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# Wheat Storage Proteins in Transgenic Rice Endosperm

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**ABSTRACT:** Transgenic rice seed expressing wheat HMW glutenin subunit was characterized to study the effects of the wheat prolamin on the protein expression pattern and protein size distribution in the endosperm and the functional and rheological properties of the rice flour and dough. Significant differences were found in the protein expression pattern between the transgenic and wild type samples. Comparing the protein expression profiles of transgenic and nontransgenic plants, combined with proteomic-based studies, indicated increased protein disulfide isomerase (PDI) levels in the transgenic rice lines. The accurate molecular size of HMW-GS in rice endosperm was identified by MALDI-TOF-MS analysis. The expressed wheat HMW (subunit 1Dx5) GS showed a positive effect on the functional properties of rice dough by significantly increasing the size distribution of the polymeric protein fraction and modifying the dough mixing parameters.

**KEYWORDS:** *transgenic rice, wheat glutenin subunit, proteomics, rice dough*

## INTRODUCTION

The unique property of wheat flour is its ability to form dough when water is added. This important feature is due to the special protein composition of the endosperm and, in particular, to the groups of glutenins and gliadins.[1,2] This property is largely determined by the ability of the component proteins to form interchain disulfide bonds within the gluten, which then stabilize the protein matrix.

Wheat prolamins are commonly divided into monomeric gliadins and polymeric glutenins, with the latter comprising subunits which are further classified on the basis of their molecular masses into two main groups: high molecular weight subunits of glutenin (HMW-GS) and low molecular weight subunits of glutenin (LMW-GS). Most wheat prolamins are sulfur-rich because they contain cysteine residues; the only exception is the $\omega$-gliadins (reviewed by Tatham and Shewry[3]). Some cysteine residues are well conserved among all sulfur-rich prolamins and form intramolecular disulfide bonds that stabilize the protein secondary structure. Glutenins, and even some types of gliadins, have additional cysteine residues that form intermolecular disulfide bonds, leading to the formation of the large polymers that are present in the mature starchy endosperm and develop into the gluten network when the flour is mixed with water to make dough.[4]

Bread wheat contains six HMW-GS genes, with linked pairs of genes encoding x- and y-type subunits present at each of the Glu1A, 1B, and 1D loci, in chromosomes 1A, 1B, and 1D, respectively.[5] The association between allelic variation of the HMW-GSs and differences in dough strength was established by Payne at al.[6] and was widely used as a marker for quality assessment in plant breeding programs.[7]

Although the HMW glutenin subunits contribute only around 12% or less of the total gluten proteins in mature wheat endosperm, they appear to have both significant quantitative and qualitative effects on gluten properties.[8] It was also established that subunit 1Dx5, which has an additional cysteine residue compared to other x-type subunits, is one of the most important components influencing the quality of wheat flour.[9]

Whereas prolamins are the major storage proteins in most cereals, rice (*Oryza sativa* L.) preferentially accumulates glutelin proteins belonging to the 11S-type globulin family.[10] In rice, 60−80% of total seed protein is composed of glutelins, and 20−30% of total seed proteins are prolamins.[11] Glutelins can be classified into four groups (GluA, GluB, GluC, and GluD) based on amino acid sequence similarity.[11] Rice glutelins are synthesized as precursor polypeptides, which are post-translationally cleaved into two smaller subunits. The two subunits of glutelins are classified as acidic (a) or basic subunits (b) with apparent molecular weights of 30−39 and 19−25 kDa, respectively. The glutelin subunits are able to form large macromolecular complexes stabilized by disulfide bonds and hydrophobic interactions in the presence of protein disulfide isomerase (PDI).[12] It is assumed that besides hydrogen and disulfide bonding, extensive aggregation and glycosylation may also be partly responsible for the limited solubility of the rice polymeric glutelin fraction.[11]

Prolamins are classified into three groups (10, 13, and 16 kDa) by their molecular mass according to their mobility on polyacrylamide gels. The 13 kDa prolamins can be further classified into three subgroups (classes I, II, and III) by their Cys residue content.[13] Only the 13 kDa prolamin is soluble in alcohol without reducing agent, whereas the other two

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prolamins are able to form long polymers, due to their high cysteine residue contents, and require reduction for solubilization.[14] Furthermore, rice prolamins have a number of characteristics (molecular weight, amino acid sequence, etc.) that differ from the prolamins of most other cereals.[15] However, similar to wheat, rice prolamins are stabilized through a sequential linking process mediated by binding proteins (BiP).[16]

The doughmaking quality of rice storage proteins is very poor compared to that of wheat.[17] A possible explanation may be that rice endosperm lacks the proteins responsible for this trait.[18] To improve the properties of rice flour dough, the gene encoding the subunit 1Dx5 HMW-GS was introduced into the rice genome by Oszvald et al.,[19] and its stable expression was confirmed over two generations (T1−T2). It was also demonstrated that due to the wheat endosperm-specific promoter used to initiate transcription, the wheat storage protein was expressed only in the rice endosperm tissue.[19]

The aim of our work was to study the effects of the introduced wheat 1Dx5 HMW glutenin subunit protein on the protein size distribution and on the protein expression pattern of the rice endosperm as well as on the functional and rheological properties of the rice flour and dough.

The effect of the wheat proteins on the protein size distributions in the transgenic flours was studied by size exclusion chromatography (SE-HPLC). The protein expression profiles of the transgenic and wild type flours were compared by two-dimensional (2D) polyacrylamide gel electrophoresis, and protein identification was performed by subsequent matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). The functional properties of rice dough were studied by using a micro z-arm mixer.

## MATERIALS AND METHODS

**Plant Material.** T2 homologous transgenic rice lines (T2#21, T2#23, and T2#26; *O. sativa* L.) expressing subunit 1Dx5 proteins reported earlier[19] were harvested and stored at 4 °C until used.

The protein content of rice flours was determined according to the Dumas method (N × 5.95), an adaptation of the AOAC Official Method (1995),[20] using an automated protein analyzer (LECO FP-528, USA).

**Total Soluble Protein Extraction and 2D Gel Electrophoresis.** Two-dimensional gel electrophoresis was carried out to compare the protein expression pattern of wild type and transgenic rice seeds. For the 2D gel electrophoresis, total soluble proteins (TSP) were extracted after the seeds had been ground in liquid nitrogen with a mortar and pestle. Powder (100 mg) was mixed with 500 μL of buffer (200 mM Tris-HCl, pH 8.0, 100 mM NaCl, 400 mM sucrose, 10 mM EDTA, and 1% dithiothreitol (DTT)), and the homogenate was centrifuged at 13000*g* at 4 °C for 15 min. The supernatant was mixed with one-fourth volume of cold acetone and kept at −20 °C overnight. The mixture was centrifuged at 15000*g* at 4 °C for 15 min, and the supernatant was discarded. The pellet was washed with cold acetone three times, centrifuged as above, and air-dried. The dried powder was solubilized in sample buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer pH 3−10.

One hundred and fifty micrograms of protein quantified by using the Bradford method was loaded onto immobilized pH gradient (IPG) strips (24 cm, pH 3−10 linear gradient). Strips were focused according to Islam et al.[21] using a Protein IEF cell (Bio-Rad). The second-dimension separation (SDS-PAGE) was carried out on 12% acrylamide gel according to the method of Laemmli.[22] After the completion of 2-DE, the protein spots were visualized by Coomassie R-250. 2D gel electrophoresis analysis of each sample was performed in three replicates.

**Analyses of Protein Spots.** Digitized images were quantitatively analyzed using the Progenesis SameSpots software (version 4.1, Nonlinear Dynamics Ltd., USA) according to protocols provided by the manufacturer. The amount of protein in each spot was normalized by total valid spot intensity. Protein spots from transgenic rice lines with >4 times the intensity of wild type samples were then selected.

Protein spots were cut from the gels and kept at −20 °C until further analysis. The proteomics analyses were performed in facilities funded by the (WA) Lotterywest State Biomedical Facility − Proteomics Node, Western Australian Institute for Medical Research, Perth, Australia. Protein samples were trypsin digested and peptides extracted according to standard techniques. Peptides were analyzed by MALDI-TOF-TOF mass spectrometry using a 5800 Proteomics Analyzer (AB Sciex). Spectra were analyzed to identify proteins of interest using the Mascot sequence matching software (Matrix Science) with the Ludwig NR Database and taxonomy set to Viridiplantae (Green Plants).

**Extraction of High Molecular Weight Proteins for MALDI-TOF-MS Analysis.** Proteins were extracted from whole rice grain according to the sequential procedure of Singh et al.[23] Rice flours (20 mg) were extracted with 55% propanol-1-ol (v/v) by shaking for 5 min followed by incubation (at 65 °C, 20 min) and centrifugation (at 10000*g*, 5 min). This step was repeated three times. The high molecular weight glutenin subunit proteins present in the pellet were reduced and solubilized in a solution containing 55% propanol-1-ol, 0.08 M Tris-HCl, and 1% DTT. Acetone (40%) was used to precipitate the high molecular weight proteins.

**MALDI-TOF-MS.** MALDI-TOF-MS was performed at the State Agriculture Biotechnology Center (SABC), Murdoch University, Australia. The dried mixtures of HMW-GS samples were dissolved in 50 mL of acetonitrile/H2O (v/v, 50:50) containing 0.05% v/v trifluoroacetic acid (TFA). Sample preparation was carried out according to the dried droplet method,[24] using sinapinic acid (SA). MALDI-TOF mass spectrometric experiments were carried out on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a UV nitrogen laser, operated in linear mode (337 nm).

The results from MALDI-TOF-MS were analyzed using the Voyager machine companion software, Data Explorer, to produce the protein spectrum profiles.

**Protein Extraction for SE-HPLC (Size Exclusion Liquid Chromatography).** The transgenic and wild type rice protein samples were prepared for SE-HPLC using the modified sonication procedure of Singh et al.[25]

"Total proteins" were extracted from 10 mg of flours in 1 mL of 0.05 M sodium phosphate buffer containing 0.5% SDS (pH 6.9) by vortex followed by sonication for 45 s, and then the supernatants were recovered by centrifugation.[26] Two step of extraction procedure was also carried out to extract "soluble" and "insoluble" proteins from rice flours. Soluble proteins were extracted from 10 mg of flours using the same phosphate buffer. The suspension was shaken for 30 min, and the soluble proteins were recovered in the supernatant by centrifugation (13000*g*, 10 min). In the second step, the so-called insoluble proteins were extracted from the pellet after resuspension (1 mL of SDS−phosphate buffer) and sonication (15 s). The solubilized insoluble proteins were in the supernatant after centrifugation (at 13000*g*, 10 min). All protein solutions were filtered through a 0.45 μm PVDF filter prior to injection on column.

**SE-HPLC Analysis.** The protein size distributions of the soluble, insoluble, and total protein fractions of rice flours were determined by SE-HPLC according to the method of Oszvald et al.[26] The proteins were separated on a Phenomenex Biosep SEC-4000 column, 300 × 7.8 mm, with an eluant consisting of acetonitrile/water (50:50) containing 1% (v/v) TFA for 10 min.

The percentage of unextractable polymeric protein (UPP%), a simple but effective measure of the size distribution of the polymeric proteins, was determined by applying the calculation method developed for rice flours and dough.[26]

**Western Blot Analysis.** Glutenin subunit 1Dx5 proteins in transgenic rice were also identified by Western blotting. The insoluble

proteins from transgenic rice (T2#26) eluted in the different SE-HPLC peaks were manually collected for electrophoresis studies. The proteins were dialyzed against distilled water and then freeze-dried and resuspended in SDS loading buffer. The proteins were separated on 12% SDS–acrylamide gel according to the method of Laemmli[22] and then transferred onto PVDF membranes (Promega, Madison, WI, USA) in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% methanol) using a semidry apparatus (Bio-Rad Inc., Hercules, CA, USA) at 130 mA for 1.5 h.

To prevent any nonspecific antibody reactions, the membranes were blocked with 5% of nonfat milk powder in TBST buffer (Tris-buffered saline with 0.05% Tween-20) overnight at room temperature. The membranes were then incubated with a 1:5000 dilution of anti-IFRN 1602 monoclonal antibody (INRA, France), which is highly specific for x-type HMW glutenin subunits developed in mice in accordance with Mills et al.,[27] in TBST buffer containing 1% of nonfat dry milk for 2 h and washed three times in TBST buffer. The membrane was then incubated with anti-mouse IgG conjugated with alkaline phosphatase as a secondary antibody (Promega S3731) at a 1:10000 dilution for 2 h and developed using BCIP/NBT (USB, Cleveland, OH, USA) in TMN buffer (10 mM Tris, 150 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

**Dough Mixing.** Microscale mixing tests were carried out on a prototype micro z-arm mixer (METEFEM Ltd., Hungary) using 4 g of flour per test. The resistance values were sampled every 0.1 s and stored electronically. The following parameters were determined from the mixing curve of rice and wheat: maximum resistance ($VU_{max}$), dough development time (DDT), breakdown (BD), and stability (ST).[26]

**Statistical Analysis.** All measurements were carried out in triplicates. Analysis of variance (ANOVA) was then carried out on the mean values. Statistica 7.0 (StatSoft Inc., 2006, USA) was used for statistical evaluation.

## RESULTS AND DISCUSSION

**2-DE Maps of Endosperm Proteins.** 2D gel electrophoretograms of the total soluble proteins from control and transgenic (T2#26) rice lines are given in Figure 1, panels A and B, respectively. An additional spot that is not present in the control line can be observed in the transgenic rice sample (Figure 1B). This protein has a molecular weight that is consistent with the presence of the mature 1Dx5 HMW-GS protein from wheat seed.[28]

**Identification of 1Dx5 HMW-GS by MALDI-TOF-MS.** The transgenic rice line with the highest level of the expressed 1Dx5 glutenin subunit (T2#26) was used for further studies. The mass spectra showing the molecular weight range of the portion of interest of the rice proteins from the two wild type and the transgenic rice lines (T2#26) are shown in Figure 2, panels A and B, respectively. Rice proteins in the molecular weight range of 75–85 kDa were very similar for both rice types. However, the MALDI-TOF-MS profiles showed differences between the transgenic and control lines. The main difference in the studied range was an additional peak (Figure 2B), which corresponded with the correct 88 kDa molecular weight of the 1Dx5 glutenin subunit protein.[29]

Blechl et al.[30] recently studied the identities of novel HMW-GS proteins in transgenic wheat by tandem mass spectroscopy (MS/MS) and found that biolistic transformation of wheat with genes encoding HMW-GS often results in changes in their coding regions that lead to production of related proteins larger or smaller in size than the native subunits. However, the transgenic rice studied here showed only one peak corresponding to the native mass of the 1Dx5 HMW glutenin subunit.

**Analysis of the 2D Gel Electrophoresis Profile and Identification of Differently Expressed Protein.** Quantitative analysis of the electrophoretogram of the total soluble proteins revealed that spots were present in the entire range (pH 3.0–10.0) of the strip and molecular mass of 20–110 kDa in both the transgenic and control samples. Among the detected spots, 21 showed differences in intensity between the control and the studied T2#21, T2#23, and T2#26 transgenic lines (see Figure 1 and Table 1).

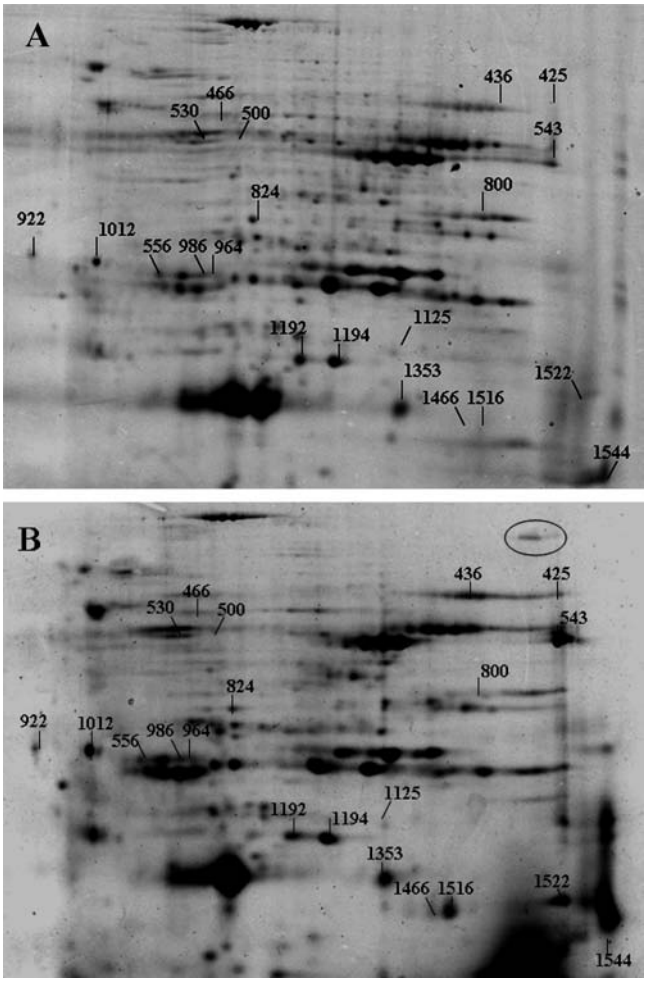


**Figure 1.** 2D gel electrophoresis profiles of the seed's total soluble proteins extracted from wild type (A) and transgenic rice (T2#26) endosperm (B). The 2D gel was visualized by CCB, and numbered spots were analyzed. The circle indicates the expressed 1Dx5 GS protein in the transgenic rice line.

On the basis of the results of the quantitative analysis of nine protein spots, which had intensities approximately 5 times or greater in the transgenic lines compared to the control line, were selected and cut from the 2D gel for MALDI-TOF-TOF-MS analysis (bold numbers in Table 1). After mapping of these polypeptides, homologous proteins were identified by the PROFOUND blast program. The number of the selected spots and the names of the identified proteins are shown in Table 2.

The identified proteins, which had shown significant differences in the expression level between the wild type and the transgenic rice lines, can be classified into three functional groups. The first group (PDI and BiP) may affect protein folding, protein stability, synthesis, and storage. The second group was associated with indigenous seed storage proteins (globulins, glutelins, and glutelin precursors). The third group contains proteins that may participate in the synthesis of

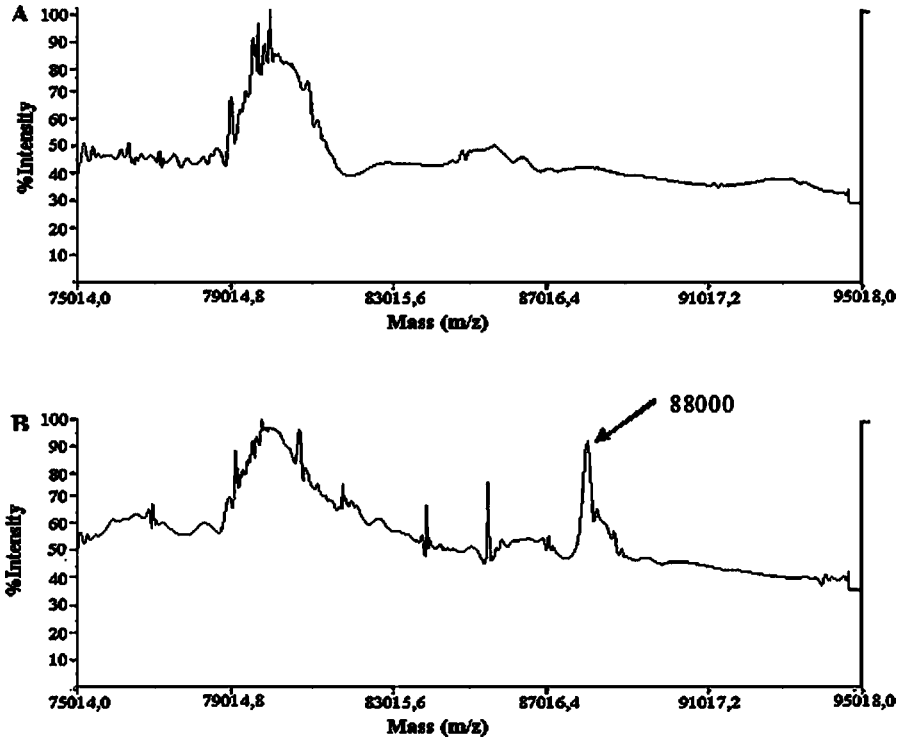


**Figure 2.** MALDI-TOF-MS protein profiles of reduced extracts showing differences between the wild type (A) and the transgenic rice line T2#26 (B) in the molecular weight range 75−95 kDa. The subunit 1Dx5 GS protein is indicated by an arrow at the corresponding molecular weight.

**Table 1. Quantitative Analysis of the Selected Protein Spots among Those Differentially Expressed in Transgenic and Wild Type Samples[a]**

| | | | av normalized volumes × $10^{4}$[b] | | | |
|---|---|---|---|---|---|---|
| spot | ANOVA ($p$) | fold | WT | T2#21 | T2#23 | T2#26 |
| **425** | **0.00648** | **5.8** | **8.540** | **8.667** | **49.12** | **11.23** |
| 436 | 0.046 | 1.8 | 104.6 | 108.1 | 106.2 | 58.93 |
| 466 | 0.015 | 3.7 | 76.80 | 32.97 | 46.36 | 21.02 |
| 500 | 0.016 | 2.3 | 48.74 | 23.50 | 23.44 | 20.77 |
| **530** | **0.027** | **8.6** | **170.7** | **30.55** | **103.8** | **19.87** |
| **543** | **0.051** | **4.8** | **9.639** | **10.84** | **46.01** | **10.76** |
| 800 | 0.040 | 1.8 | 97.60 | 55.06 | 58.72 | 83.21 |
| 824 | 0.003 | 2.6 | 1.307 | 1.046 | 2.715 | 1.296 |
| **922** | **0.005** | **5.7** | **10.50** | **29.65** | **5.742** | **5.196** |
| 955 | 0.022 | 2.4 | 7.626 | 6.538 | 15.21 | 6.216 |
| **964** | **0.032** | **4.9** | **2.388** | **3.559** | **11.66** | **3.571** |
| **986** | **0.005** | **4.9** | **7.208** | **34.25** | **20.35** | **6.995** |
| 1012 | 0.034 | 4.3 | 136.8 | 437.7 | 128.9 | 102.5 |
| **1125** | **0.505** | **15.1** | **16.21** | **14.315** | **20.82** | **215.7** |
| 1192 | 0.996 | 1.5 | 19.26 | 26.18 | 24.85 | 28.10 |
| 1194 | 0.084 | 2.2 | 27.27 | 14.67 | 32.25 | 20.41 |
| 1353 | 0.995 | 1.2 | 380.9 | 396.5 | 381.1 | 447.26 |
| **1466** | **0.005** | **4.9** | **199.92** | **989.27** | **518.22** | **681.21** |
| **1516** | **0.029** | **5.4** | **736.24** | **6197.55** | **3.332** | **1.016** |
| 1522 | 0.021 | 1.9 | 1.779 | 2.452 | 1.380 | 2.657 |
| 1544 | 0.018 | 4.4 | 88.45 | 51.45 | 199.6 | 44.90 |

[a]The values were calculated from the intensity of different spots on the gels after 2D gel electrophoresis using Progenesis SameSpots software. The "fold" numbers refer to any transgenic lines that have a higher spot ratio compared to the wild type sample. [b]WT, wild type; T2#21, T2#23, T2#26, transgenic rice lines.

**Table 2. Selected Seed Proteins of Transgenic Rice Lines Showing Significant Differences in the Protein Amount Compared to Wild Type Rice and Identified by MALDI-TOF-TOF-MS**

| spot | % Cov[a] | $M_r$/p$I$[b] | accession no. | protein name |
|---|---|---|---|---|
| **425** | 27 | 73345/5.09 | Q2Z7BO | DnaK type molecular chaperone BiP[49] |
| **543** | 16 | 57984/8.96 | Q9MBI3 | protein disulfide isomerase[50,51] |
| **530** | 27 | 63389/5.35 | Q75GX9 | putative globulin[52] |
| **922** | 22 | 56028/5.11 | A1YQH4 | glutelin protein[11] |
| **986** | 29 | 48013/5.41 | B8BFV2 | enolase protein[53] |
| **964** | 34 | 38799/3.35 | Q5N725 | fructose bisphosphate aldolase |
| **1125** | 14 | 25345/6.13 | BAA09308 | 26 kDa globulin[52] |
| **1466** | 9 | 21041/7.48 | P29835 | 19 kDa globulin precursor[54] |
| **1516** | 8 | 20502/5.89 | POC5A4 | late embryogenesis abundant protein |

[a]% cov, percentage of sequence coverage. [b]$M_r$, experimental molecular mass; p$I$, experimental isoelectric point.

secondary metabolites (fructose bisphosphate aldolase, enolase, and LEA proteins).

The analysis of specific spots on the 2D gels revealed clear differences between the profiles of wild type and transgenic rice expressing the wheat 1Dx5 HMW glutenin subunit protein. This indicates that the genetic transformation of rice had an effect on the protein distribution of the rice endosperm, although no changes could be observed on the total protein content (7.24%).

In previous studies series of transgenic wheat lines carrying one of the wheat storage protein genes coding for HMW glutenin subunits have been produced and analyzed.[31−34] Statistical analyses on field-grown samples showed that the

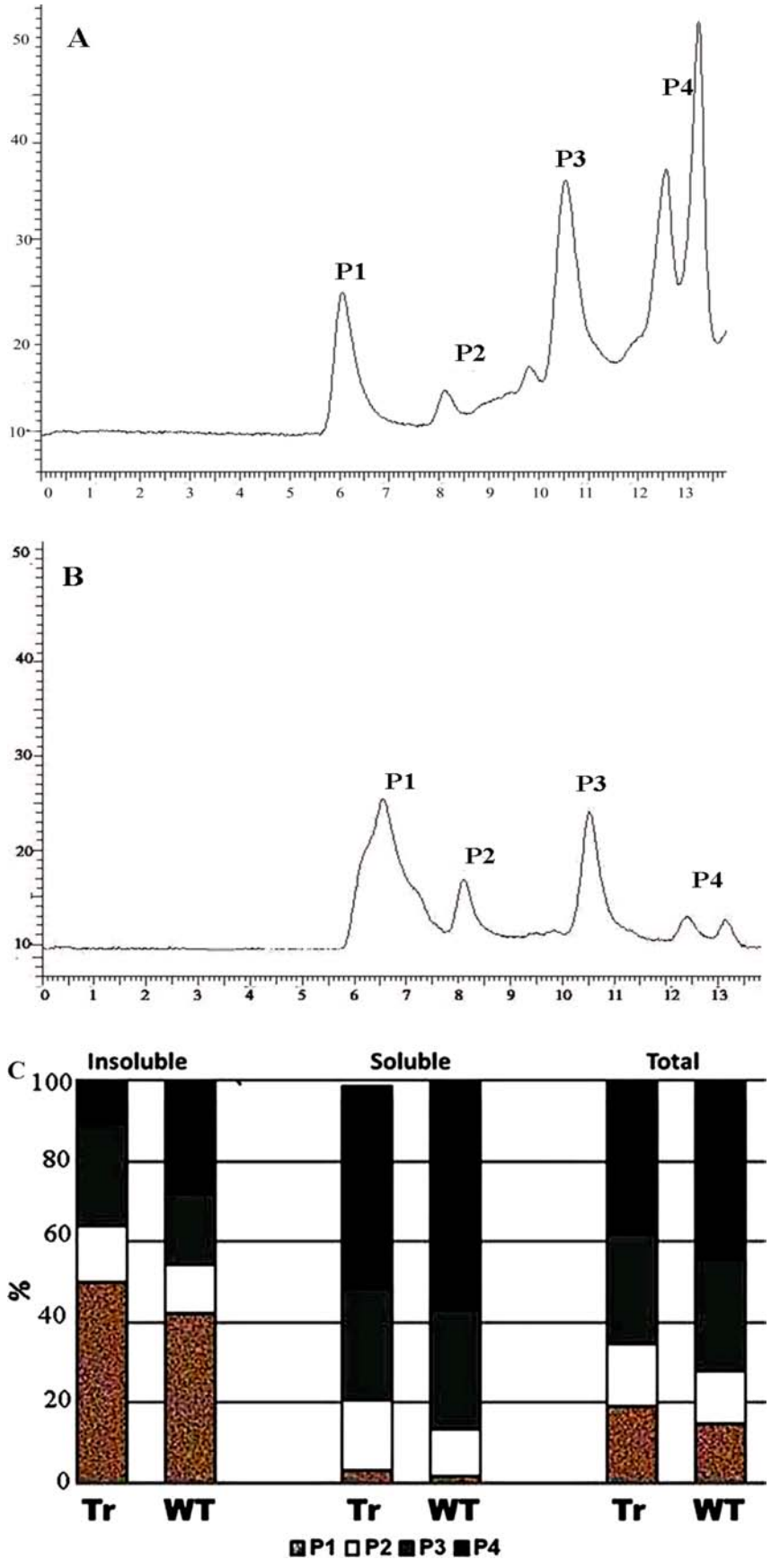


**Figure 3.** (A, B) SE-HPLC chromatograms of "soluble" and "insoluble" proteins of transgenic rice line (T#26), respectively. (C) P1−P4, peak areas of elution profile of the "soluble", "insoluble", and "total" protein extract from the transgenic rice line compared to the wild type. Tr, transgenic rice line T2#26; WT, wild type rice. P1−P4, protein fractions.


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transgenic and nontransgenic wheat lines did not differ in terms of stability of HMW subunit gene expression, in the stability of grain nitrogen, in the dry weight, or in dough strength, either between harvest years or between sites and plots. Baudo et al.[35] produced strong evidence that the presence of the transgenes did not significantly alter the gene expression pattern of the transgenic wheat plants.

One of the identified proteins, which had shown a significant difference in the expression level between the transgenic rice and control endosperm (4.8 times higher in the transgenic line), was the protein disulfide isomerase (PDI). This protein is a ubiquitous, multifunctional enzyme that is not only present in the lumen of the endoplasmic reticulum (ER) but also colocalized with seed storage proteins in dense protein bodies. PDI is likely to be involved in storage protein folding and intracellular transport. It also catalyzes the right formation and disruption of disulfide bonds between cysteine residues within proteins as they fold in ER lumen by leading the transportation into the Golgi apparatus.[4,36]

The level of PDI involved in the formation of disulfide bonds has already been studied in mature transgenic rice seeds accumulating the 7Crp peptide.[37] PDI levels in these transgenic rice seeds were also increased (2.1−3.1-fold). On the basis of previous studies of transgenic rice expressing recombinant proteins with Cys residues, we hypothesize that the detected increase in the amount of PDI can be explained by the expression of the wheat 1Dx5 glutenin subunit protein.

This correlation between the increased expression level of the PDI enzyme and the expressed wheat HMW-GS proteins has not yet been observed in other transgenic crops such as wheat or barley.[31−34] However, Wang et al.[38] recently used substituted lines of spring wheat cv. Chinese Spring to study the molecular mechanism of its superior quality conformation. A variety of aspects were investigated including PDI protein expression. The introduction of the HMW subunit genes resulted in increased HMW-GS accumulation and a higher abundance of PDI and PDI-like proteins in the mature grains.

Alternatively, the increased PDI enzyme level in transgenic rice endosperm could be caused by the retention of the 1Dx5 subunit and other Cys-rich rice proteins within the ER lumen or may be caused by ER stress in rice endosperm.

**SE-HPLC Analysis of Rice Flours.** The transgenic rice line T2#26 was also used to investigate whether the introduced wheat 1Dx5 HMW glutenin subunit protein was deposited in monomeric form or, as in wheat, became part of the polymeric protein fraction formed through disulfide bridges among the storage proteins.

All of the soluble, insoluble, and total proteins extracted from both types of flours were separated by SE-HPLC, and four peaks were differentiated on the chromatogram (Figure 3A, B). The transgenic line showed significant differences in the protein content of each peak, indicating changes in the size distribution of the rice storage proteins (Figure 3C). P1 and P2 fractions represent the polymeric and high molecular weight proteins, P3 represents the oligomers, and P4 represents the monomer proteins. The molecular weight ranges of the protein in the P1, P2, P3, and P4 fractions were approximately 200−151, 150−80, 62−52, and 32−10 kDa, respectively.

The greatest difference between the two lines was found within the P1 and P4 fractions of the insoluble protein extract (Figure 3C). The P1 fraction of the transgenic line showed an 8% increase in the amount of proteins compared to the wild type; meanwhile, the amount of proteins in the P4 fraction of the transgenic sample contained 18% less monomeric proteins (Figure 3C).

A similar change to the protein size distribution was observed with the soluble protein extracts. The polymeric protein fraction (P1) was 3% higher and the P4 fraction 9% lower in the transgenic line.

The protein size distribution of the total protein extract prepared from transgenic rice flour was also affected by the 1Dx5 HMW-GS in a manner similar to the soluble and insoluble protein samples (Figure 3C).

No significant differences were observed in the amounts of P2 and P3 fractions between the two lines (Figure 3) in any of the soluble, insoluble, or total proteins.

The subunit compositions of the four (P1−P4) fractions collected from the insoluble protein extracts from the transgenic rice sample were separated by SDS-PAGE under reducing conditions (Figure 4A). SDS-PAGE analysis of the collected samples demonstrated that the subunit 1Dx5 GS protein was located only in the P1 (polymeric) fraction of the transgenic rice. The rice polymeric and high molecular weight proteins (glutelins) were also observed in the reduced gels of P1−P2 (Figure 4A); the oligomeric proteins were in the P3 fraction, whereas the monomeric albumin, globulin, and prolamins were predominantly in the P4 fraction.[26] Western blot analysis using IFRN 1602 monoclonal antibody specific to the x-type of HMW subunits[19] verified the result of the gel electrophoresis (Figure 4B). Wheat glutenin subunit protein was present in the P1 fractions but was not detected in P2−P4. These results clearly demonstrated that the wheat glutenin protein is present only in polymerized form in the large polymeric fraction of the transgenic rice endosperm.

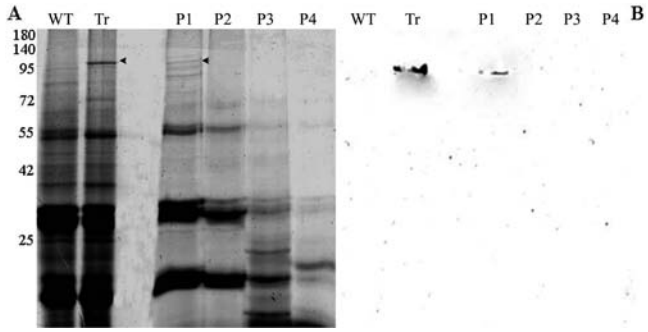


**Figure 4.** SDS-PAGE (A) and Western blot analysis (B) of the collected SE-HPLC fractions (P1−P4) from protein extract of transgenic rice line (T2#26) expressing subunit 1Dx5 HMW-GS. WT, total soluble proteins from wild type rice; Tr, total soluble proteins from transgenic rice line (T2#26); P1−P4, collected "insoluble" protein fractions from the transgenic rice line (T2#26). Western blot analysis was carried out using IFRN 1062 antibody. Arrows indicate the 1Dx5 GS proteins.

**Functional Properties of Rice Doughs.** The mixing curves of the dough made from wild type and transgenic rice flours (T2#26) are shown in Figure 5, panels A and B, respectively. Significant differences were observed between the mixing parameters of the dough formed from the transgenic and control flours (Figure 5C). The dough development time (DDT) was 15% higher in the case of transgenic flour compared to nontransgenic flour. The stability (ST) was also increased by 35%, whereas the value of the resistance breakdown (BD) dropped by 16%. The increased DDT and


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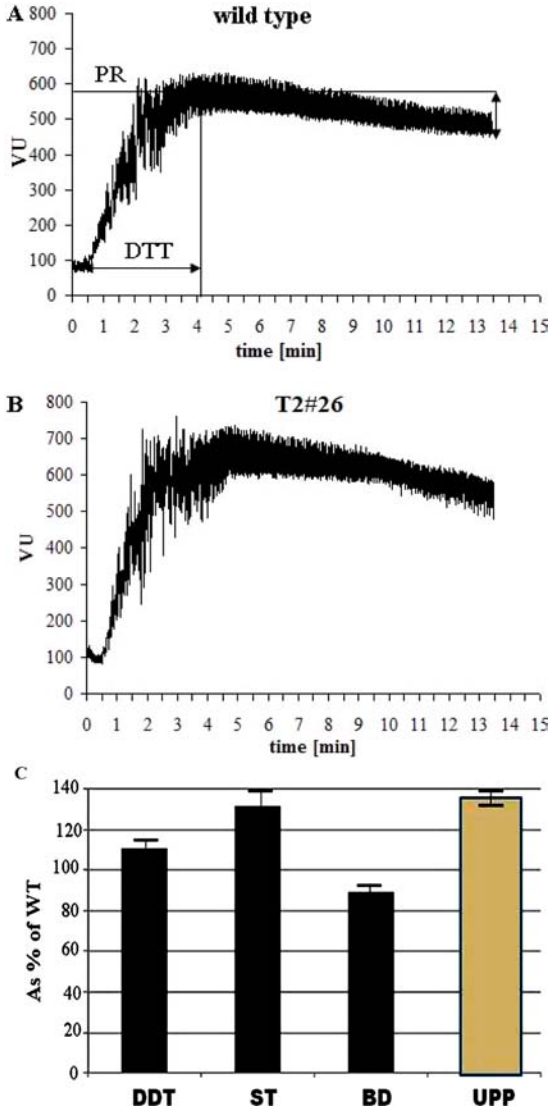


**Figure 5.** Micro z-arm mixer curve of wild type (A) and transgenic rice (T2#26) (B) dough. PR, peak resistance; DDT, dough development time; BD, resistance breakdown. (C) Mixing parameters of dough (DDT, BD, ST, dough stability) made from transgenic rice (T2#26) flour in the percent of wild type determined with prototype micro z-arm mixer. UPP%, unextractable polymeric protein percentage; the ratio of polymeric proteins extractable only by using sonication compared with the total amount of polymeric protein.

ST values in the transgenic rice dough indicate that the expressed wheat 1Dx5 HMW glutenin subunit made the dough stronger and more stable. The recombinant protein in the transgenic rice endosperm had a positive effect on the tolerance to overmixing indicated by the significantly lower BD value (Figure 5C).

The observed changes in the mixing properties were proportional with the extent of the alteration in the polymer size distribution of rice dough. Size distribution was studied in the dough as well, and the percentage of unextractable polymeric protein (UPP%) was determined. A significantly higher value of the UPP% (38%) was calculated in the case of transgenic rice dough carrying the expressed 1Dx5 HMW glutenin subunit (Figure 5C). This increased UPP% value indicated that the storage proteins formed polymers in larger numbers or larger sizes in the transgenic than in the wild type rice dough. Similar to our previous in vitro experiments, when individual HMW glutenin subunit proteins were incorporated into rice dough,[39] the improved functional parameters of dough made from transgenic rice flour can be associated with the presence of the wheat 1Dx5 HMW glutenin subunit.

Dough mixing and development are critical processes in breadmaking. Many studies have therefore been carried out to determine the parameters that influence them to facilitate the production of high-quality bread.[39,40] Several laboratories use HMW subunit genes to transform cultivars of bread wheat.[30,31] The expression of subunits 1Dx5 and 1Ax1 clearly improved the dough quality of transgenic tritordeum and triticale, increasing the tenacity and the deformation energy and providing a more balanced dough quality.[31,41] The expressed 1Dx5 subunit in transgenic wheat lines unexpectedly resulted in overly strong doughs unsuitable for breadmaking.[42,43] Similar to the effect observed in transgenic wheat, the alteration of the gliadin/glutenin ratio by in vitro incorporation of HMW-GS proteins had a positive effect on the rheological properties of the dough and resulted in stronger and more stable dough. However, the behavior of individual subunits may also be affected by the HMW-GS composition of the base flour in which they are expressed, as subunit interactions are crucial in forming the glutenin polymers.[41] Using rice as a base flour could therefore provide a gluten-free environment to study the behavior of these HMW-GS proteins.

The observed increase in the level of the PDI enzyme in transgenic rice endosperm suggests that PDI may have affected the polymerization of the storage proteins. Therefore, PDI may indirectly have a positive effect on the doughmaking quality of rice flour. The effect of PDI on doughmaking has previously been reported in in vitro studies when glutenin subunits were incorporated into the polymeric glutenin of wheat dough in the presence of the PDI enzyme.[44]

Watanabe et al.[45] have reported that the addition of exogenous PDI to wheat dough resulted in a more elastic product; however, the function of endogenous PDI activity in flour for breadmaking has still not been proven. Mixograph analysis by Koh et al.[46] revealed that the addition of one of the PDI inhibitor enzymes, bacitracin, had decreased both the development time and stability time of wheat dough and therefore weakened the strength of the dough. It was observed that the addition of the PDI inhibitor enzyme increased depolymerization of gluten macropolymer during mixing. However, further studies are necessary to elucidate the polymerization mechanism of the PDI enzyme in the presence of increased substrate and its function during breadmaking.

The increased PDI level could therefore be partially responsible for the observed changes in the protein distribution in the transgenic rice samples (Figure 3C). The rice prolamin and globulin proteins that appeared in monomeric form in the wild type rice seem to be a part of the polymeric proteins in the transgenic samples.

In agreement with our previous studies using wheat and rice as base flour in in vitro incorporation experiments, it was found that the major governing factor determining the mixing requirement of the dough is the size distribution of the polymeric proteins (the value of UPP%).[47] The in vivo expression of 1Dx5 GS protein in transgenic rice reveals possible effects of other factors in the starchy endosperm. The observed increase in the PDI level could affect the polymer formation of rice monomer proteins or the polymer/monomer

ratio resulting in changes to the UPP% value and mixing properties.

It was confirmed that the wheat 1Dx5 HMW glutenin subunit in transgenic rice endosperm was present in polymeric form. This study has also confirmed the positive effects of the in vivo expressed wheat 1Dx5 HMW-GS protein in transgenic rice on the mixing properties of rice dough. Similar effects were observed in in vitro experiments using rice flour as a base flour for incorporation of HMW-GS proteins.[47] Due to its improved protein content and doughmaking properties, this rice could be used to produce rice-based baking products for people who suffer from certain forms of wheat-related diseases or allergies.[48] This kind of product seems suitable for people suffering from gluten sensitivity.

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The authors declare no competing financial interest.

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