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Gil-Humanes, J., Piston, F., Shewry, P. R., Tosi, P. and Barro, F. 2011. Suppression of gliadins results in altered protein body morphology in wheat. *Journal of Experimental Botany*. 62 (12), pp. 4203-4213.

The publisher's version can be accessed at:

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The output can be accessed at:

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RESEARCH PAPER

Suppression of gliadins results in altered protein body morphology in wheat

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Received 11 January 2011; Revised 24 February 2011; Accepted 21 March 2011

Abstract

Wheat gluten proteins, gliadins and glutenins, are of great importance in determining the unique biomechanical properties of wheat. Studies have therefore been carried out to determine their pathways and mechanisms of synthesis, folding, and deposition in protein bodies. In the present work, a set of transgenic wheat lines has been studied with strongly suppressed levels of γ -gliadins and/or all groups of gliadins, using light and fluorescence microscopy combined with immunodetection using specific antibodies for γ -gliadins and HMW glutenin subunits. These lines represent a unique material to study the formation and fusion of protein bodies in developing seeds of wheat. Higher amounts of HMW subunits were present in most of the transgenic lines but only the lines with suppression of all gliadins showed differences in the formation and fusion of the protein bodies. Large rounded protein bodies were found in the wild-type lines and the transgenic lines with reduced levels of γ -gliadins, while the lines with all gliadins down-regulated had protein bodies of irregular shape and irregular formation. The size and number of inclusions, which have been reported to contain triticins, were also higher in the protein bodies in the lines with all the gliadins down-regulated. Changes in the protein composition and PB morphology reported in the transgenic lines with all gliadins down-regulated did not result in marked changes in the total protein content or instability of the different fractions.

Key words: Gluten proteins, immunolocalization, protein bodies, RNAi, transgenic wheat, wheat endosperm.

Introduction

Wheat is the most widely cultivated cereal in the world due to its adaptability to different environments and high yields, as well as to the unique biomechanical properties of the gluten proteins, which allow the production of bread and many other food products. Wheat gluten proteins, called prolamins, are classically divided into two fractions, the glutenins and gliadins. Although their proportions may vary, the total gluten proteins generally account for around 80% of the total grain proteins, with about 30% being gliadins and 50% glutenins (Shewry *et al.*, 2009). The glutenins are present as polymeric complexes comprising two types of subunit, called high molecular weight (HMW) subunits and low molecular weight (LMW) subunits, while the gliadins are monomeric and are classified into three structural types: α -, γ - and ω -gliadins. These fractions also

differ in their functional properties with the glutenins contributing mainly to dough elasticity and the gliadins mainly to the dough extensibility and viscosity. The gliadin and glutenin proteins interact in dough to form a network that confers the characteristic cohesive viscoelastic properties that allow the entrapment of carbon dioxide during fermentation and expansion of the dough to give leavened bread. However, the amount and composition of the gluten proteins also vary with genotype and environment resulting in differences in dough properties which are exploited to allow the manufacture of a wide range of products including bread, cakes, cookies (biscuits), pasta, and noodles.

The synthesis of gluten proteins occurs on polyribosomes attached to the endoplasmic reticulum (ER). However, the

subsequent transport, deposition, and accumulation of gluten proteins into protein bodies (PB) is not completely understood and is thought to depend on the properties of the proteins themselves (particularly their insolubility in aqueous solvents) and also on the developmental stage of the endosperm (Tosi *et al.*, 2009). Two separate pathways appear to operate in the developing wheat endosperm, with assembly of storage proteins into PB occurring both within the ER and within the vacuoles. Furthermore, it has been proposed that transport from the ER to vacuoles may occur via the Golgi apparatus or via a Golgi bypass pathway (Kim, 1988; Levanony, 1992). Rubin (1992) reported the existence of two different types of PB with different densities, called light PB and dense PB, which accumulated simultaneously and independently in wheat endosperm cells. In the early stages of grain development, gliadins were found within the light PB, while in the later stages of grain development they were found in both PB types. However, HMW subunits were highly enriched in the dense PB during the entire period of grain development. The authors concluded that during early grain development most of the gliadins were transported from the ER to the vacuoles forming the light PB, and the remainder aggregated within the ER forming the dense PB. In addition, they suggested that the HMW subunits, and possibly also the LMW subunits, aggregated mostly within the ER to form dense PB.

The mechanisms that determine whether gluten proteins are transported to the vacuoles or stored in the ER are not known, but the accumulation of glutenins in the ER lumen could be a consequence of their ability to form insoluble aggregates, which would be less easily transported than the monomeric gliadins (Shewry, 1999).

RNA interference (RNAi) technology has been used to down-regulate multigene families (Travella *et al.*, 2006) including reducing the content of α -gliadins in lines of bread wheat cv. Florida (Becker *et al.*, 2006). Gil-Humanes *et al.* (2008) also reported the reduction of γ -gliadins by up to 80% in bread wheat lines of cv. 'Bobwhite'. More recently, all ω -, α -, and γ -gliadins have been strongly down-regulated by RNAi, drastically altering the protein content of the wheat endosperm (Gil-Humanes *et al.*, 2010). These wheat lines are therefore a useful tool to study the deposition of gluten proteins and formation and fusion of PB in developing seeds with low contents of gliadins.

In the present work, light and fluorescence microscopy, combined with immunodetection with specific antibodies for the γ -gliadins and HMW glutenin subunits, were used to study the formation and fusion of PB in developing seeds of wild-type and transgenic lines with reduced levels of gliadins.

Materials and methods

Plant material

Three transgenic wheat lines (*Triticum aestivum*) with down-regulation of the γ -gliadins fraction (γ -hpRNA lines) that contained the γ -hpRNA constructs pghpg8.1 (lines C655 and D445) and pGghpg8.1 (line D577); and six transgenic lines with all the groups of gliadins down-regulated (ω/α -hpRNA lines) that carried the

ω/α -hpRNA constructs pDhp- ω/α (line 28A), pGhp- ω/α (lines D894 and D793) and both constructs (E140, D911, and D874), as well as their untransformed wild-types BW208 and BW2003, were grown in a greenhouse at Rothamsted Research. For each line, three individual seeds were sown in different pots. Mature seeds were collected and seeds from the same genotype combined for further analysis. Growth conditions were as described by (Tosi *et al.*, 2004). Plasmids pGhp- ω/α and pGghpg8.1 contain the γ -gliadin promoter (Pistón *et al.*, 2009), while plasmids pDhp- ω/α and pghpg8.1 contain the D-hordein promoter (Pistón *et al.*, 2008) (Table 1). All transgenic lines were reported previously or obtained as described (Gil-Humanes *et al.*, 2008; Gil-Humanes *et al.*, 2010).

SDS-PAGE and Western assays

Total protein for SDS-PAGE and Western blot analysis was extracted from mature seeds. Flour of individual seeds was incubated for 30 min at 70 °C in an extraction buffer (62.5 mM TRIS-HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate (SDS), 1.5% (w/v) dithiothreitol (DTT), 10% (v/v) glycerol, and 0.002% Bromophenol Blue) in the proportion of 25 μ l of solution mg^{-1} of flour. Samples were centrifuged for 2 min at 14 000 *g* and the supernatant was collected. Extracted proteins were separated in Nu-PAGE 4–12% BIS-TRIS SDS precast gels (Invitrogen) at 200 V for 45 min. Protein transfer to Hybond-c Extra membranes (Amersham) was carried out in a Transblot (Bio-Rad) at 14 V for 30 min. Membranes were rinsed in TRIS-buffered saline (TBS) solution (20 mM TRIS-HCl, 500 mM NaCl, pH 7.5) and blocked in a TBS solution containing 5% (w/v) of non-fat powdered milk (Marvel) for 1 h. Membranes were then washed in TBS and incubated for 1 h in the corresponding primary antibody solution. Two primary antibodies were used diluted in a solution of TBS containing 1% bovine serum albumin (BSA) and 0.05% Tween20 (Sigma-Aldrich): a mouse monoclonal anti- γ -gliadins (S3B512, INRA, Nantes) diluted 1:4000 and a rabbit polyclonal anti-HMW-glutenins (HMW R2) diluted 1:8000. Membranes were then rinsed three times in 0.05% Tween 20 TBS solution and incubated for 1 h in the secondary antibody solution: anti-mouse or anti-rabbit IgG (whole molecule)–Alkaline Phosphatase (Sigma-Aldrich) diluted 1:6250 in the same TBS solution used for primary antibodies. Two washes with 0.05% (v/v) Tween20 TBS and a final wash with TBS solution were made before adding the BCIP[®]/NBT-Purple Liquid Substrate System for Membranes (Sigma-Aldrich). The reaction was stopped when bands were visible by rinsing the membranes in distilled water.

Gels were stained with a solution of 0.05% (w/v) Coomassie Brilliant Blue R-250, 5% (v/v) ethanol, 12% (w/v) Trichloroacetic acid to identify different protein bands. To remove the excess of staining, gels were immersed in tap water overnight.

Quantification of total protein

Total protein was determined in mature seeds according to the Kjeldahl method described in the American Association of Cereal Chemists (AACC) Method 46-10.01. The assay was performed in duplicate and results are expressed as the protein percentage ($\text{N} \times 5.7$) of the total dry matter.

Quantification of albumins and globulins

Albumins and globulins were extracted from 100 mg of flour. Albumins were extracted with 500 μ l of Milli-Q water (Millipore) and incubated at room temperature (RT) for 15 min with shaking. Samples were then centrifuged for 10 min at 13 000 *g*, and supernatants containing the albumin fraction collected and combined. Pellets were washed twice with 500 μ l of Milli-Q water (Millipore) and centrifuged for 10 min at 13 000 *g*. Globulins were extracted from the insoluble material of the previous step with 700 μ l of a 0.5 M NaCl solution at RT for 15 min with shaking. Samples were centrifuged for 10 min at 13 000 *g* and supernatants

Table 1. Plant material and seed protein characteristics

Genotypes and target genes of the RNAi mediated silencing. Total protein and gluten protein are expressed as % of protein in total dry matter; Gliadin and glutenin content is expressed as $\mu\text{g mg}^{-1}$ flour. Numbers in bold indicate that the mean is significantly different to control as determined by Dunnett's multiple comparisons at $P < 0.05$.

Line	Genotype	Target genes	Total protein (%)	Gluten protein (%)	Gliadins ($\mu\text{g mg}^{-1}$ flour)				Glutenins ($\mu\text{g mg}^{-1}$ flour)			Albumins ($\mu\text{g mg}^{-1}$ flour)	Globulins ($\mu\text{g mg}^{-1}$ flour)
					ω	α	γ	Total	HMW	LMW	Total		
BW208 wild type	BW208	N/A	16.1	11.5	13.4	43.3	33.5	90.2	8.8	16.1	24.9	12.2	9.4
C655	BW208	γ -gliadins	17.9	13.6	24.7	65.2	7.8	97.7	13.9	24.1	38.0	14.4	11.6
D445	BW208	γ -gliadins	19.2	12.9	25.2	59.8	0.4	85.4	17.5	26.0	43.5	14.3	10.7
D577	BW208	γ -gliadins	16.0	9.6	14.4	46.5	4.9	65.8	10.6	19.3	29.9	14.6	11.4
28A	BW208	All gliadins	17.7	7.2	4.5	14.3	4.0	22.8	22.7	26.2	48.8	17.1	14.6
D894	BW208	All gliadins	16.6	6.4	6.5	14.2	3.0	23.7	20.1	20.5	40.6	18.3	15.7
D793	BW208	All gliadins	16.0	3.9	5.1	6.2	0.7	11.9	17.8	8.9	26.8	20.3	16.6
BW2003 wild type	BW2003	N/A	15.3	9.8	12.9	32.9	27.9	73.8	10.8	13.3	24.1	12.7	9.7
E140	BW2003	All gliadins	16.6	6.3	7.8	13.3	8.6	29.7	20.4	12.8	33.1	18.0	15.5
D911	BW2003	All gliadins	15.4	4.3	9.8	7.5	3.7	20.9	16.9	5.4	22.4	18.8	16.3
D874	BW2003	All gliadins	15.9	4.3	6.6	5.8	3.5	15.8	21.0	6.1	27.1	22.2	17.2

containing the globulin fraction collected and combined. Two independent extractions were made for each sample. Albumin and globulin contents were determined in triplicate in the above extracts using a Bradford assay system (Bradford, 1976) using bovine serum albumin (Sigma-Aldrich) as standard.

RP-HPLC

The gliadin fraction from 100 mg of flour was extracted with $2 \times 670 \mu\text{l}$ of 60% (v/v) ethanol, vortexing for 2 min at room temperature, followed by incubation at RT for 10 min with shaking. The samples were centrifuged at 6000 g for 20 min and the supernatants were collected and combined. The glutenin fraction was extracted from the insoluble material of the previous step with $2 \times 500 \mu\text{l}$ of 50% (v/v) 1-propanol, 2 M urea, 0.05 M TRIS-HCl (pH 7.5), and 2% (w/v) DTT, vortexing for 2 min at RT, and incubation for 15 min at 60 °C with shaking. Samples were centrifuged at 6000 g for 20 min and the supernatants were collected and combined. Finally, samples were filtered through a 0.45 μm nylon filter (Teknokroma).

Gliadin (40 μl) and glutenin (40 μl) extracts were applied to a 300SB-C8 reverse phase analytical column (4.6 \times 250 mm, 5 μm particle size, 300 Å pore size; Agilent Technologies) using a 1200 Series Quaternary LC System liquid chromatograph (Agilent Technologies) with a diode array ultraviolet-visible (DAD UV-V) detector, as described in Gil-Humanes *et al.* (2010). The software with some minor manual adjustment handled the integration procedure automatically. Three replicate analyses were carried out on separate extracts for each transgenic line and control.

Statistics

Data were analysed using the SPSS version 11.0 statistical software package (SPSS). Analysis of variance (ANOVA) and two-sided Dunnett's test for median multiple comparisons with a control were used to analyse the results. *P*-values less than 0.05 were considered to be significant.

Preparation of samples for microscopy

Two grains of each individual plant were harvested from the central part of the spike at 21 days post anthesis (dpa) and 28 dpa. Collected grains were cut into 1 mm thick transverse slices and transferred immediately for fixation into a solution of 4% (w/v) paraformaldehyde and 2.5% (w/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.4, for 4 h. Then, samples were dehydrated in a 10% graded ethanol series (10% to 100% ethanol) and infiltrated in increasing concentrations of LR White resin (TAAB) diluted in ethanol (from 20% to 100%). Two changes of resin a day were made for 4 d, placing the samples in a rotator at RT during the day and at 4 °C overnight. When the samples were completely embedded with the resin they were transferred into capsules, filled with fresh LR White resin, and polymerized in the oven in an oxygen-free environment at 55 °C for 20 h.

Sections for microscopy studies were prepared using a Reichert–Jung Ultracut microtome. Sections of 1 μm thick were taken from polymerized samples and placed on Poly-lysine coated multi-well slides for immunofluorescence experiments, and on glass slides for protein staining with Naphthol Blue Black.

Immunofluorescence and light microscopy

For immunofluorescence studies, grain sections collected on Poly-lysine coated slides were pre-incubated for 30 min in a blocking solution made of 0.3% Tween20 (Sigma-Aldrich) and 3% BSA (Sigma-Aldrich) in phosphate buffered saline (PBS) (Sigma-Aldrich) solution at pH 7.4. Samples were then incubated for 1.5 h in primary antibody solution. The two primary antibodies described above were used in combination, diluted in antibody buffer (1% BSA, 0.05% Tween 20 (Sigma-Aldrich) PBS solution) in a relation of 1:50 and 1:100, for the mouse monoclonal anti- γ -gliadins (S3B512) and the rabbit polyclonal anti-HMW-glutenins (HMW R2), respectively. After three washes in a 0.3% Tween 20 PBS solution, samples were incubated for 1.5 h in the dark in secondary antibody solution of anti-rabbit Alexa Fluor 488 conjugated and anti-mouse Alexa Fluor 568 conjugated

(Invitrogen) diluted 1:250 in the same PBS solution used for the primary antibodies. Slides were rinsed three times in a 0.3% Tween 20 PBS solution followed by a wash in PBS solution. Samples were analysed on a Zeiss Axiophot fluorescence microscope equipped with a Retiga EXi CCD (Qimaging) camera and the MetaMorph® v7.5.5.50 software. For light microscopy, proteins of the grain sections were stained with Naphthol Blue Black solution (Sigma-Aldrich) for 30 s, washed with water, and air-dried.

Results

Nine homozygous transgenic lines of wheat (*Triticum aestivum*) cv. 'Bobwhite' (BW208 and BW2003) were selected for study (Table 1), five of which have been reported previously (Gil-Humanes *et al.*, 2010). Lines expressing the γ -hpRNA constructs (pGghpg8.1 and pghpg8.1) (γ -hpRNA lines) showed specific and strong reduction of the γ -gliadin fraction, while lines expressing the ω/α -hpRNA constructs (pGhp- ω/α and pDhp- ω/α) (ω/α -hpRNA lines) showed a strong reduction of all the gliadin fractions (Gil-Humanes *et al.*, 2010). The transgenic and wild-type plants were grown in the same conditions and the fertility and morphology of the plants and seeds did not differ markedly (see Supplementary Fig. S1 at *JXB* online). The total protein contents of the wild-type lines BW208 and BW2003 were 16.1% and 15.3% of total dry matter, respectively (Table 1). Most of the transgenic lines did not differ significantly from the wild-type lines in their protein contents that ranged between 15–16%. However, the γ -hpRNA lines C655 and D445 and the ω/α -hpRNA line 28A had significantly higher total protein contents of 17.9, 19.2, and 17.7%, respectively.

Reversed-phase high performance liquid chromatography (RP-HPLC) was used to quantify the contents of gliadins and glutenins in both the transgenic and wild-type lines (Table 1). The total gluten protein content (gliadins and glutenins) was significantly lower in all of the ω/α -hpRNA transgenic lines, with levels ranging from 3.9% (line D793) to 7.2% (line 28A), compared with 9.8% and 11.5%, respectively, in the corresponding wild-type lines, BW2003 and BW208. By contrast, the gluten protein contents of the lines in which only the γ -gliadins were suppressed were not significantly different from that of the wild-type BW208 line (Table 1). The total gliadin content was significantly reduced in all of the ω/α -hpRNA lines but not in the γ -hpRNA lines, compared with the wild-type lines (Table 1). Although the γ -hpRNA lines showed significantly decreased amounts of γ -gliadins, this was compensated for by increases in the amounts of other gliadin fractions (Table 1). This is evident for lines C655 and D445 in which the amounts of ω - and α -gliadins were increased and the total content of gliadins was comparable to that of the wild-type line. However, line D577 (γ -hpRNA line) showed a strong reduction in γ -gliadin content which was not compensated for by increases in the other gliadin fractions. The greatest reduction in gliadins was in the ω/α -hpRNA line D793 that contained only 11.9 $\mu\text{g mg}^{-1}$ flour: this represents a decrease of 86.8% in the total gliadin content

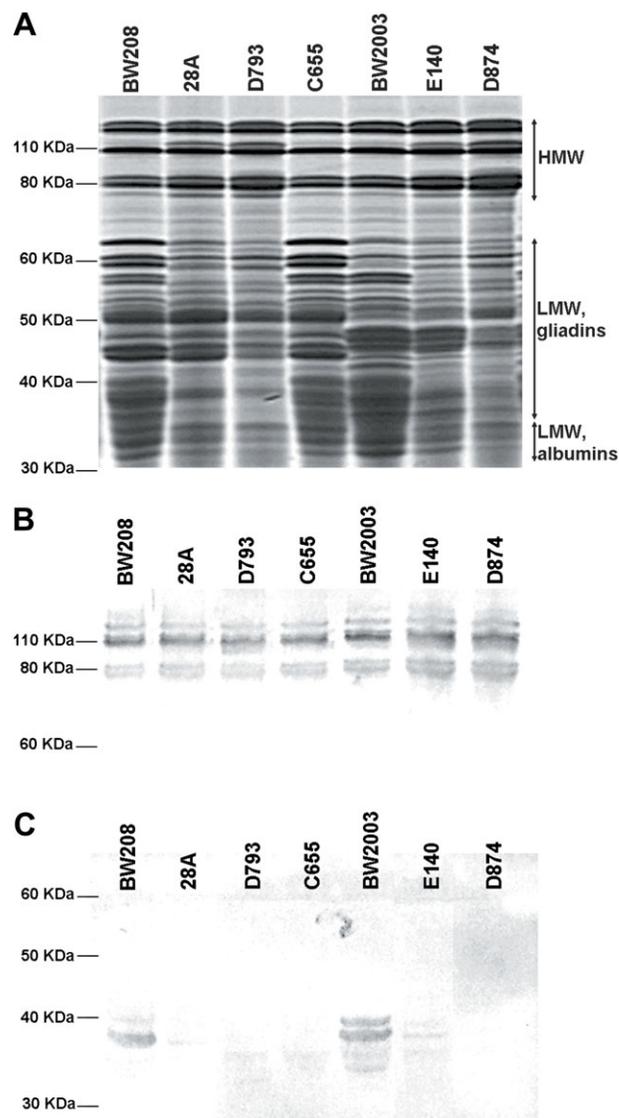


Fig. 1. SDS-PAGE gel and Western blot of wild-type and transgenic lines. (A) SDS-PAGE gel of total protein extracts stained with Coomassie Blue; (B, C) Western blot of total protein extracts separated in a SDS-PAGE gel, labelled with the HMW R2 pAb specific for HMW glutenins (B), and the S3B512 mAb specific for γ -gliadins (C).

with respect to the wild-type line BW208. A more detailed analysis of transgenic lines transformed with the ω/α -hpRNA construct showed that not all gliadin fractions were reduced to the same extent. All of the ω/α -hpRNA transgenic lines showed a significant reduction in the γ -gliadin fraction, with contents ranging from 0.7 $\mu\text{g mg}^{-1}$ flour (line D793) to 8.6 $\mu\text{g mg}^{-1}$ flour (line E140), representing decreases of 97.9% and 69.1%, respectively. By contrast, the ω - and α -gliadins were reduced by a smaller extent (Table 1). Lines D793 and D874 showed the greatest reduction in all gliadin fractions compared with the respective wild-type lines.

In most lines the suppression of gliadins was also associated with compensatory increases in the glutenin fraction. Lines D445 and C655 (γ -hpRNA) and lines 28A

and D894 (ω/α -hpRNA) showed significant increases in the amounts of both HMW and LMW glutenin subunits and their total glutenin content were therefore much higher than that of the wild-type line. Line D577 (γ -hpRNA) did not show any compensatory effects and the total glutenin content was comparable with that of the wild-type line (Table 1). Lines D793, D911, and D874 (ω/α -hpRNA) showed significant increases in the contents of HMW subunits but also significant decreases in LMW subunits. Overall, the total glutenin contents of these lines were comparable to those of their corresponding wild-type lines (Table 1). Line E140 (ω/α -hpRNA) showed a significant increase only in HMW subunits, and the total amount of glutenin subunits was higher than in the wild-type line (Table 1). Compensatory effects were also observed in the albumin and globulin fractions. Albumin contents were $12.2 \mu\text{g mg}^{-1}$ flour and $12.7 \mu\text{g mg}^{-1}$ flour for the BW208 and BW2003 wild-type lines, respectively; while globulins were $9.4 \mu\text{g mg}^{-1}$ flour and $9.7 \mu\text{g mg}^{-1}$ flour for the BW208 and BW2003 wild-type lines, respectively. Albumins and globulins showed significantly increased amounts in most of the ω/α -hpRNA lines (with only line E140 showing albumins levels in the range of the wild type), while all γ -hpRNA lines showed albumin and globulin contents comparable with that of the wild type (Table 1).

Total protein extracts from the two wild-type lines (BW208 and BW2003) and the transgenic lines 28A, D793, C655, E140, and D874 were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for Coomassie Blue staining (Fig. 1A) and Western blot analysis (Fig. 1B, C). The ω/α -hpRNA lines 28A, D793, E140, and D874 showed decreases in the number and staining intensity of the protein bands between 30 kDa and 70 kDa. However, these decreases were not clearly observed in the γ -hpRNA line C655, possibly due to overlapping with other protein bands in the region around 35 kDa and 40 kDa (Fig. 1A). Western blotting with the HMW R2 polyclonal antibody (pAb) showed that all of the lines gave similar reactions with only bands corresponding to HMW subunits (about 80–110 kDa) being detected (Fig. 1B). Western blotting with the S3B512 monoclonal antibody (mAb) specific for γ -gliadins showed two reactive bands between 35 kDa and 40 kDa in the wild-type BW208 line and at least four bands in the wild-type BW2003 line, while either no bands (28A, D793, C655, and D874) or weak signals only (E140) were detected in the transgenic lines (Fig. 1C).

The pattern of deposition of the HMW glutenins and γ -gliadins was studied in all the transgenic lines described in Table 1 and compared with the corresponding wild-type lines. Sections of developing caryopses at 21 dpa and 28 dpa

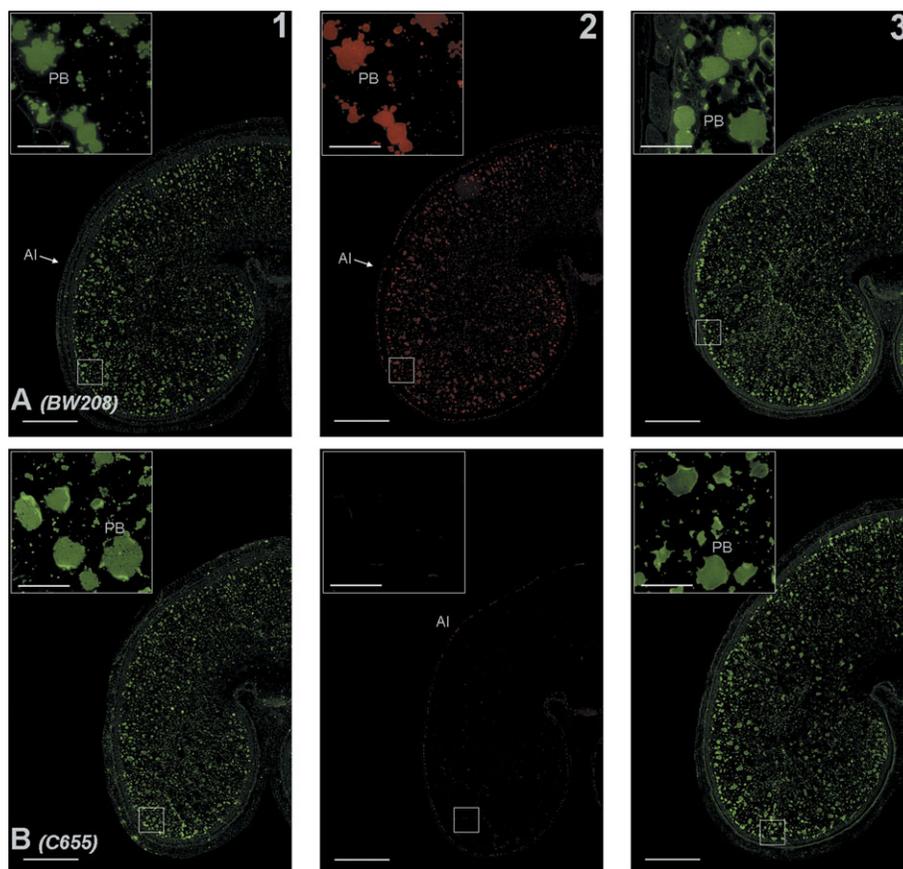


Fig. 2. Immunolocalization of HMW and γ -gliadins in seed sections of BW208 wild-type line (A) and transgenic line C655 (B). Columns 1 and 2 correspond to 21 dpa sections labelled with the HMW R2 pAb specific for HMW glutenins (1) and the S3B512 mAb specific for γ -gliadins (2). Column 3 corresponds to 28 dpa sections labelled with the HMW R2 pAb specific for HMW glutenins. Bars in sections of column 1, 2, and 3 correspond to 500 μm , and bars in magnifications correspond to 50 μm . PB, protein bodies; Al, aleurone layer.

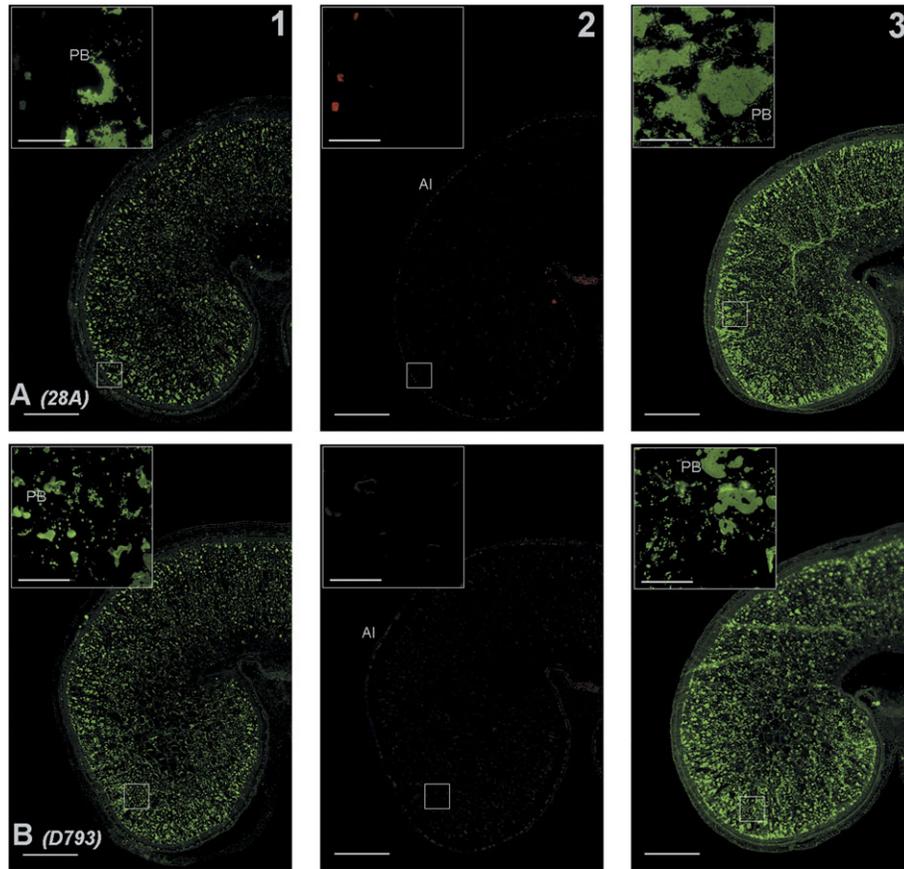


Fig. 3. Immunolocalization of HMW and γ -gliadins in seed sections of transgenic lines 28A (A) and D793 (B). Columns 1 and 2 correspond to 21 dpa sections labelled with the HMW R2 pAb specific for HMW glutenins (1) and the S3B512 mAb specific for γ -gliadins (2). Column 3 corresponds to 28 dpa sections labelled with the HMW R2 pAb specific for HMW glutenins. Bars in sections of column 1, 2, and 3 correspond to 500 μ m, and bars in magnifications correspond to 50 μ m. PB, protein bodies; AI, aleurone layer.

were labelled with the HMW R2 pAb and the S3B512 mAb (Figs 2, 3; see Supplementary Figs S2 and S3 at *JXB* online). Figure 2 shows the single channel images of the double labelling of HMW subunits and γ -gliadins in the BW208 wild-type line and the γ -hpRNA transgenic line C655 with decreased levels of γ -gliadins, while Fig. 3 shows the ω/α -hpRNA transgenic lines 28A and D793 in which all gliadins are decreased. Double labelling with the HMW R2 pAb (specific for HMW subunits) and S3B512 mAb (specific for γ -gliadins) was carried out on sections at 21 dpa (Fig. 2, columns 1 and 2; Fig. 3, columns 1 and 2; see Supplementary Fig. S2, columns 1 and 2, at *JXB* online) and 28 dpa (Fig. 2, column 3; Fig. 3, column 3; see Supplementary Fig. S2, column 3 and Supplementary Fig. S3 at *JXB* online). Two fluorochromes, AlexaFluor 488 and 568 that emitted in the green and the red region of the spectra, respectively, with no overlapping in their excitation or emission spectra, were used. Immunofluorescence with the HMW R2 pAb was detected in 21 dpa and 28 dpa sections in both the wild-type and transgenic lines (Fig. 2, columns 1 and 3; Fig. 3, columns 1 and 3; see Supplementary Fig. S2, columns 1 and 3, at *JXB* online). This showed that HMW subunits accumulated mainly in the cells of the subaleurone layer, showing a gradient decreasing from the cells of the aleurone layer to the cells of the inner

endosperm. Labelling of the HMW subunits in sections at 28 dpa (Fig. 2, column 3; Fig. 3; column 3; see Supplementary Fig. S2, column 3, at *JXB* online) gave a stronger signal than in 21 dpa sections in all the genotypes, probably due to higher protein accumulation at 28 dpa than at 21 dpa. In addition, the signals observed with the HMW R2 pAb in the wild-type line BW208 and in the γ -hpRNA transgenic line C655 (Fig. 2, A3 and B3, respectively), were weaker than that observed in the ω/α -hpRNA transgenic lines (28A and D793; Fig. 3, A3 and B3, respectively). Figure 2 and Fig. 3 (column 2) show immunolocalization of the γ -gliadins in sections at 21 dpa. The S3B512 mAb showed immunofluorescence only in the wild-type BW208 line (Fig. 2, A2; see Supplementary Fig. S2, A2, at *JXB* online), while none of the transgenic lines showed any signal (Fig. 2, A2 and B2; Fig. 3, A2 and B2; see Supplementary Fig. S2, B2 and C2, at *JXB* online). No differences were observed between the sections at 21 dpa and 28 dpa labelled with the anti- γ -gliadin S3B512 mAb (Fig. 2, column 2; Fig. 3, column 2; see Supplementary Fig. S3 at *JXB* online).

Observation of specific areas of the endosperm close to the aleurone layer at higher magnification confirmed the presence of the HMW subunit in the control and transgenic lines (insets Fig. 2, columns 1 and 3; insets Fig. 3, columns 1 and 3; see insets Supplementary Fig. S2, columns 1 and 3,

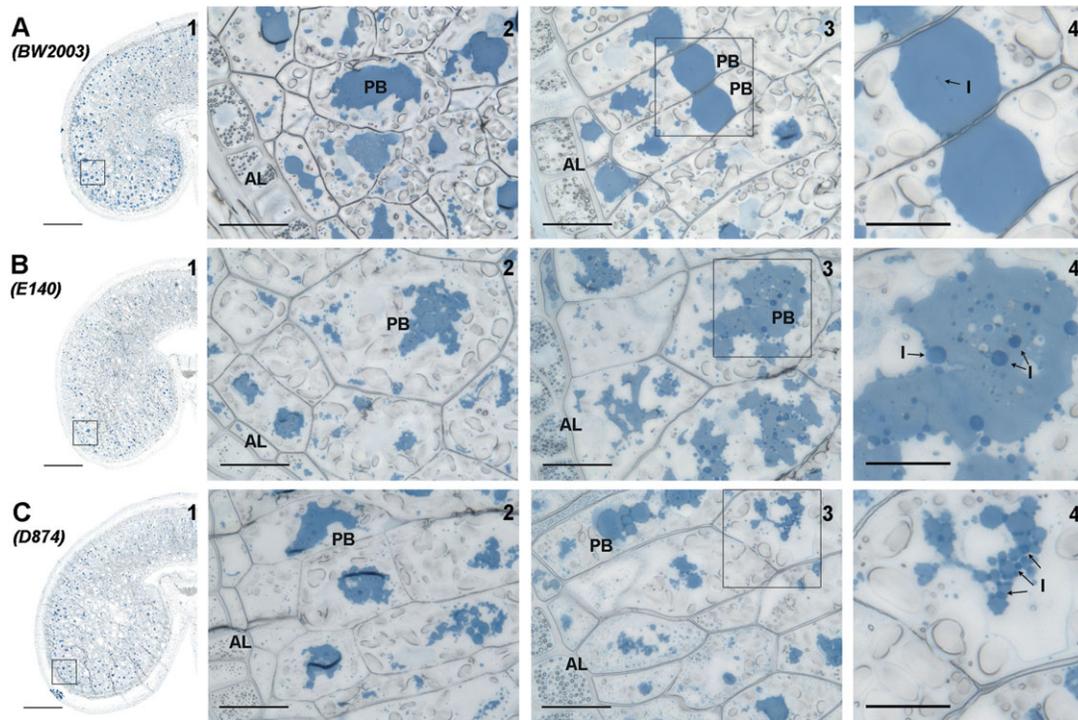


Fig. 4. Naphthol Blue Black-stained sections of developing wheat seeds and magnification of specific areas of BW2003 lines: wild-type line (A), E140 (B), and D874 (C). Column 1, half-seed sections of 21 dpa developing seeds. Squares show the magnified region of column 2 pictures; column 2, magnification of specific areas of 21 dpa seed sections; column 3, magnification of 28 dpa seed sections; column 4, inclusions on protein bodies of 28 dpa sections. Bars in sections of column 1 correspond to 500 μm ; bars in magnifications of columns 2 and 3 correspond to 50 μm , and bars in pictures of column 4 correspond to 25 μm . PB, protein bodies; AL, aleurone layer; I, inclusion.

at *JXB* online) while γ -gliadins were only detected in the wild-type BW208 line (insets Fig. 2, column 2; see insets Supplementary Fig. S2, column 2, at *JXB* online). Close examination of the protein bodies in the ω/α -hpRNA transgenic lines, in which all of the gliadins were silenced, showed that they also differed in shape and size from those in the wild-type lines and in the γ -hpRNA lines lacking γ -gliadins and did not merge in the same way. The diameter of the PB located in the sub-aleurone cells was measured in all the lines and expressed as the mean \pm standard error. At 21 dpa, the BW208 wild-type line showed PB diameters of $30.7 \pm 1.9 \mu\text{m}$, while for γ -hpRNA lines C655, D445, and D577 the diameters were $35.1 \pm 1.9 \mu\text{m}$, $25.0 \pm 3.1 \mu\text{m}$, and $28.3 \pm 1.7 \mu\text{m}$, respectively; and for the ω/α -hpRNA lines 28A, D894, and D793 were $29.4 \pm 2.3 \mu\text{m}$, $24.4 \pm 1.5 \mu\text{m}$, and $16.1 \pm 1.1 \mu\text{m}$, respectively. For BW2003 lines, the differences between the wild-type and ω/α -hpRNA transgenic lines at 21 dpa were greater than observed for the BW208 lines. The BW2003 wild type showed PB with $43.5 \pm 2.1 \mu\text{m}$ diameter, while line E140 had $16.0 \pm 1.1 \mu\text{m}$ and line D874 $15.6 \pm 1.4 \mu\text{m}$ (line D911 was not determined). Higher magnification images of the sections at 21 dpa showed that the PB in the wild-type BW208 line and in the γ -hpRNA C655 line were rounded and merged to form large PB (insets Fig. 2, A1 and B1), while the PB in the ω/α -hpRNA lines were smaller (insets Fig. 3, A1 and B1; see insets Supplementary Fig. S2, B1 and C1, at *JXB* online), irregular in shape and did not merge to form PB but

remained as dispersed small PB. By contrast, at 28 dpa the PB in the BW208 ω/α -hpRNA transgenic lines were similar, or even larger, $50.7 \pm 4.6 \mu\text{m}$ in line 28A and $27.8 \pm 1.7 \mu\text{m}$ in line D793, than those in the wild-type line and the γ -hpRNA line C655, which had diameters of $36.2 \pm 1.7 \mu\text{m}$ and $34.7 \pm 1.9 \mu\text{m}$, respectively (insets Fig. 2, column 3; insets Fig. 3, column 3; see insets Supplementary Fig. S2, column 3, at *JXB* online), although their shape remained irregular. In BW2003 ω/α -hpRNA transgenic lines the PB at 28 dpa were smaller than the wild type ($43.3 \pm 2.2 \mu\text{m}$ diameter), with diameters of $34.9 \pm 2.2 \mu\text{m}$ in line E140, and $21.5 \pm 0.7 \mu\text{m}$ in line D874.

Sections of the transgenic and wild-type lines at 21 dpa and 28 dpa were stained with Naphthol Blue Black to highlight the differences in PB merging, size and shape (Fig. 4; see Supplementary Fig. S4 at *JXB* online). This confirmed the previous observations and showed the PB in the wild-type lines (Fig. 4, A2; see Supplementary Fig. S4, A2, at *JXB* online) and the γ -hpRNA line C655 (see Supplementary Fig. S4, B2, at *JXB* online) at 21 dpa were similar, being large and rounded. However, the PB in the ω/α -hpRNA transgenic lines, in which all of the gliadins were suppressed, were irregular in shape and did not merge (Fig. 4, B2 and C2; see Supplementary Fig. S4, C2, at *JXB* online).

At 28 dpa the PB in the wild-type lines (Fig. 4, A3; see Supplementary Fig. S4, A3, at *JXB* online) and the γ -hpRNA line C655 (see Supplementary Fig. S4, B3, at *JXB* online) were similar in shape and size to those

observed at 21 dpa. By contrast, the PB in the ω/α -hpRNA transgenic lines E140 and 28A (Fig. 4 B3; see Supplementary Fig. S4, C3, at JXB online) were larger but still irregular. Line D874 (Fig. 4, C3) showed small dispersed PB, similar to those observed at 21 dpa. The PB in the ω/α -hpRNA transgenic lines (Fig. 4, column 4; see Supplementary Fig. S4, column 4, at JXB online) at both 21 d and 28 d also contained small inclusions close to the PB surface. These inclusions were also larger and more numerous in line E140 (Fig. 4 B4) than in lines D874 and 28A (Fig. 4, C4; see Supplementary Fig. S4, C4, at JXB online). These may correspond to the inclusions that have previously been reported to contain the storage globulin triticin (Bechtel *et al.*, 1991).

Discussion

The unique characteristics of the transgenic lines studied here, and the high specificity of the two antibodies used to detect HMW subunits and γ -gliadins allow the relationship between protein composition and deposition in developing seeds to be studied. Western blotting of total protein extracts from mature seeds double-labelled with antibodies specific for HMW subunits and γ -gliadins showed that γ -gliadins but not HMW subunits were suppressed in all lines (Fig. 1). The total protein contents of both the wild-type and transgenic lines were higher than those described previously (Gil-Humanes *et al.*, 2010). However, the plants were grown in different environmental conditions in the two studies, and both the total protein content and the protein composition are known to be strongly influenced by the environment, and, in particular, by the availability of mineral nutrients (Wieser and Seilmeier, 1998). Quantification of extracted gluten proteins showed that compensatory increases in glutenin subunits occurred in most lines but not in line D577 (Table 1). In most cases the contents of both the HMW and LMW subunits were increased, but, in lines D911 and D874, the contents of HMW subunits were increased and of LMW subunits decreased. The globulins were significantly increased in all ω/α -hpRNA transgenic lines while albumins were increased in all lines except one ω/α -hpRNA transgenic line. Compensatory effects were observed previously by Lange *et al.* (2007) who reported increases in the amounts of B hordeins and glutelins in transgenic lines of barley with reduced contents of C hordeins. Hansen *et al.* (2007) performed microarray analyses of the same lines and showed up-regulation of B and γ -hordein genes and of the gene encoding the barley prolamins-binding factor (BPBF), a transcription factor that regulates B hordein gene expression.

Rubin (1992) reported two different types of PB in wheat with different densities, called light PB and dense PB. They showed that both types of PB accumulated simultaneously and independently in wheat endosperm cells, with the light PB containing mainly gliadins and the dense PB glutenins and gliadins. However, other authors have reported the existence of only one kind of PB, containing both gliadins

and glutenins (Stenram, 1991; Loussert *et al.*, 2008). In addition, Loussert *et al.* (2008) showed an even distribution of gliadins and glutenins in PB, with no internal organization or micro-domains. The results reported here similarly show that HMW subunits and γ -gliadins are co-localized in most PB in the wild-type line (insets Fig. 2, A1 and A2; see insets Supplementary Fig S2, A1 and A2, at JXB online).

The content of HMW subunits was higher in most of the transgenic lines, compared with the wild-type lines, irrespective of the construct. The transgenic lines shown in Figs 2 and 3 showed increases in the HMW subunit contents of 58% in the γ -hpRNA line C655, and of 158% and 103% in the ω/α -hpRNA lines 28A and D793, respectively, compared with the wild-type lines. However, the intensity of immunofluorescence with the HMW pAb was higher in the ω/α -hpRNA transgenic lines, which had decreased contents, of all gliadins (Fig. 3, A3 and B3), than in the γ -hpRNA line C655 (Fig. 2, B3), which showed a similar level of intensity to the wild-type line (Fig. 2, A3). Similar results were obtained in single-labelled sections using the HMW R2 pAb (data not shown). Consequently, the higher intensity of immunofluorescence found in the ω/α -hpRNA lines can be explained by a higher proportion of HMW subunits within the PB and not only by a higher total amount of HMW subunits. In addition, the size and shape of the PB differed in the lines, with large rounded PB in the wild-type lines and the γ -hpRNA lines, and smaller PB of irregular shape in the ω/α -hpRNA lines (mainly in BW2003 transgenic lines). Irregular PB formation has previously been described in mutants and transgenic lines of maize with altered levels of prolamins (Zhang and Boston, 1992; Wu and Messing, 2010), in RNAi transgenic lines of rice (Kawakatsu *et al.*, 2010), and transgenic lines of tobacco expressing a modified 10 kDa δ -zein protein (Randall *et al.*, 2000). Maize prolamins, which are called zeins, are specifically synthesized on polyribosomes of the ER and translocated into the lumen of the ER, where they assemble into PB (Wolf *et al.*, 1967). The maize PB have a highly ordered architecture, with the α - and δ -zeins deposited in the centre of the PB and existing as monomers, and the β - and γ -zeins located in the peripheral layer and forming polymers stabilized by disulphide bonds (Ludevid *et al.*, 1984; Lending and Larkins, 1989). Wu and Messing (2010) reported the effects on PB morphology caused by RNAi-mediated silencing of different zeins. They concluded that, although decreases in individual β - and γ -zeins had little effect on PB formation, their simultaneous decrease severely distorted the PB structure, suggesting a redundant function of β - and γ -zeins for stabilizing the formation of PB, and the importance of disulphide bonds in maintaining the normal PB shape. Changes in the morphology of the PB have also been observed in rice lines with RNAi-mediated down-regulation of the different seed storage proteins (Kawakatsu *et al.*, 2010). Rice prolamins are exclusively stored in ER PB, while vacuolar PB contain α -globulins and glutelins (Bechtel and Juliano, 1980). The authors observed changes in the formation of PB of both vacuolar and ER origin in the different RNAi

lines, and related these changes to the reduction of globulins and 13 kDa prolamins, respectively. They proposed that the cysteine-rich 13 kDa prolamins may play a role in the PB stabilization by disulphide bonds (similarly to the β - and γ -zeins in maize), whereas the cysteine-poor 13 kDa prolamins may be involved in enlarging PB. They also suggested that the ratio of cysteine-poor to cysteine-rich prolamins may be important for proper aggregation/folding of prolamins.

The present study showed that changes in PB morphology were only observed in lines that showed reductions in all gliadins (ω/α -hpRNA lines), but not in lines with the down-regulation of γ -gliadins only. All the ω/α -hpRNA lines also showed reduction in total gluten protein content, although the content of HMW subunits was increased. Consequently, the ratio of glutenins:gliadins in these lines was much higher than in the wild-type lines and the γ -hpRNA lines, and thus the proportions of glutenins and gliadins within the ER PB were also altered. The fact that the γ -hpRNA lines formed normal PB does not mean necessarily that γ -gliadins are not involved in the determination of PB morphology, as the up-regulation of the other groups of gliadins compensated for the reduction of γ -gliadins resulting in normal levels of total gliadins. Gliadins may play an important role in the formation and stabilization of PB, as the β - and γ -zeins in maize and the 13 kDa prolamins in rice, and, in particular, the S-rich gliadins (α - and γ -gliadins) that can form intra-molecular disulphide bonds. In addition, the repetitive domains of the α - and γ -gliadins are rich in β -reverse turns and may form extended structures (Gianibelli *et al.*, 2001). Furthermore, the decreased proportion of PB of vacuolar origin in the starchy endosperm may also affect the merging of the PB of ER origin. This is supported by the suggestion that gliadins are deposited preferentially in vacuolar PB which would then merge with ER PB (Rubin, 1992). As a result, the PB in the ω/α -hpRNA lines (which lacked gliadins) would originate mainly by the fusion of smaller PB derived from the ER.

Therefore, the changes in PB morphology in the ω/α -hpRNA lines can be explained by (i) the reduction of the total amount of gliadins, which may be involved in the PB stabilization, (ii) the increased amounts of HMW subunits and decreased amounts of gliadins in the ω/α -hpRNA lines that resulted in altered ratios of glutenins:gliadins, (iii) the significant reduction in the total content of gluten proteins observed in all the ω/α -hpRNA lines, and/or (iv) the decreased proportion of PB of vacuolar origin in the starchy endosperm.

Staining of sections of the developing seeds for protein with Naphthol Blue Black (Fig. 4; see Supplementary Fig. S4 at *JXB* online) also revealed an increase in the number and size of the inclusions present at the surface of the PB of the ω/α -hpRNA lines. These inclusions have been reported to contain triticin (Bechtel *et al.*, 1991), an 11S globulin homologue which is deposited in the PB of the endosperm between 8 d and 21 d after anthesis (Singh and Shepherd, 1987). In the present study all the ω/α -hpRNA transgenic lines had significant increases in total globulins, while none of the γ -hpRNA transgenic lines showed

significant differences compared with the wild-type lines. Therefore, the strong reduction of all the gliadins results in a compensatory effect on the synthesis of non-gluten proteins, which may include triticins. Although triticins represent only about 5% of the total seed protein (Singh *et al.*, 1988; Bechtel *et al.*, 1991), they have a significant effect on the nutritional quality of the flour due to their good balance of essential amino acids with a high content of lysine (Singh *et al.*, 1993). In addition, triticins may also affect the functionality of wheat flour doughs by participating in thiol disulphide interchange reactions (Sievert *et al.*, 1991).

In summary, the suppression of gliadin synthesis by RNAi in transgenic wheat resulted in changes in the protein balance in the endosperm, with an increase in the glutenin fraction in most lines. Immunolocalization studies carried out with a HMW subunit-specific pAb showed more intensive staining of PB in the lines which showed the greatest reduction in gliadins and the greatest increase in HMW subunits. Lines with reduced levels of all gliadin groups (ω/α -hpRNA lines) also showed increases in the number and size of the inclusions distributed at the surface of the PB. Finally, differences in the size, shape, and extent of fusion of the PB were observed in the ω/α -hpRNA lines. However, these changes resulted in little or no effect on total grain protein content and did not appear to affect gluten protein assembly or protein body stability.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Fig. S1. Mature seeds from BW208 lines: wild-type BW208 (A) and transgenic lines C655 (B), 28A (C), and BW2003 lines: wild-type BW2003 (D) and transgenic lines E140 (E), and D874 (F).

Supplementary Fig. S2. Immunolocalization of HMW and γ -gliadins in seed sections of BW2003 wild-type line (A), and transgenic lines E140 (B) and D874 (C).

Supplementary Fig. S3. Immunolocalization of γ -gliadins in 28 dpa seed sections of wild-type BW208 (A), and transgenic lines C655 (B), D793 (C) and D874 (D).

Supplementary Fig. S4. Naphthol Blue Black-stained sections of developing wheat seeds and magnification of specific areas of BW208 lines: wild-type line (A), C655 (B), and 28A (C).

Acknowledgements

This work was supported by the Spanish Comision Interministerial de Ciencia y Tecnologia (AGL2010-19643-C02-02 and TRA2009_0047), the European Regional Development Fund (FEDER), and Junta de Andalucía (Project P09-AGR-4783). Javier Gil-Humanes acknowledges financial support from the I3P Program from the Consejo Superior de Investigaciones Científicas, which is co-financed by the European Social Fund. Rothamsted

Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK. Authors thank Dr Allison van de Meene, Mrs Jean Devonshire (Bioimaging Unit at Rothamsted), and Dr Cristina Sanchis Gritsch for their help and support with the immunofluorescence and light microscopy studies. Authors are very grateful to Dr Oliver Tranquet at INRA-Nantes, for providing us with the γ -gliadin antibody.

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