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Decreased accumulation of glutelin types in rice grains constitutively expressing a sunflower seed albumin gene

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Abstract

Previous studies have shown differential accumulation of sulfur rich glutelins and sulfur poor prolamins in transgenic rice seeds expressing a sunflower seed albumin gene [Hagan, N.D., Upadhyaya, N., Tabe, L.M., Higgins, T.J., 2003. The redistribution of protein sulfur in transgenic rice expressing a gene for a foreign, sulfur-rich protein. Plant J 34, 1–11]. Here, we show, by two-dimensional electrophoresis, differential accumulation of three classes of glutelin proteins – type I, II and III – and a globulin, not previously resolved, in transgenic seeds grown under low and high sulfur nutrition. Several glutelin polypeptides were resolved and four identified as a type I glutelin, two type II glutelins and a type III glutelin. Although sulfur nutrition did not affect the accumulation of sunflower seed albumin, the levels of all four identified glutelins and the globulin were lower in mature seeds derived from transgenic plants grown under sulfur-optimum or sulfur limited conditions compared to non-transgenic rice seeds. The reduction of all four glutelin polypeptides and the globulin varied from 21% to 68%. The re-allocation of sulfur reserves from endogenous proteins to the sulfur sink in transgenic grain is suggestive of a transcriptional control of sulfur mobilization in plants.

Keywords: Transgenic rice; Sunflower gene in transgenic rice; Sulfur rich proteins; Rice; Pleiotropy in transgenic plants; Proteome of transgenic plants

1. Introduction

The organic sulfur content in seed is an important determinant of seed protein quality. The sulfur containing amino acids, cysteine and methionine, are a vital part of most proteins and cysteine is involved in stabilising protein structure by the formation of disulfide bonds.

Over the last two decades, several efforts have been made to improve the sulfur content in field crops by incorporating gene(s) from the same or different species, with some notable success in legumes and oil seeds (Tabe and Droux, 2002). Efforts have been made to improve grain protein quality by inserting a sunflower seed albumin (SSA) gene encoding a sulfur-rich protein (Hagan et al., 2003; Molvig et al., 1997; Tabe and Droux, 2002). A significant increase in organic sulfur content was observed in transgenic lupin containing the SSA gene (Molvig et al., 1997). However, no such increase was observed in rice, despite high accumulation of transgenic SSA (7% of rice seed protein), suggesting that there might be a change in sulfur utilization in the transgenic rice. Hagan et al. (2003) observed that the demand for sulfur appears to exceed supply in transgenic rice, and that the SSA in transgenic rice was produced at the expense of other sulfur pools. Changes in the relative levels of two seed storage proteins – glutelins and prolamins – were obvious in one dimensional SDS–PAGE, but the different products of the glutelin and prolamin gene families could not be resolved (Hagan et al., 2003). In this study, we analysed changes in profiles of different glutelin polypeptides and a major sulfur rich protein

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($M_r = 19$ kDa) identified as globulin in transgenic and non-transgenic rice seeds grown under low and high sulfur nutrition by two-dimensional gel electrophoresis. We found that the levels of different glutelin polypeptides and the globulin protein were lower in mature seeds derived from transgenic plants grown under both sulfur-optimum and sulfur limiting conditions compared to the non-transgenic parent lines.

2. Results and discussion

2.1. Protein expression in transgenic rice expressing a sunflower seed albumin gene

The SSA gene was introduced into rice with a view to increasing methionine and cysteine in the endosperm. However, over-expression of this sulfur rich protein did not increase the total sulfur amino acid content of the grain (Hagan et al., 2003). Redistribution of protein sulfur from endogenous sulfur rich proteins to SSA was apparent from one-dimensional SDS–PAGE which showed an increase in sulfur poor prolamins and a decrease in sulfur rich glutelins (Hagan et al., 2003). However, the identities and quantities different glutelins could not be resolved by one-dimensional SDS–PAGE.

In this study, we analysed proteins from mature seeds of transgenic and non-transgenic rice lines by 2-DE. The power of the 2-DE analysis is that it is possible to separate closely related proteins of the type expected from a multigenic family and to check whether all are affected in the same way. The 2-DE gels used here effectively resolved polypeptides in the range of 10–75 kDa and in the pI range of 4–10. Thus, prolamins and other low molecular weight proteins such as the basic polypeptide chains of the glutelins that were found earlier by Hagan et al. (2003), were not resolved here.

An abundant protein appeared in gels of the transgenic samples near the predicted molecular weight (14 kDa) and pI (5.9) of SSA (indicated by an arrow in Fig. 1) which was absent in non-transgenic proteome. Kortt et al. (1991) reported that the mature SSA protein consists of a single polypeptide chain of 103 amino acids which contains 16 residues of methionine and 8 residues of cysteine (MARFSIVFAA AGVLLLVAMA PVSEASTTTI ITTIIEENPY GRGRTESGCY QQMEEAEMLN HCGMYLMKNL GER**SQVSPRM** REEDHKQLCC MQLKNLDEKC MCPAIMMMLN PMWIRMRDQV MSMAHNLPI E CNLMSQPCQ M), indicating SSA is a very high sulfur rich protein (24%). The SSA spot was not identified by simple MALDI-TOF peptide mass fingerprinting, possibly because most of the predicted tryptic peptides could have many permutations of oxidation or other modifications of methionine and cysteine residues. Natively expressed SSA is reported to have a signal peptide (residues 1–25) followed by a further 13-residue pro-sequence that are removed in the mature protein (Kortt et al., 1991). To confirm the identity of the putative SSA spot, we performed an in-gel digestion with trypsin followed by reversed phase separation of the peptides coupled with electrospray/ion-trap mass spectrometry. Fragmentation of peptides in the ion trap unequivocally identified the spot as SSA by showing extensive sets of B and Y-series ions consistent with sequences from SSA; the 'distinct summed MS/MS score' was 62, where a score over 20 is accepted by the analysis software for automatic validation of the identification. One such fragmented ion was a simple, unmodified tryptic peptide ((R)SQVSPR). Other ions derived from the N-terminal tryptic peptide predicted by removal of the signal peptide but not the pro-sequence. Further peptides also derived from the N-terminus indicated that some of the SSA molecules lacked a further three, four residues or a further eight residues after the

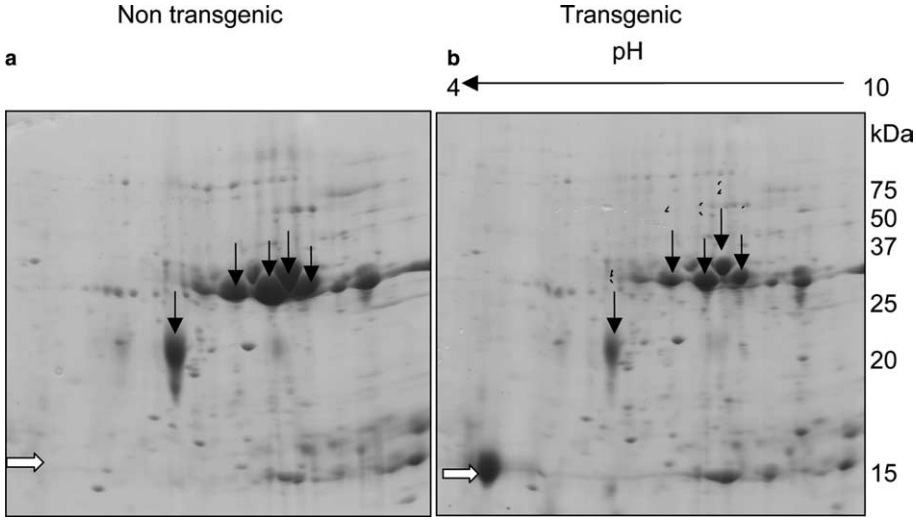

Fig. 1. Comparison of protein profiles in grains at maturity between: (a) a non-transgenic and (b) a transgenic line of rice expressing an SSA gene. Arrow marked proteins were downregulated; numbered proteins were identified by MALDI-TOF-MS for spots 1–4 and by Ion-trap LC/MS/MS for spot 5. SSA appeared only in the transgenic proteome and it's position is shown by horizontal arrow.

signal peptide. All these N-terminus-derived peptides showed similar spectral intensities suggesting similar abundance among the forms of SSA with these various N-terminal cleavages. As with MALDI-TOF MS the methionine and cysteine-rich peptides were not recovered and/or difficult to interpret.

As expected, some abundant endogenous proteins were reduced (indicated by down arrows in Fig. 1) in the transgenic grains. The observation that the levels of some proteins were reduced in concert with the expression of the SSA transgene indicates complexity in the mechanism by which SSA transgene expression is orchestrated. Although the actual mechanism of the downregulation of the protein level is unknown, it is anticipated that it could result from the metabolic or signalling pathways responsible for sulfur amino acid accumulation and utilization and could be mediated at transcriptional, post-transcriptional, translational or post translational levels.

2.2. Four glutelins and a globulin protein (M_r = 19 kDa) are reduced in SSA transgenic seeds

Results of quantitative image analyses of polypeptides in transgenic and non-transgenic grains are presented in Table 1. Selected polypeptides were identified by their peptide mass fingerprints after tryptic digestion. Differentially expressed polypeptides (spots 1–4), were identified as different glutelins – the most abundant storage proteins in rice endosperm – and the 5th spot corresponded to a globulin ($M_r = 19$ kDa). Spot 1 was identified as a Type I glutelin. It is most likely one of the 33 kDa proteins previously reported to be downregulated (Hagan et al., 2003). Although the ProFound search of the NCBI non-redundant protein database identified it as a unique glutelin, a search of the TIGR genome browser (http://www.tigr.org/tdb/e2k1/osa1/) showed that two closely linked genes on Chromosome 2 could encode the same glutelin. Spots 2 and 3 were identified as two isoforms of a type II glutelin most likely encoded by *OsGluII* on rice chromosome 10. The mobility shift observed in these two isoforms in the 2-DE gel suggests that these two polypeptides differ slightly in their pI which may be explained by the expected small changes in amino acid composition of the products of a multigene family. Spot 4 was identified as glutelin type III, and the gene encoding this protein is located at chromosome 3.

A quantitative analysis of the spot intensities showed a 50% reduction in spot 2. Spot 1 was reduced by 28% whereas spots 3 and 4 were reduced by 37% and 45%, respectively. Type II and type III glutelins are known to contain higher levels of cysteine and methionine (2.3%) compared to type I glutelins (1.9%) (Wen and Luthe, 1985). In addition to the downregulation of glutelin proteins, a major protein (spot 5) identified as globulin was also downregulated. According to Rice Proteome Database (http://gene64.dna.affrc.go.jp/RPD), a spot that appeared at a comparable position are globulin fragments. The gene encoding this globulin protein is located in Chromosome 5. Interestingly, this protein is also a sulfur rich protein with 10.2% sulfur-containing amino acids (methionine and cysteine). The significant downregulation of the glutelin and globulin proteins in relation to SSA could be explained by competition for sulfur as all of them are sulfur rich proteins.

Glutelins are highly polymeric proteins with intermolecular disulphide bonds. It is believed that glutelin proteins are transported via Golgi and deposited in storage vacuoles. It is possible that, in our study, the sulfur amino acids destined for glutelin were reallocated to SSA, suggesting that SSA competes more effectively than the endoneous proteins for the limited available sulfur. However, the downregulation of these proteins in the presence of SSA gene might also be explained by reduced level of mRNA as found in other legumes (Spencer et al., 1990).

2.3. Protein expression under sulfur stress conditions

To further analyse the changes in glutelin expression when SSA is expressed, non-transgenic and transgenic rice were grown under sulfur rich and sulfur limiting conditions. A clear phenotypic difference (short plant height and yellowish leaves) between the plants grown under low and high sulfur was observed with both non-transgenic and transgenic plants (data not shown). Sulfur nutrition did not affect the level of glutelins in non-transgenic rice

Table 1
Identification of proteins expressed differentially in the endosperm of transgenic and non-transgenic plants

Spot no.	Spot vol.			Protein identified and their related genes	Matched peptides (no.)	Est'd Z/MS/MS score	Sequence coverage (%)	Accession no.
	SSA−	SSA+	*t* value					
1	7.5	5.4	1.7	Glutelin subunit (Osglu × 14393, chromosome 2)	12	2.38	37	BAC77349
2	9.2	5.1	2.6*	Glutelin subunit (Osglu II × 05564, chromosome 10)	7	2.33	24	1311273A
3	12.9	8.1	2.1	Glutelin type II (Osglu II × 05564, chromosome 10)	7	1.34	28	P07730
4	7.7	4.7	1.9	Glutelin type III (Osglu III × 54313, chromosome 3)	7	1.79	23	Q09151
5	10.1	4.9	2.4*	Globulin (OS19KDGLO × 63990, chromosome 5)	2	28.78	10	CAA45400

Quantitative analyses of protein expression in mature seeds were performed by ImageMaster 2D Platinum software using four CBB stained gels from each of the transgenic and non-transgenic lines. The student's *t*-test was used to analyse the data; significantly ($p < 0.1$) downregulated spots are indicated by *. To identity spot no. 5, we performed an in-gel digestion with trypsin followed by reversed phase separation of the peptides coupled with electrospray/ion-trap mass spectrometry (LC/MS/MS). The sequence information was searched against NCBInr using Spectrum Mill (Spectrum Mill MS Proteomics Workbench, Agilent Technology).

(bottom panel) or SSA in transgenic rice (top panel). In contrast, a decrease in all resolved glutelin polypeptides was observed when transgenic plants were grown under limited sulfur supply (Fig. 2). Based on the four glutelin-specific spots intensity reduction varied between glutelin types. Type II glutelins were reduced by 50–68% whereas Type I and Type III glutelins were reduced by 20% and 50%, respectively. In addition, another major sulfur rich protein ($M_r = 19$ kDa, spot 5) identified as globulin was also reduced by 65%. The reduced levels of glutelin and globulin proteins were accompanied with no change in SSA level under low sulfur supply. We speculate that the level of sulfur stress on pool sulfur reserves in rice was intensified in presence of SSA gene, which contributed to the low expression of glutelin and globulin proteins. Also, the strong endosperm-specific promoter from a wheat high molecular weight glutelin gene used for transgenic SSA expression is not responsive to sulfur stress.

As pointed out by Spencer et al. (1990), the reduced level of mRNA in sulfur stressed legumes could be the result of transcriptional or post-transcriptional processing. It is also worthwhile noting our previous glutelin mRNA expression data where we observed a reduction in the transcript levels of glutelin A (Hagan et al., 2003). It is also conceivable that a translational control is in operation. This translational control model postulates that the sulfur stress can lead to a shortage of cysteine and methionine residues for sulfur rich proteins. Hagan et al. (2003) also speculated that changes in the accumulation of glutelins in the transgenic SSA rice may be attributable to a post translational mechanism. Decreased accumulation of legumin and albumin in peas grown under limited sulfur also seems to be due to a post-transcriptional mechanism (Beach et al., 1985; Chandler et al., 1984). Although the actual mechanism of the interference is unknown, it is obvious from our results that the expression of SSA has a negative impact on the level of glutelin proteins.

Genetic modification of plants for better products such as improved seed protein quality has been sought. An in-depth analysis of transgene effects using proteomic analysis will assist in unravelling the complex transcriptional, post-transcriptional and post-translational controls that may come into play as a result of adding even a single transgene as illustrated here from SSA.

3. Experimental

3.1. Plant materials

Transgenic rice expressing SSA was produced by micro-projectile bombardment of japonica rice (*Oryza sativa* L. cv Taipei) (Hagan et al., 2003), A homozygous transgenic line (T3) was identified by hygromycin phosphotransferase gene (*hph*, co-segregating selectable marker gene) segregation analysis of progeny seeds (T4) using polymerase chain reaction (PCR) as described by Hagan et al. (2003). Transgenic and non-transgenic seeds were sown in pots containing 75% potting mix/25% perlite supplemented with 1 g/L gypsum (230 mg S kg^{-1} – S optimum condition) to provide adequate supply of sulfur and raised in a glasshouse

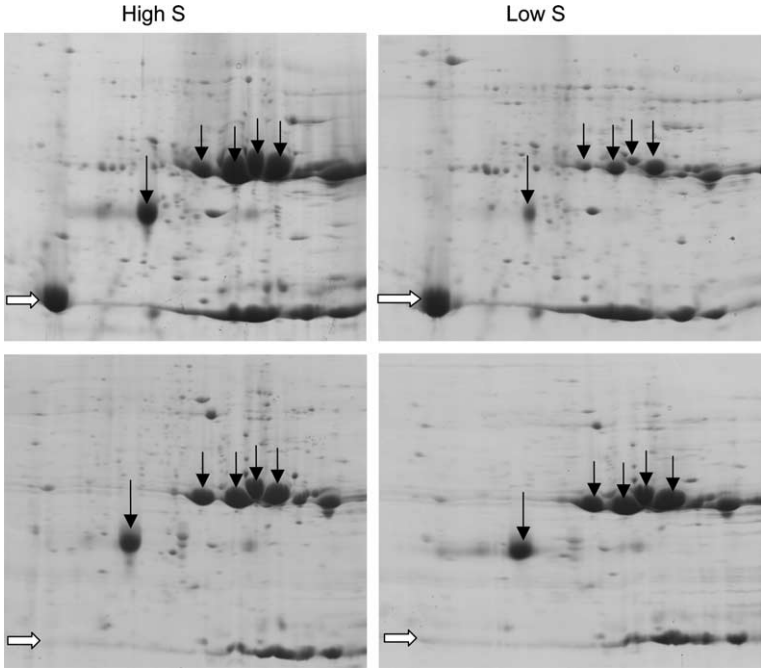

Fig. 2. 2-DE proteome pattern of transgenic (top) and non-transgenic (bottom) rice seed grown under high- and low-sulfur supply. Plants were grown in flooded conditions in a pot containing either 10 mg S kg^{-1} soil (for low S treatment) or 320 mg S kg^{-1} (for high sulfur treatment). Proteins were extracted from mature endosperm following the procedure described in materials and methods. Solid arrow marked proteins were different types of glutelin proteins and globulin. The position of the transgenic product SSA is shown by the horizontal arrow.

maintained at 25 °C day and 22 °C night temperatures under natural light conditions as described previously. To study the effect of sulfur nutrition on seed sulfur rich protein profile, rice plants were grown under flooded conditions in pots containing either 10 mg S kg^{-1} soil for low sulfur treatment or 320 mg S kg^{-1} for high sulfur treatment as described by Randall et al. (2003).

3.2. Protein extraction

Proteins were extracted from mature rice seeds by the method described by Islam et al. (2004). Briefly, a portion of fine powder (600 mg) was taken into a pre-chilled Falcon tube (13 × 51 mm) with pre-chilled glass beads (about 200 mg, 0.04 mm, Grade-20, HLS Scientific, Sydney, Australia). Freshly prepared 10% trichloroacetic acid (TCA) in acetone containing 0.07% DTT (5 ml) was added. After a brief vortexing, the tube was placed on dry ice and sonicated (MSE 100 probe sonicator, Thomas Optical and Scientific, Sydney, Australia) in six bursts of 10 s each with 60 s intervals. The solution was then placed at −20 °C for 1 h, centrifuged (30,000*g*, 15 min.), and the supernatant was discarded. The pellet was crushed using a clean and pre-chilled glass rod and resuspended in another 5 ml of 10% TCA in acetone containing 0.07% DTT. The precipitate, after being treated two times with TCA, was washed three times with cooled acetone containing 0.07% DTT followed by centrifugation. The final pellet was freeze dried for 10 min in Flexi-DryTM μP (FTS Systems, Stone Ridge, NY, USA). Although this method of protein extraction was developed for green rice leaves, we found that it is equally effective for protein extraction from mature grains.

The lyophilized sample was transferred into a pre-chilled Eppendorf tube and briefly ground. The sample was dissolved with 300 μL extraction solution comprising 8 M urea, 2% (v/v) Nonidet P-40 (NP-40), 0.8% (v/v) amopholines, pH 3.5–10, 5% (v/v) 2-mercaptoethanol (ME) and 5% (w/v) polyvinylpyrrolidone-40 by brief sonication, then vortexed for 1 h (O'Farrell, 1975).

3.3. Two dimensional electrophoresis

After 1 h in extraction buffer, the samples were centrifuged (15,000 *g*, 10 min) and 150 μl of the supernatant, containing 800 mg proteins, was applied to the acidic side of an isoelectric focusing (IEF) (pH 3–10) rod gel 20 cm long and 3 mm in diameter (Hirano, 1989). About 20 μl of 50% extraction buffer was overlayed on the top of the sample to protect it from the anodic electrode solution. Electrophoresis proceeded at 200 V for 30 min., 300 V for 30 min., and 500 V for 16 h. The rod gel was then removed from the tube and equilibrated for 15 min in a solution containing glycerol (10%), Tris–HCl give final conc. and pH, sodium dodecyl sulfate (SDS) (2.5%) and 2-mercaptoethanol (5%). SDS–polyacrylamide gel electrophoresis (PAGE) in the second dimension was performed with 15% separating and 5% stacking gels at a constant temperature (10 °C) and current (25 mA) in a ProteanIIxi apparatus (Bio-Rad). Protein spots in 2-DE gels were visualized by 0.1% Coomassie brilliant blue (CBB) G-250 staining. Extraction was performed from four replicates grown under similar conditions.

3.4. Image analysis

Following staining, the 2-DE gel patterns were scanned using a flatbed scanner, and analysed using ImageMaster 2D Platinum software (GE Bioscience). After scanning, spots in eight gels, four from each of the transgenic and non-transgenic, were detected using auto-detection parameters and then matching is adjusted manually, if necessary. Gels were then normalised, and statistical analysis was performed using the Student's *t*-test; mean differences were compared using *t* value at $p < 0.1$.

3.5. Protein identification

Differentially expressed proteins visualised by 2-DE were identified from peptide mass fingerprints. Pieces of gel containing protein were excised, placed in 500 μl microfuge tubes, washed twice in 100 μl of 30% methanol with constant shaking for 30 min and then washed repeatedly in 100 μl of 1:1 acetonitrile and 10 mM ammonium bicarbonate for 30 min until the stain disappeared or faded sufficiently. The gel pieces were then dried under vacuum for 15 min. Proteins in the gel pieces were digested by the addition of trypsin solution (5 μl of Promega stock solution-cat. no. V511C 11652008- diluted 30-fold in 10 mM ammonium bicarbonate) and overnight incubation in a heat block at 37 °C. The following day condensed fluid under the lid of the tubes was removed carefully (Li et al., 1997), and the gel pieces were rehydrated with 2 μl of 0.2% trifluoroacetic acid (TFA) for at least 15 min. Matrix solution (2μl of a saturated solution of α-cyano-4-hydroxycinnamic acid in 67% acetonitrile/33% of 0.2% TFA) was then added and incubated for 30 min at room temperature. Two microlitres of the solution surrounding the gel pieces was allowed to dry on the sample plate of a Voyager Elite matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/MS, Applied Biosystems, Framingham, MA) which was then operated in delayed-extraction/reflector mode. Peptides from a trypsin digest of bovine serum albumin (MH^+ 927.490 and 2045.0285) were used for close external calibration of each sample spot.

The peptide mass fingerprint obtained from each gel sample was used to search the entire NCBI non-redundant protein database using the ProFound search engine available at http://www.unb.br/cbsp/paginiciais/profound.htm allowing up to two missed cleavages, partial oxidation of methionine, complete modification of cysteine residues by acrylamide, and a mass tolerance of 50 ppm.

To confirm the identity of the putative SSA and spot no 5, we performed an in-gel digestion with trypsin followed by reversed C18 phase separation of the peptides (Agilent

1100 capillary LC) coupled with electrospray/ion-trap mass spectrometry (Agilent XCT). Peptide fragmentation data were used to search the nonredundant database of NCBI. For SSA spot, the SSA sequence was matched with "no enzyme" to interpret N-terminal cleavages using Spectrum Mill software (Spectrum Mill MS Proteomics Workbench, Agilent Technology).

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