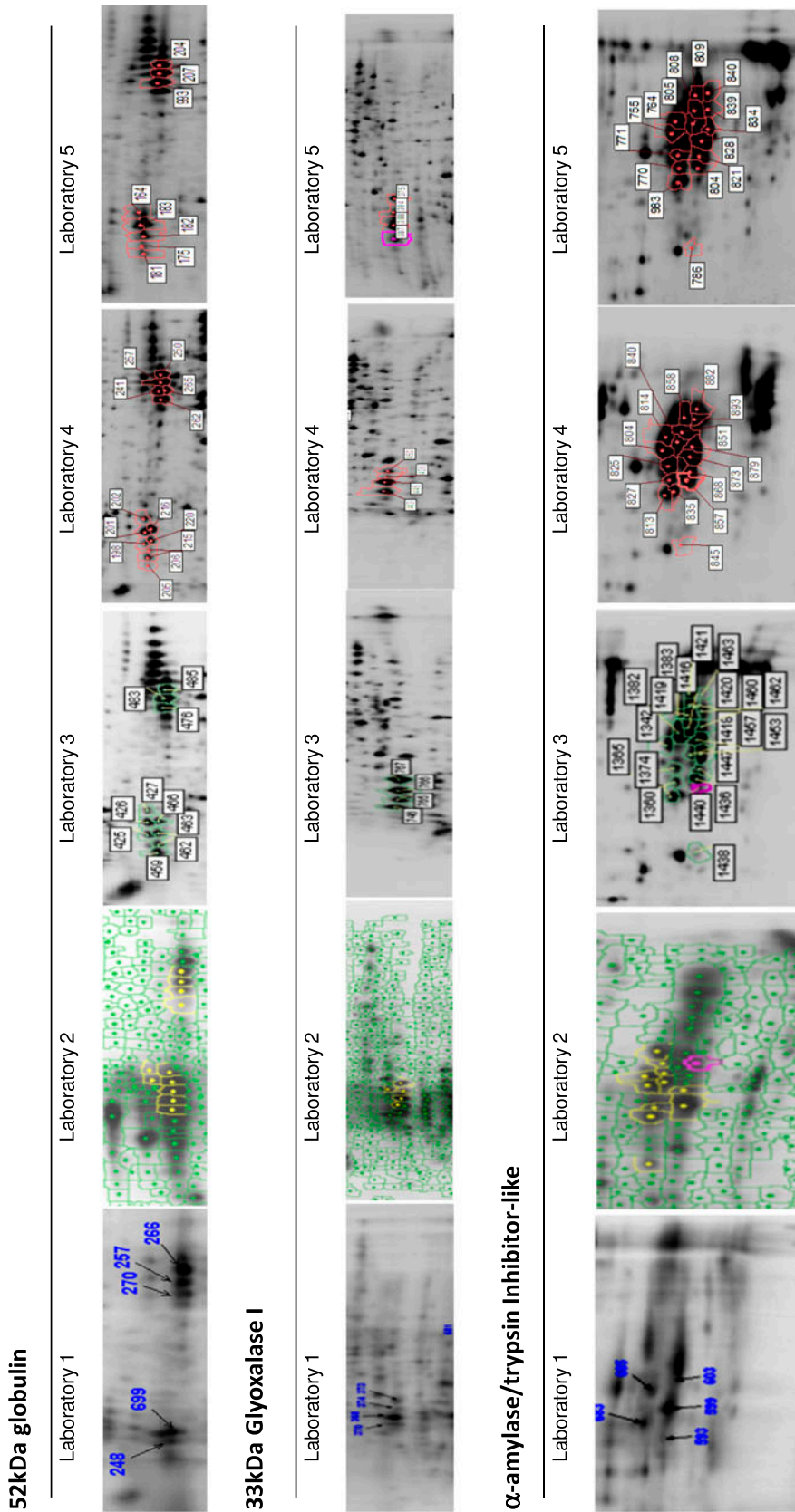


Fig. 3. 2D-DIGE analysis of rice seed proteins.

Notes: Representative allergen detection in the five participating laboratories obtained on the Nipponbare variety. For 52 kDa globulin, spots No. 182, 183, 193, 207, and 204 in Fig. 2(A) are Glo52k-1, 2, 3, 4, and 5, respectively. For 33 kDa glyoxalase I, spots No. 387, 388, 394, and 375 in Fig. 2(A) are Gly-1, 2, 3, and 4, respectively. For 19 kDa globulin, spot No. 567 in Fig. 2(A) is Glo19k-I. For alpha-amylase/trypsin inhibitor, 771, 764, 804, 828, and 834 in Fig. 2(A) are RAG-1, 2, 3, 4, and 5, respectively.

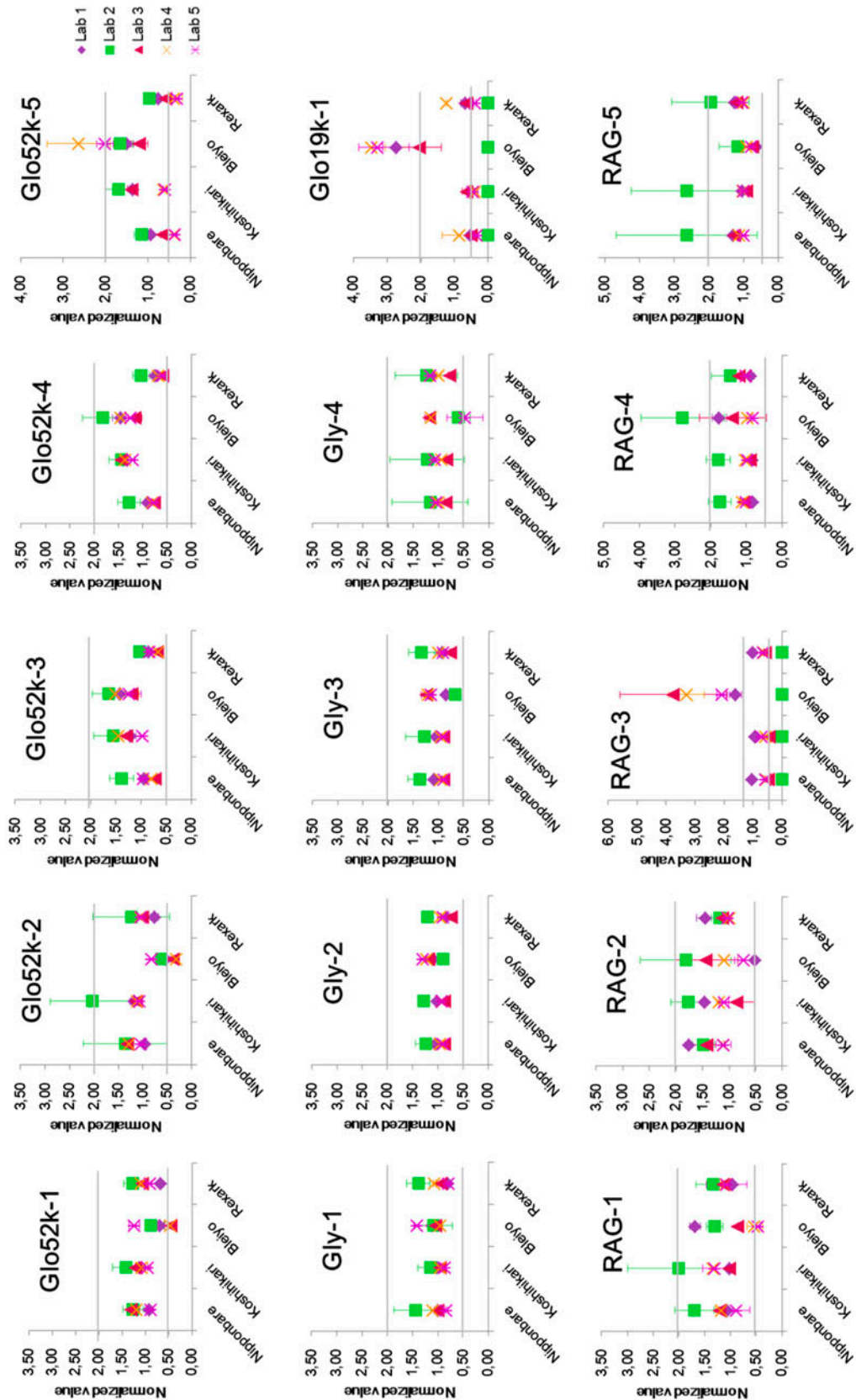


Fig. 4. (Colour in print)Quantification of representative rice allergen spots.

Notes: Fluorescence intensities of each spot from four gels were revised with the internal standard and averaged normalized ratios with error bars of 15 allergens in four rice cultivars obtained from five different labs were plotted. Statistical significance was analyzed using the Tukey HSD multiple comparison test with R (ver. 3.2.0), a 0.05 level of probability was used as the criterion for significance (see supplemental data file).

As the NaCl-based extraction method provided a better protein allergen detection capability in the 1D-PAGE (with Coomassie staining), we decided to use NaCl-based buffer for extraction of allergenic proteins and apply those proteins to subsequent 2D-DIGE analyses.²⁰⁾

Inter-laboratory 2D-DIGE analysis of rice seed allergens from different cultivars

An inter-laboratory validation of the 2D-DIGE rice protein detection method, initially developed by Teshima et al. (2010),¹⁷⁾ was conducted in five laboratories, two located in Japan and three in USA (Table 1(B)). Each laboratory measured the content of four rice allergen families: the 52 kDa globulin, the 33 kDa Gly I, the 14–16 kDa α -amylase/trypsin inhibitor family members, and the 19 kDa globulin. This analysis was done in two varieties of Japonica rice (*Oryza sativa* cv. Nipponbare and Koshihikari), one Indica rice (Kasalath), and one Thai rice (Bleiyoy). In order to evaluate the results from all laboratories in a manner that would indicate whether substantive differences in protein detection were observed between laboratories, each participant extracted proteins from the same starting material consisting of 200 mg of rice seed powder for each variety. The 2D-DIGE analysis was performed on 25 μ g of salt-soluble protein extracts in quadruplicate experiments as described in Table 3, and spot fluorescence intensity was measured using an image analyzer.

Inter-laboratory identification of the rice allergen spots

A representative 2D-DIGE profile of rice proteins and allergens from the Nipponbare cultivar is presented in Fig. 2. The mapping of each rice allergen spot was based on previous work from Teshima et al.¹⁷⁾ Spots were identified using image analysis software as described in the Materials and methods section. Between 765 and 1359 spots were detected for each rice cultivar (data not shown) which is in line with previous work from Teshima et al.¹⁷⁾ where approximately 700 spots were detected.

Rice allergen spots were then manually identified on each gel in order to compare spot intensities in each laboratory. A typical identification of the rice allergen spots in the five participating laboratories is presented in Fig. 3. The 52 kDa globulin was detected in each laboratory, appearing as a minimum of two acidic spots (Glo52 k-1 and Glo52 k-2) and three basic spots (Glo52 k-3 to Glo52 k-5). The 33 kDa Gly I was also detected in each laboratory, appearing as four spots (Gly-1 to Gly-4). In contrast to the consistency observed for the other allergens, the α -amylase/trypsin inhibitor family was not reliably detected in each laboratory. There was variation in the number of spots representing the α -amylase/trypsin inhibitors which ranged from 5 to 15 spots. Among the five participating laboratories, five common spots could be identified (these were named as RAG-1 to RAG-5).

Inter-laboratory comparison of expression levels of rice allergen spots using 2D-DIGE

For each rice cultivar, four gel replicates were run. The 52 kDa globulin (Glo52 k-1 to Glo52 k-5), the 33 kDa Gly I (Gly-1 to Gly-4), and the 14–16 kDa α -amylase/trypsin inhibitor family members (RAG-1 to RAG-5) were identified and their fluorescence intensity quantified with image analysis software. The mean of four replicate spot fluorescence intensities were calculated, and ratio of the mean value against the corresponding mean internal standard value (pool of equal protein quantities of the four rice cultivars) was calculated (Fig. 4). *p*-values between four cultivars were calculated by Tukey's HSD test and compare across laboratories (data not shown, see supplementary file)).

While the acidic spots (Glo52 k-1 to Glo52 k-2) of the 52 kDa globulin showed similar fluorescence levels among the four rice cultivars, the basic spots (Glo52 k-3 to Glo52 k-5) showed statistically significant differences in their expressions between the Nipponbare and Bleiyoy varieties in three out of five laboratories. The four spots of the 33 kDa Gly I (Gly-1 to Gly-4) showed no differential expression among the different cultivars. For these two allergen families, the relative apparent expression appears to be consistent in the five participating laboratories. On the contrary, the α -amylase/trypsin inhibitor family was not detected in a consistent manner in each laboratory. As mentioned previously, between 5 and 15 spots were observed, with variable intensities within each laboratory, preventing precise measures of their concentrations. It is known that the precise separation of basic proteins on 2D polyacrylamide gels is not straightforward. One explanation for the inconsistency is that once the basic proteins reach their PI on the first dimension, disulfide bridges are regenerated. This phenomenon induces the concomitant formation of macro-aggregates which then become entangled and cannot easily enter the polyacrylamide gel fibers and migrate at their respective molecular mass and charge in the second dimension.²¹⁾

Conclusion

The goal of the present study was to define an analytical method that would allow sensitive and reproducible detection of several classes of known rice allergens. In a three-laboratory ring-trial, the one-dimensional electrophoresis platform was used to determine an optimal rice grain extraction buffer that could support broad identification of rice allergen families. The results demonstrated that the NaCl-based extraction method had the broadest capacity to support multiple protein separation and identification. This conclusion was based on the quality of the rice allergen immunodetection which was characterized by high-quality separation (band separation) and band clarity of proteins with a wide range of molecular weights and at varying concentrations. In addition, for those proteins known to be allergens, the concentration of protein extracted and available for detection was greatest for this buffer (Fig. 1(B)). In terms of providing representation of allergens that might be expected to be

exposed through digestion, the NaCl buffer was also considered a better representative of *in vivo* gastrointestinal physiological conditions.²²⁾

An inter-laboratory validation of 2D-DIGE analysis was then conducted in five independent laboratories, focusing on three rice allergens (52 kDa globulin, 33 kDa glyoxalase I, and the 14–16 kDa α -amylase/trypsin inhibitor family members). The intent was to extend the separation and detection capabilities of electrophoresis and to build upon the buffer optimization testing. The results indicated that the 2D-DIGE method is a valid approach for the 52 kDa globulin and 33 kDa Gly I allergens. The combination of very precise separation of proteins in two dimensions coupled with very sensitive fluorochrome labeling allowed for reproducible detection of several spots per allergen. This detection was also demonstrated to provide a basis for quantifying the amount of each allergen; a distinct advantage over the less precise 1D platform. In effect, multiple spots per identified protein were accounting for endogenous expression of allergen isoforms. In quantifying each allergen, the 2D-DIGE data were suitable for a statistical assessment; this was deemed desirable for means comparison between treatment groups. In our studies, we could determine differences for each allergen across cultivars. This was demonstrated as a platform that would allow the type of comparative assessment across rice cultivar grain samples expected for endogenous allergen evaluation for GM crops.

The methods approach outlined here provides precision to quantify known rice allergens with a level of reproducibility that is suitable for use as a standardized platform, regardless of laboratory location. Although variation in one of the three allergen families (α -amylase/trypsin inhibitor) limited full validation across all of the laboratories, the indication was that optimizing detection of this allergen remained possible, as it was for the other two families. The data supports the use of a combination of the 1D multiplexed SDS-PAGE and 2D-DIGE for qualitative and quantitative analysis of rice allergens and provides an acceptable platform to determine measurable impacts on allergen content.

Author contributions

Rie Satoh, Kazumi Kitta, and Gang-Hua Lan (National Agriculture and Food Research Organization, Japan) participated in the experiments testing for the optimal salt-soluble rice proteins extraction method, as well as in the 2D-DIGE experiments on rice seed allergens from different cultivars. Rie Satoh participated in the data analysis and interpretation.

Reiko Teshima (National Institute of Health Sciences, Japan) participated in the experiments testing for the optimal salt-soluble rice proteins extraction method, as well as in the 2D-DIGE experiments on rice seed allergens from different cultivars. She also conducted the analysis of the 2D-DIGE data produced by the five participating laboratories, and participated in the data analysis and interpretation.

Kathleen Schegg (Nevada Proteomics Center, USA) participated in the 2D-DIGE experiments on rice seed allergens from different cultivars.

Kenneth Blumenthal (Formerly at Department of Biochemistry, USA) participated in the 2D-DIGE experiments on rice seed allergens from different cultivars.

Leslie Hicks (Formerly at Donald Danforth Plant Science Center, USA) participated in the 2D-DIGE experiments on rice seed allergens from different cultivars.

Bénédicte Labory-Carcenac and David Rouquié (Bayer S.A.S., France) participated in the experiments testing for the optimal salt-soluble rice proteins extraction method.

Rod A. Herman (Dow AgroSciences, USA), Corinne Herouet-Guicheney (Formerly at Bayer S.A.S., France), Gregory S. Ladics (DuPont Co., USA), Scott McClain (Syngenta Crop Protection, USA), Lars K. Poulsen (Copenhagen University Hospital at Gentofte, Denmark), Laura Privalle (Formerly at BASF Plant Science, USA), Jason M. Ward (Formerly at Monsanto, USA), and Nancy Doerrer (ILSI Health and Environmental Sciences Institute, USA) participated in the evaluation of this project and in the review of the manuscript.

Jean-Baptiste Rasclé (Bayer S.A.S., France) designed and coordinated this project. He also took care of writing the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Supplementary material

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